Correlation between Field Formation, Proton Translocation, and the Light Reactions in Photosynthesis *

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Electrical field changes across the function membrane of photosynthesis have been measured via special absorption changes. These occur by the action of the field on pigments which are located within the membrane. — Proton translocation has been measured by absorption changes of added pH indicators. Repetitive flash technique has been used for the sensitive detection of these absorption changes.

1. Each of the two light reactions of photosynthesis sets on one half of the electrical field.
2. The action spectra indicate that the two light reactions which set on the field are identical with those which promote the electron transfer.
3. The voltage set on in the elementary act both light reactions is $V = 50 \text{ mV}$.
4. Each of the two light reactions translocates one proton across the membrane.
5. The proton uptake is faster than $8 \times 10^{-3} \text{ sec}$.
6. Two protons are translocated per one electron transferred to a terminal acceptor.
7. A reaction scheme compatible with the measured correlation between the light reactions, electron transfer, field formation, and proton translocation is depicted in Fig. 7.

1. In the primary processes of photosynthesis electrons are transferred from water to NADP through at least ten electron carriers by the energy of two light reactions. The results obtained in this respect especially by the pulse techniques are discussed in I.c. 1.

2. Simultaneously with the electron transfer ATP is synthesized from ADP and inorganic phosphate 2.

3. Furthermore with the electron transfer ion translocations have been observed. Protons are taken up in exchange with other cations 3, 4.

4. Moreover, evidence has been provided that with the electron transfer an onset of an electrical field across a membrane takes place 5.

5. The function unit of phosphorylation, ion translocations, and field changes is the membrane of one thylakloid 5, 6.

6. Mitchell postulated that the free energy necessary for the synthesis of ATP may be gained with the translocation of protons “down hill” a proton gradient and a voltage gradient across a membrane 7. This idea is supported by several facts.

a) If an artificial pH-gradient is set up on chloroplasts, a formation of ATP can be observed in the dark 8.

b) In vivo, it has been demonstrated that the synthesis of ATP in the light is coupled with a field driven extra proton efflux out of the thylakoid 9, 5.

c) Furthermore, it has been shown that the synthesis of ATP is coupled with a diffusion driven extra efflux of protons out of the thylakoid 10.

d) A number of other results giving experimental evidence in photosynthesis for the hypothesis of Mitchell and results which extend the concept, have been presented in I.c. 5, 6 and in I.c. 9–15. In the


further development of the concept the following questions are of interest.

a) Does there exist a definite ratio of electron transfer and $H^\circ$-translocation which demonstrates the coupling of both?

b) What is the correlation of the $H^\circ$-translocation with respect to the two light reactions?

c) What is the correlation of the electrical field formation with respect to the two light reactions?

d) What is the mechanism of the coupling between these four events?

These questions are the subject of the following experiments.

Only the first question concerning the coupling between electron transfer and proton translocation has been studied by several authors. They determined the stoichiometric ratio between the number of protons taken up by chloroplasts (Δ$H^\circ$) and the number of electrons transferred to a terminal electron acceptor (Δ$e^\circ$) in steady illumination. The results of Izawa et al. in l.c.16 showed an uptake of 2 protons per 1 electron transfer. This was confirmed in l.c.13. However in l.c.18 it is reported on 2—6 protons per 1 electron transfer. This result have been quoted by Karlish and Avron against a fixed coupling between electron transfer and proton translocation.

As will be shown in l.c.13 the discrepancy in the ratio of Δ$H^\circ$/Δ$e^\circ$ cited above is caused through the change of the rate of the electron transfer in the beginning of the illumination before the steady state is reached. This has not been regarded in l.c.18.

To avoid these difficulties, in the following the Δ$H^\circ$/Δ$e^\circ$ ratio is measured in the elementary act. In the elementary act which can be realized by excitation of the photochemical active pigments with flashes shorter than $\leq 6 \cdot 10^{-4}$ s, see l.c., each light reaction acts only on one electron. Therefore, only one electron is transported across each of the possible coupling sites between electron transfer and proton translocation. Measurements of rates can be completely avoided and therefore also the complications mentioned above for steady illumination.

Materials and Methods

The experiments were carried out on suspensions of spinach chloroplasts prepared by a method proposed by Winget et al.19. The repetitive flash photometry was used as measuring technique. Details of the apparatus and of the methods have been described elsewhere 20, 21.

The number of protons taken up by the thylakoids on flash-light excitation was calculated from the pH change in the chloroplasts suspension. The pH-change was measured photometrically by pH indicators as brom-thymol-blue BTB 22. The improvement of the signal to noise ratio by repetitive measuring techniques resulted in a resolution of pH-jumps of less than $10^{-4}$ units (at pH 6.8) with a time resolution of $2 \cdot 10^{-4}$ sec. The time resolution of the whole apparatus corresponds to $7 \cdot 10^{-5}$ sec.

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**Fig. 1.** Top: Change in the absorption of the pH indicator bromthymol blue (BTB) at 605 nm as a function of the pH. Bottom: Change of the proton concentration in dependence of the pH in a chloroplast suspension of spinach. Chlorophyll concentration: $10^{-5}$ M/l, activity of O$_2$ production 7.2/(24) mm (O$_2$)/M(Chl)^-s, activity of ATP-production 20.2 mm (ATP)/M(Chl)^-s. Electron acceptor: Benzylviologen 5*10^-5 M/l. Further additions: KCl 3*10^-5 M/l, Saccharose 6*10^-1 M/l, BTB 3*10^-5 M/l.

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605 nm on pH is depicted. To check that the BTB absorption change is a true indication of the pH-changes in chloroplast suspensions it was confirmed that:

a) The electron transport and the phosphorylation activity of chloroplasts were practically not altered (less than 10 per cent) on addition of BTB to a final concentration of $3 \times 10^{-5}$ M/l.
b) The absorption change of BTB at 605 nm was clearly separable from other slow optical effects due to light scattering, intrinsic absorption changes, etc. at this wavelength (see fig. 2).

c) The pH-changes in chloroplast suspensions indicated by the absorption change of BTB in long flashes and steady illumination respectively have been controlled by pH-measurements with a glass electrode*. Sufficient agreement of the steady state values of pH-changes has been found.

The number of electron transfers has been measured by the oxidation of chlorophyll-ai via the absorption change of Chl-ai at 705 nm. The calibration of the change in the extinction coefficient by the oxidation of chlorophyll-ai at 705 nm has been carried out by a comparison with the absorption changes of the electron acceptor 2,6-dichlorophenolindophenol (DPIP) at 605 nm. It has been shown that the rapid phase of the reduction of DPIP takes place at the end of the whole electron transport chain.

Using the change of the extinction coefficient of DPIP at its reduction as determined by Punnett, the change of the extinction coefficient of chlorophyll-ai at its oxidation has been determined as $\Delta E_{705} = 4.2 \times 10^4/\text{M} \cdot \text{cm}$.

This value is about 20 per cent bigger than the value published by Rumberg and Schmidt-Mende which has been determined with $K_2\text{Fe(CN)}_6$ as acceptor that is known to accept electrons at higher concentration also between the two light reactions.

**Results**

1. **The Stoichiometry of Proton Translocation and Electron Transfer in the Elementary Act**

The $\Delta H^0/\Delta e^0 = \Delta H^0/\Delta \text{Chl-ai}$ ratio has been measured for electron acceptors that are known to accept one proton per electron: benzylviologen, thymol-indophenol (TIP), safranine T, indigo carmine. The number of protons taken up by the chloroplasts in the elementary act has been calculated from the absorption change of BTP (fig. 3, top, left side), the number of electrons transported from the absorption changes of chlorophyll-ai (fig. 3, bottom, left side).

The results are depicted in table 1. For all these acceptors about two protons were taken up per one electron transported across chlorophyll-ai. This result is in agreement with those values of Iwawa and Hind and Rumberg et al. which have been extrapolated fort he beginning of steady illumination.

2. **Correlation between the Proton Translocation and the two Light Reactions**

The value of two protons translocated per one electron transported across chlorophyll aI may indicate the existence of two univalent coupling sites,
Table 1. Ration between the number of protons taken up by the chloroplasts (ΔH°) and the number of electrons transferred to a terminal electron acceptor (Δe°) in the elementary act. Experimental conditions as in fig. 3, left. (The measurements were carried out on chloroplast preparations of various activities of O2- and ATP-formation. Concentration of the electron acceptors 5·10^{-5} M/l).

<table>
<thead>
<tr>
<th>Acceptor A</th>
<th>ΔH°/Δe°</th>
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<tbody>
<tr>
<td>Benzylviologen</td>
<td>1,93</td>
</tr>
<tr>
<td></td>
<td>2,23</td>
</tr>
<tr>
<td></td>
<td>1,65</td>
</tr>
<tr>
<td></td>
<td>2,05</td>
</tr>
<tr>
<td></td>
<td>2,08</td>
</tr>
<tr>
<td>TIP</td>
<td>2,32</td>
</tr>
<tr>
<td></td>
<td>2,06</td>
</tr>
<tr>
<td></td>
<td>2,09</td>
</tr>
<tr>
<td></td>
<td>2,04</td>
</tr>
<tr>
<td>Safranine T</td>
<td>1,65</td>
</tr>
<tr>
<td></td>
<td>2,01</td>
</tr>
<tr>
<td>Indigocarmine</td>
<td>2,19</td>
</tr>
<tr>
<td></td>
<td>1,85</td>
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</tbody>
</table>

A discrimination between the two alternative possibilities can be achieved by the measurement of the ΔH°/Δe° ratio when light reaction I has been chemically separated by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) with TIP. DCMU desactivates light reaction II. Under these conditions light reaction I is full active as can be seen from the absorption change of chlorophyll-a1 in fig. 3 (bottom, right side). Fig. 3 (top, right side) demonstrates that the proton uptake is decreased by a factor of 2 on addition of DCMU. From the ΔH°/Δe° ratio of 1 under these conditions it can be seen that the light reaction I mediated by TIP translocates one proton only. Very probably this means that when light reactions I + II are in action the second proton is translocated by light reaction II. Therefore, in the elementary act each of the two light reactions translocates one proton across the thylakoid membrane.

3. Correlation between the Field Formation and the two Light Reactions.

Evidence has been provided that the characteristic absorption changes at 478, 515, and 648 nm (chlorophyll-b) on spinach chloroplasts are a molecular indicator for an onset of an electrical field across the thylakoid membrane.

This field is set on in illumination in less than 2·10^{-8} sec and decays by ion fluxes in the range of 10–100 msec, depending on temperature, pH and the state of the membrane. The translocation of two protonic charges across the thylakoid membrane and the onset of an electrical field may be coupled with each other. In this case it has to be expected that both light reactions participate in equal parts in charging the membrane capacity, as they do so in proton translocation. Indeed, a corresponding result to that of fig. 3 has been obtained comparing the absorption change of chlorophyll-b at 515 nm in fig. 4 in three cases a) when both light reactions were active, b) when only light reaction I was active, and c) when only light reaction II was active.

Light reaction I has been separated from the overall reaction by addition of TIP and DCMU. Light reaction II has been separated from the overall reaction by addition of ferricyanide (Fecy) and DPIP.

light reactions I + II (TIP+DCMU)  
light reaction I (TIP+DCMU)  
light reaction II (DCPIP+FeCy)

Fig. 4. Time course of the absorption change of chlorophyll-b at 515 nm. Left: Light reaction I and II (TIP). Center: Light reaction I only (TIP+DCMU). Right: Light reaction II only (DCPIP+FeCy).

From fig. 4 it can be seen that the absorption changes at 515 nm with light reaction I only, or light reaction II only, are by a factor 2 smaller than those with light reaction I + II. This has been proved for each of the three characteristic changes of Chl-b at 478, 515, and 648 nm. This demonstrates clearly that in the elementary act each of the two light reactions set on one half of the field.

That the absorption changes at 515 nm can be excited by each of both light reactions is a well-known phenomenon. It has been demonstrated in different ways by WITT et al. 28, RUMBERG 29, and WITT et al. 30.

WITT et al. 28 compared the absorption changes of chlorophyll-b together with those of cytochrome-f oxidation by excitation with \( h\nu_1 \)-light (\( \lambda = 720 \text{ nm} \)) and \( h\nu_1 + h\nu_1 \)-light (\( \lambda < 700 \text{ nm} \)) respectively. In \( h\nu_1 \)-light the changes of chlorophyll-b are strongly decreased compared with the changes in \( h\nu_1 + h\nu_1 \). The absorption changes of cytochrome-f are, however, the same in both lights. From this effect (and the characteristic kinetics) it was derived that photosynthesis is run by two light reactions coupled in series see l.c. 28. RUMBERG 29 demonstrated that the contribution of each light reaction to the changes of chlorophyll-b is one half, using also \( h\nu_1 \)- and \( h\nu_1 + h\nu_1 \)-light respectively. WITT et al. 30 came to the same conclusion by a chemical separation of light reaction II from the overall reaction with addition of ferricyanide + DPIP.

An new feature in the result of fig. 4 is that the absorption changes at 515 nm show the same decay rate, no matter which of the two light reactions is active or both together. In fig. 4 the half time of the decay has been accelerated by addition of gramicidin D to the ad lib. value of 0.7 msec. In table 2 the half times or the decay are compared at different concentrations of gramicidin.

<table>
<thead>
<tr>
<th>Gramicidin D M/l</th>
<th>( \tau_{1/2} \text{(515 nm)} ) [msec]</th>
<th>( \tau_{1/2} \text{(515 nm)} ) [msec]</th>
<th>( \tau_{1/2} \text{(515 nm)} ) [msec]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.2</td>
<td>11.5</td>
<td>12.5</td>
</tr>
<tr>
<td>( 10^{-10} )</td>
<td>5.3</td>
<td>4.8</td>
<td>4.9</td>
</tr>
<tr>
<td>( 3 \cdot 10^{-10} )</td>
<td>1.9</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>( 10^{-9} )</td>
<td>0.6</td>
<td>0.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 2. Rate of the decay of the absorption change of chlorophyll-b at 515 nm in dependence of the concentration of gramicidin D if the light reactions I + II (TIP) are active, if light reaction I (TIP+DCMU) is active solely and light reaction II (DPPIP+FeCy) is active solely respectively. Experimental conditions as in fig. 4.

Thus, it can be concluded from these experiments that the two absorption changes at 515 nm, one excitable by light reaction I and the other by light reaction II, reflect the same physical event (an electrical field across the thylakoid membrane).

4. Action Spectra of the Field Formation

The identity of the light reactions which set on the electrical field with those that promote the electron transport could be checked by measurements of the action spectra. Fig. 5 shows the action spectra for the field changes (absorption change at 515 nm) if light reaction I was separated and when, on the other hand, light reaction II was separated. Light reaction I has been separated from the overall reaction as in fig. 4 by TIP and DCMU. Light reaction II has been separated from the overall reaction as in fig. 4 by addition of ferricyanide and DPIP. The action spectra for the field changes (absorption

Fig. 5. Normalized action spectra of the absorption change of chlorophyll-b at 515 nm with light reaction I (TIP + DCMU) and light reaction II (DPIP + FecY) respectively are separated. Chlorophyll-concentration 10⁻⁴ M/l, activity of O₂-production 6/(35.8) mM(O₂)/M(Chl) -s, activity of ATP-production 34.6 mM(ATP)/M(Chl) -s. Tricine-NaOH-buffer: pH 4; 5-10⁻³ mM(O₂)/M(Chl) -s, activity of O₂-production 34,6 mM/l. Electron acceptor: O₂ TIP, resp. • DPIP each 10⁻⁴ M/l. Further additions: KC1 10⁻² mM/l. Electron concentration 10⁻⁴ M/l. Reaction volume 1 ml. Path length through the cuvette 1.4 mm. Excitation: sharp cut off bandfilters DAL (Schott and Gen.), halfwidth 18 nm, duration 15 µs, frequency 2 cps. Measuring beam: wavelength 515 nm, grating monochromator, optical bandwidth Δλ = 2 nm, intensity <400 erg/cm²s. T = 22 °C. Electrical bandwidth Δν = 4,5 kcps. Repetitive flash photometry, 500—1000 flashes were fired.

change at 515nm) with light reaction II in action has been published already by Müller et al. in l. c. 31, that with light reaction I in action by Fork et al. in l. c. 32. The two action spectra for the field changes in fig. 5 correspond to the well-known action spectra for the electron transfer with light reaction I in action and light reaction II in action respectively (see e. c. fig. 14 in l. c. 1).

5. The Field Strength

From the result that two protons are translocated across the membrane per one turn-over of both light reactions in one electron chain, the value of the difference of the electrical potential \( V_0 \) across the membrane can be calculated by

\[
V_0 = \frac{2 \cdot e \cdot l}{\epsilon_a \cdot \epsilon_0 \cdot A}
\]

The none aqueous insulating layer in the thylakoid membrane, the lipid layer, has a thickness of about \( l = 30 \) Å see l. c. 33. One electron chain covers an area of \( A = 300 \) Å x 300 Å 33. The effective dielectric constant of a phospholipid membrane is \( \epsilon = 2 \) 34. With these figure it results for the voltage across the thylakoid membrane

\[
V_0 \approx 50 \text{ mV.}
\]

This corresponds to a field strength of about \( 1,7 \cdot 10^9 \text{ V/cm.} \)

Because the absorption change of the chlorophyll-b can increase from short flashes to longer, maximal by a factor 4 see l. c. 35, and because the relationship between the absorption changes of chl-b is linear, see l. c. 12, the maximal voltage across the thylakoid membrane can reach a value up to

\[
V_{\text{max}} \approx 200 \text{ mV.}
\]

From the magnitude of the changes of chl-b in chloroplasts in permanent light 37 it follows a steady state value of \( V \approx 100 \text{ mV.} \)

Discussion

It has been shown that each of the two light reactions translocates one proton across the thylakoid membrane if one electron is transferred from H₂O to a terminal acceptor A (NADP⁺). Thus, the \( \Delta H^+ / \Delta e^0 \) ratio equals 2 for the elementary act. Moreover, it has been shown that each of the two light reaction sets on one half of the electrical field strength across the thylakoid membrane.

The participation of both light reactions in the electron transfer, in the field onset and in the proton translocation makes it probable that these four processes are directly coupled with each other.

Two possibilities can be discussed for the latter two.

a) Primary the field is set on and than this field causes the proton translocation. Or

b) primary the proton translocation takes place which is automatically accompanied by an onset of the field.

For a discussion the following facts have to be considered. The electrical field is set on in \( \leq 2 \cdot 10^{-8} \) sec 1.14. The proton uptake is probably much slower.

Fig. 3 reflects a pH-rise time of about 70 msec for stripped chloroplasts. A rise time of 8 msec is indicated if the measurements are carried out on broken chloroplasts isolated in a density gradient. This is shown in fig. 6. The difference in the response time of the pH indicator BTB in fig. 3 and fig. 6 may be due to a shielding of BTB molecules from the thylakoid membranes at which the primary proton uptake takes place. It is possible that even this value on broken chloroplasts is not the true one but delayed by unknown effects. So it can be concluded that the proton uptake takes place in 8 msec or faster, but probably much slower than $2 \cdot 10^{-8}$ sec. So possibly first the field is set on and than this field causes the $H^+$-uptake and -translocation. This can in principle be realized by one of the different possibilities discussed by Mitchell in his hypothesis $^7$.

![Graph showing pH-rise time](image)

The field may be set on by a rapid transfer of one electron from a donor molecule at the inner side of a membrane to a carrier molecule at the outer side. The reduced carrier molecule may take up a proton from the outer phase, and diffuse as a neutral molecule to the inner side of the membrane. Here it may be reoxidized by an electron acceptor molecule releasing a proton into the inner phase.

According to the results reported above and regarding all the facts which are known on the electron transfer system in photosynthesis — see for instance l. c. $^1$-$^5$ —, the coupling between the two light reactions, electron transfer, field formation, and proton translocation can be formulated in more detail in the following way (see fig. 7).

![Diagram of electron transfer and proton translocation](image)

Each of the two photoactive chlorophylls $a_1$ and $a_1$ absorbs in a short flash ($\ll 6 \cdot 10^{-4}$ sec) one quantum $h\nu_1$ and $h\nu_1$ respect. This absorption is followed by one electron transfer from the chlorophyll to an acceptor. This transfer is assumed to occur at each chlorophyll-a reaction diagonal through the thylakoid membrane from the inner to the outer surface. This can occur in $\leq 2 \cdot 10^{-8}$ sec. The component of this transfer which is perpendicular to the membrane causes the formation of an electrical potential $V_0$. At the end of this process at each light reaction one negative charge $\square$ is located at the outer surface of the membrane and one positive charge $\square$ at the inner surface. In the following the names of the charged molecules known from l. c. $^1$-$^5$ are put in brackets.

The negative charge $\square$ ($X^-$) of light reaction II reduces plastoquinone PQ within $\leq 6 \cdot 10^{-4}$ sec. This is accompanied by an uptake of one $H^+$ from the outer phase.

$$1/2 \text{PQ} + \square \rightarrow 1/2 \text{PQH}_2 + \square - H^+_{\text{extern}}.$$  

The neutral molecules $1/2 \text{PQH}_2$ (symbolized in fig. 7 with PQH) translocate a hydrogen diagonal from the outer surface of the membrane to the positive charge $\square$ (Chl-$a_1^+$) of the light reaction I at the inner surface which oxidizes (via cytochrome f) $1/2 \text{PQH}_2$. This reaction is accompanied by a release of one $H^+$ into the inner phase. This takes place in $2 \cdot 10^{-2}$ sec.

$$1/2 \text{PQH}_2 + \square \rightarrow 1/2 \text{PO} + \square + H^+_{\text{intern}}.$$  

The positive charge $\square$ (Chl-$a_1^+$) 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.

$$1/2 H_2O + \square \rightarrow 1/4 O_2 + \square + H^+_{\text{intern}}.$$  

![Diagram of electron transfer, field formation, plastoquinone reaction, and proton translocation](image)
The negative charge □ (Chl-\(a_1^\ominus\)) of light reaction I at the outer phase of the membrane reduces (via intermediates) the acceptor A (NADP\(\ominus\)) which is accompanied with a H\(\ominus\) uptake from the outer phase.

\[
\frac{1}{2} A + □ \rightarrow \frac{1}{2} AH_2 + □ - H_\text{extern}^\ominus.
\]

This can be summarized as follows:

a) An electrical field is set on across the thylakoid membrane in \(\leq 2 \cdot 10^{-8}\) sec.

b) Two protons (without counter-ions) are translocated from the outer into the inner phase of the thylakoid within \(\leq 8 \cdot 10^{-3}\) sec.

c) In toto one electron is transferred from H\(_2\)O to A (NADP\(\ominus\)). This takes place in \(2 \cdot 10^{-2}\) sec which is the time of the rate-limiting step which is the oxidation of 1/2 PQH\(_2\).

This concept is in accordance with all the results reported above. A consequence of fig. 7 is regarding for instance only the events at light reaction II — that with a transfer of one electron — realized in a flash shorter than \(\leq 6 \cdot 10^{-4}\) s — the set on of one half of the electrical field and one H\(\ominus\)-translocation should be accompanied with the formation of one half PQH\(_2\). This has been shown in l.c.\(^{36,25}\). This 1:1:1 corresponds between the on-set of one half of the field, one H\(\ominus\)-translocation and one half PQH\(_2\) formation mediated by light reaction II has been proved in a wide range in the following way.

There exists not only 1 PQ molecule between the two light reactions as electron carrier but a pool of about 3 – 5 PQ molecules with an electron capacity of \(6 – 10^{36,25}\). According to fig. 7 simultaneously with the PQ-pool reduction through \(n\) electrons, the uptake of \(n\) H\(\ominus\) and a \(n\)-fold increase of the field (absorption change of e.g. 515 nm) should occur. A corresponding strict coincidence of all these three components has indeed been shown up to \(n = 6\). This is demonstrated in the following paper\(^{12}\).

In fig. 7 additionally the results on the proton efflux out of the thylakoid are indicated. The efflux is realized by the intrinsic efflux \(k_0\), the extra efflux \(k_{ATP}\) during phosphorylation and by the accelerated efflux \(k_U\) through an increase of the H\(\ominus\) permeability of the membrane by uncouplers.

A discussion of the whole concept is presented in l.c.\(^{15}\).

The authors wish to thank Miss J. MANN for her excellent technical assistance.
