The Necessity of an Electric Potential Difference
and its Use for Photophosphorylation in Short Flash Groups

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The ATP-synthesis and the electric phenomena on the thylakoid membrane have been studied under excitation of photosynthesis with short flash groups. The light induced electric potential difference has been measured by means of field indicating absorption changes around 523 nm.

It is shown that upon excitation with short flash groups a certain electric potential difference is required before ATP can be synthesized. Moreover it is confirmed that the ATP-synthesis is accompanied by an additional flux of protons down their electrochemical potential gradient. Correlation between the number of protons which have flowed across the ATPase coupled pathway, and the number of ATP-molecules which have been formed, yields a ratio $H^+/ATP = 3$.

Since this ratio includes only those protons, which have interacted with the ATPase complex, it represents the lower limit for H^+/ATP-ratios determined by any other method.

The necessity of a certain electric potential difference and the fact that 3 H^+ have to be translocated down their electrochemical potential difference before one molecule of ATP can be synthesized is consistent with the chemiosmotic postulate, that the ADP $\sim$ P bond energy is derived from the electrochemical potential difference of the proton across the coupling membrane.

With respect to the alternative hypothesis for phosphorylation, the results impose grave restriction on a phosphorylation driven only by the free energy of a chemical intermediate, if this does exist at all.

It is still an open question, in which way the free energy necessary for the ATP-synthesis may be conserved from the electron transport in photosynthesis. The current discussion is centered around two hypotheses. The chemical hypothesis assumes, that the energy is conserved as bond energy of a chemical intermediate [1,2].

$$e^-\text{-transport} \rightleftharpoons (\sim) \rightleftharpoons ATP$$
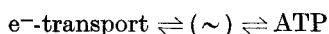
(In this scheme the squiggle indicates a chemical high energy intermediate and the arrows indicate energy fluxes.) The chemiosmotic hypothesis assumes energy conservation in an electrochemical potential difference of the proton across a membrane [3,4].

$$e^-\text{-transport} \rightleftharpoons (\Delta pH, \Delta\psi) \rightleftharpoons ATP$$
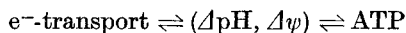
That a pH-gradient [5,6] and an electric field [7] can be induced across the photosynthetic membrane on illumination, has been demonstrated. In the last three years the correlation between the pH-difference and the ATP-synthesis has been extensively studied by several authors. It has been shown, that an artificially induced pH-difference across the photosynthetic membrane can induce ATP synthesis [5,8]. On the other hand it has been demonstrated that the dissipation of a pH difference competes with phos-

phorylation [9—11]. These results are fully consistent with the chemiosmotic hypothesis. But, if the chemical hypothesis is modified, they can also be understood from this modified hypothesis. An appropriate modification has been proposed by several authors [12,13]. It can be summarized in the following scheme:

$$e^-\text{-transport} \rightleftharpoons (\sim) \rightleftharpoons ATP$$
$$\Updownarrow$$
$$(\Delta pH, \Delta\psi)$$
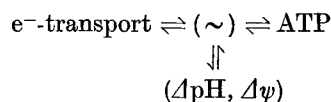
The main difference between this modified chemical hypothesis and the chemiosmotic one lies in the possibility of a "direct squiggle phosphorylation" in the former one, while the latter predicts that phosphorylation occurs only with an intermediate electrochemical potential difference.

In this paper we question whether such a "direct squiggle phosphorylation" does exist, or if this cannot be answered conclusively, we shall try, to derive limiting properties for the as yet unspecified "direct squiggle phosphorylation".

This paper will not be concerned with any squiggle which may operate in series with or cooperate in parallel with and necessarily linked to a chemiosmotic mechanism. Moreover it shall be completely ignored

whether the mechanism of an energy storage which is indicated by the squiggle sign may be based on covalent bonding or on conformational changes.

In order to test if a "direct squiggle phosphorylation" exists, one may proceed along two different lines:

a) One may try to establish extreme experimental conditions where phosphorylation takes place although no electrochemical potential difference can be detected.

b) In those cases where phosphorylation occurs together with electrochemical potential differences, one may study the dynamics of ion fluxes and of phosphorylation, correlate these data and try to find out if there is any ATP synthesis which is not linked to the decay of an electrochemical potential difference.

An important feature in this context is that the chemiosmotic hypothesis predicts a constant stoichiometry between the number of protons which are translocated by an anisotropic ATPase and the number of ATP molecules synthesized. Although the proponents of the squiggle hypothesis have not specified their postulates as to this question, as yet, it is evident that the finding of a constant stoichiometry would reduce the probability for a "direct squiggle phosphorylation", while the finding of a variable one would give evidence for it.

Experiments of the first type have been carried out on subchloroplast particles [14]. Under appropriate conditions it has been shown that these particles synthesize ATP in the light without establishing a measurable pH difference. Unfortunately, no information on the magnitude of the electric potential difference has been obtained from these experiments. Thus one may argue that a high electric potential difference compensates for the low pH difference and therefore these experiments cannot be regarded as conclusive as to the possibility of a "direct squiggle phosphorylation".

In this paper the problem in question will be attacked in the second way. The relationship between the ATP synthesis and the decay of the electrochemical potential difference will be studied. These studies are an extension of those which have been reported previously [15,7].

The light induced electric potential difference across the thylakoid membrane can be measured by means of special absorption changes of chloroplast bulk pigments [7,16]; for a summary see [17]. These absorption changes have been interpreted from the fact that an absorption band undergoes a shift, if a dye is exposed to a strong electric field (electrochroism). Further evidence for this method has arisen from the finding that the dye rhodamin B reveals electrochromic absorption changes when incorporated into the thylakoid membrane [18]. The

spectrum of this response resembles the electrochromic difference spectrum measured in vitro [19].

By means of field indicating absorption changes several properties of the light induced electric potential difference have been evaluated previously [7,16, 21,26], for a summary see [20]. Details which are important in the context of this paper are the following.

On excitation with a short flash of light ($\leqslant 6 \times 10^{-4}$ sec) each of the light reactions, I and II, translocates one proton across the thylakoid membrane [21]. This is accompanied by the onset of an electric potential difference of about 50 mV [21]. If intact thylakoids are excited with longer flashes ($\leqslant 20$ msec) the number of protons taken up and the electric potential difference can increase about four-fold without losing their proportionality [22].

Under appropriate non-phosphorylating conditions (see Materials and Methods), the electric potential difference decreases rather slowly due to the low basic conductivity of the thylakoid membrane [7]. However, the decay is accelerated if phosphorylation occurs [15]. From indirect arguments it has been concluded that this acceleration is due to a proton conductivity of the membrane [7]. This finding fits well into Mitchell's concept of an ATPase which translocates protons down their electrochemical potential gradient [3,4]. To test if there is additionally any "direct squiggle phosphorylation", the relation between the ATP synthesis and the decay of the electrochemical potential difference should be studied quantitatively.

MATERIALS AND METHODS

Freshly isolated chloroplasts from marked spinach were used; the method for isolation has been described by Winget et al. [23]. Additionally 10 mM ascorbate was added during grinding. The ATP-productivity in continuous saturating light was about 50 mmoles $\times$ moles chlorophyll$^{-1} \times$ sec^{-1}.

It has to be pointed out, that the results obtained in our experiments refer to chloroplasts with a very low basic conductivity of the thylakoid membrane. This low conductivity results in decay half-times of the electric potential difference of some 100 msec. To get a good preparation, the avoidance of strong preillumination and of aging of more than 2 hours (at 0°) are necessary.

The complete reaction mixture contained in a volume of 1 ml: chloroplasts containing 0.15 μmole chlorophyll/ml, 20 mM tricine pH 8, 50 μM benzylviologen, 10 mM KCl, 3 mM $MgCl_2$, 5 mM K_2HPO_4, 0.5 mM ADP, 0.5 mM ATP, temperature: 21°. The optical path length was 1.3 mm.

The ATP production has been determined with ^{32}P following a procedure given by Avron [24].

The light induced electric potential difference across the photosynthetic membrane has been measured via the electrochromic absorption changes at 523 nm. In the range of our experiments this absorption change is a linear indicator of the electric potential difference (for a summary of this method see [17]). The signal to noise ratio in the absorption measurements has been increased by averaging repetitive signals [25]. Optical band width of the measuring light: $\Delta\lambda = 5$ nm, intensity: 45 erg $\times$ cm^{-2} $\times$ sec^{-1}. Electrical band-width: 4.5 msec^{-1}.

Photosynthesis has been excited by saturating short flashes, which were given in groups of at most four in a time interval of less than 15 msec (flash duration: $t_{1/2} = 15$ μsec, wavelength: 630—730 nm). The repetition rate of these flash groups was 0.1 sec^{-1}, the repetition number was about 50.

When the absorption change at 523 nm and the phosphorylation have been compared with each other, the measurements have been carried out on the same probe. The probe has been illuminated by short flash groups and the absorption change has been registered. Immediately after the end of excitation photosynthesis has been stopped by addition of trichloroacetic acid and the ATP-production has been determined.

The ATP-production has been related to the number of active electron transport chains. The number of active electron transport chains has been determined from the maximum oxygen yield per flash on excitation with periodic short flashes ($t_{1/2} = 20$ μsec). The measurements have been carried out under conditions, where the transients occurring at the onset of periodic flashlight have been eliminated (repetition rate 2 sec^{-1}, repetition number 150).

RESULTS

The time course of the absorption change at 523 nm which is induced by a group of four short flashes is depicted in the first figure. From the above cited studies it is known that the absorption change is proportional to the electric potential difference. Moreover, its amplitude is proportional to the number of protons taken up by the thylakoids.

In the left half of Fig. 1 this absorption change has been measured under non-phosphorylating conditions. The decay of the electric potential difference is rather slow ($t_{1/2}$ approx. 350 msec). In contrast to this, the decay is markedly (about 5-fold) accelerated if ADP and inorganic phosphate are added (Fig. 1 B).

That the decay is accelerated if phosphorylation occurs, is a known fact [15]. The striking feature in Fig. 1 is that the acceleration obviously "stops" at a certain value of the electric potential difference. This value comes close to the potential difference which is induced in a single short flash of light ($\Delta I/I = 3 \times 10^{-3}$).

This phenomenon has not been observed in the experiments reported previously. This has obviously been due to both unfavourable chloroplast preparations and unfavourable experimental conditions giving rise to a fast decay even in the absence of phosphorylation. In order to get a basic decay as slow as possible here we have used only chloroplast preparations favourable in this respect and in addition we employed a very weak monitoring light and took care to avoid any other strong preillumination.

The absorption change at 523 nm represents the average electric potential difference over all the thylakoids in the cuvette. Thus the biphasic decay may be interpreted in two different ways: either it
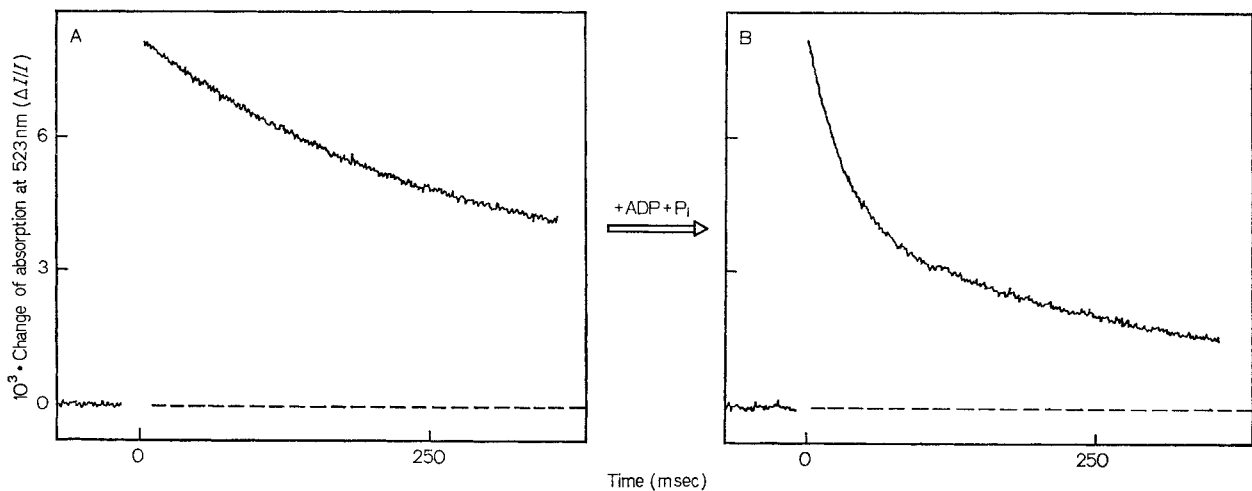

Fig. 1. *Time course of the field indicating absorption changes at 523 nm on excitation with a short flash group.* (A) without ADP and P$_i$; (B) on Addition of ADP and P$_i$. A biphasic decay of the electric potential difference is observed under phosphorylating conditions
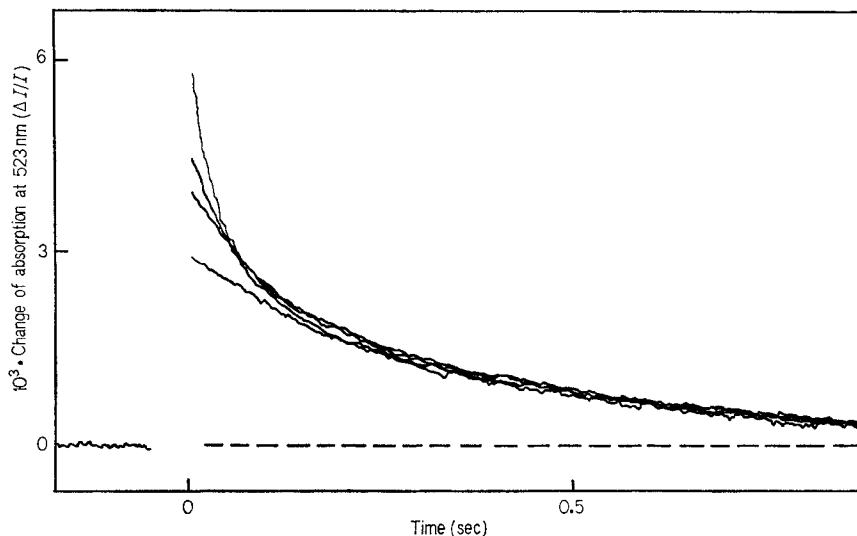
Fig. 2. *Time course of the absorption change at 523 nm on addition of ADP and P_i.* The four curves correspond to four different energies of the exciting short flash group. The fast decay phase of the electric potential decay is observed above a certain "critical value", only
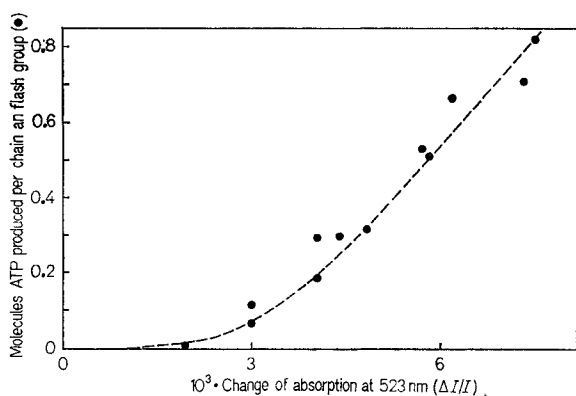

Fig. 3. *The dependence of the ATP production in short flash groups on the initial value of the absorption change at 523 nm.* There is practically no ATP synthesized above the "critical electric potential difference"

represents the biphasic decay of the electric potential difference on each thylakoid, or the rapid phase is due to those thylakoids which are phosphorylating and the slow phase to those which are not.

These alternatives can easily be discriminated between if the initial value of the electric potential difference is varied. This has been achieved by varying the energy of the exciting flash group. The time course of the electric potential difference starting with four different initial values is depicted in Fig. 2. In all these curves the acceleration stops at the same value of the electric potential difference. This gives support to the first interpretation, and it seems obvious that the "critical potential difference",

where the transition from the rapid to the slow decay occurs, has a physical meaning.

One may argue that the transition in the decay curve signals that phosphorylation stops. To test this, the dependence of the ATP yield on the initial value of the electric potential difference has been measured. The result is indicated by the points in Fig. 3. Indeed, below the "critical potential difference", which here corresponds to about $\Delta I/I = 3 \times 10^{-3}$, practically no ATP is synthesized. Above this value there is an approximately linear relationship between the amount of ATP synthesized and the excess amplitude of the electric potential over its "critical value".

This demonstrates again, that the ATP synthesis is accompanied by an accelerated decay of the electric potential difference. Probably this accelerated decay indicates, that the electric potential difference is necessary for phosphorylation. A probe for this can be based on the following consideration. If the electric potential difference is necessary for phosphorylation, then phosphorylation should be inhibited when the electric energy is dissipated by an artificially induced permeability of the membrane.

This has been tested making use of the antibiotic valinomycin which is known to increase the membrane conductivity selectively for K^+, Cs^+ and Rb^+-ions [27]. The acceleration of the electric potential decay induced by low concentrations of valinomycin is depicted in Fig. 4. The acceleration is obvious if one looks at the slow decay phase below the "critical potential difference". The corresponding dependence of the phosphorylation activity on the valinomycin concentration is depicted by squares in
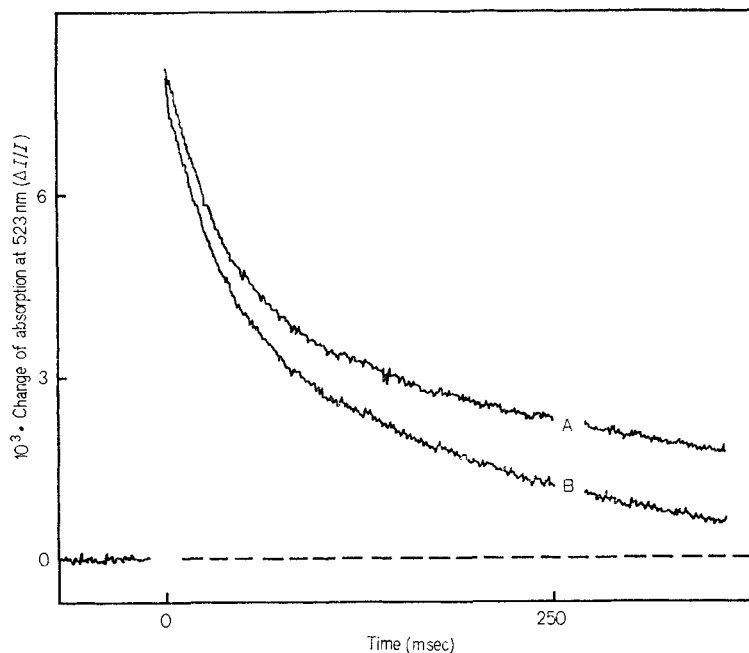
Fig.4. *The acceleration of the decay of the absorption change at 523 nm on addition of valinomycin in the presence of ADP and*
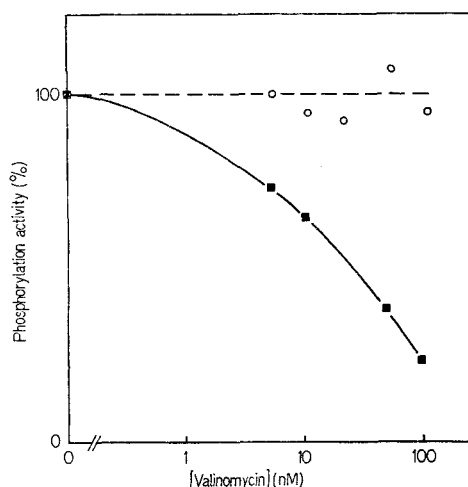P_i. (A) no valinomycin; (B) 10 mM valinomycin

Fig.5. *The dependence of the relative ATP production on the
concentration of valinomycin.* ■, *excitation with a short flash
group;* O, *excitation with saturating continuous light*

Fig.5. It is obvious that the phosphorylation is inhibited if the electric potential difference is dissipated *via* valinomycin mediated potassium currents. Thus, it follows that a certain electric potential difference is necessary for phosphorylation in short flash groups.

To avoid confusion in comparing Fig.5 with the results of other authors [28,29] it has to be pointed out, that the experiments of these authors were carried out under continuous illumination. Under these conditions and in the same concentration range we find practically no influence of valinomycin on the phosphorylation rate either (Fig.5, open circles). In contrast to this, the experiments indicated by the squares in Fig.5 have been carried out in short flash excitation, where the ratio of the electric field and pH-gradient is balanced towards the electric field. This will be discussed elsewhere.

DISCUSSION

The experimental results presented above will be discussed in two steps. At first, a conclusion will be drawn which does not involve any assumption and then the discussion will proceed to more detailed questions, where the use of assumptions is inevitable at the moment.

A conclusion which can be drawn from the experiments directly is the necessity of a certain electric potential difference for phosphorylation in short flash groups. This follows from the valinomycin-sensitivity of the ATP-synthesis in short flash groups and from the existence of a "critical electric potential difference" below which no phosphorylation takes place.

The necessity of an electric potential difference for photophosphorylation is fully consistent with the chemiosmotic hypothesis. However, it does not

provide a sufficient test for it. Thus one may come back to the quantitative question, which was put forward in the introduction: in which way is the decay of the electrochemical energy, stored on the thylakoid membrane, quantitatively linked to the production of ATP. This general question will here be specified in the following form: what is the relation between the number of protons, which have flowed across the ATPase coupled pathway, and the number of ATP molecules synthesized?

The ATPase coupled proton current can be measured from the time course of the electric potential decay under the following assumptions:

a) The thylakoid membrane is basically permeable to ions. The basic conductivity alone gives rise to a first order decay of the electric potential difference [7].

b) If phosphorylation occurs, there is an additional (ATPase coupled) proton conductivity of the membrane (see [7,15]).

c) The decay of the electric potential difference under phosphorylating conditions results from the currents which pass across these two conductivities.

d) A decay, which corresponds to the value of the electric potential difference which is set on in a single short flash $(\Delta I/I = 3\times10^{-3})$, is due to the backflux of two elementary charges per electron transport chain across the membrane [21].

These four assumptions are based on earlier results which have been summarized in the introduction.

From measured decay curves similar to Fig.2, the ATPase coupled proton current has been separated from the basic current. This separation has been carried out based on the following relations. The decay rate of the electric potential difference $\left(\dfrac{\mathrm{d}}{\mathrm{d}t}\,\Delta\psi\right)$ is due to the ATPase coupled proton current $(J^*_{\mathrm{H^+}})$ and the basic current (J_{B}):

$$\frac{\mathrm{d}}{\mathrm{d}t}\,\Delta\psi = \alpha\times(J^*_{\mathrm{H^+}} + J_{\mathrm{B}}).$$

(The proportionality constant α will be specified in the following paper.)

This part of the decay rate, which is due to the basic current, is proportional to the electric potential difference, with a time constant k:

$$\alpha\times J_{\mathrm{B}} = -\,k\Delta\psi.$$

Thus the proton current can be separated:

$$J^*_{\mathrm{H^+}} = \alpha^{-1}\left(\frac{\mathrm{d}}{\mathrm{d}t}\,\Delta\psi - k\Delta\psi\right).$$

The value of the proportionality constant follows from the fourth assumption. Integration of the separated proton current over the time yields the number of protons which have flowed across the ATPase coupled pathway during phosphorylation.

Table. *Proton translocation and ATP production on excitation with short flash groups of different energy*
$\Delta I/\Delta I^{(1)}$ = Initial value of the absorption change at 523 nm on excitation with a short flash group, normalized to the value on excitation with a single short flash of light. $(\Delta\mathrm{H^+})^*/\Delta\mathrm{e^{-(1)}}$ = Number of protons which have flowed across the ATPase coupled pathway after excitation with a short flash group in relation to the number of electrons transported in a single short flash; $\Delta\mathrm{ATP}/\Delta\mathrm{e^{-(1)}}$ = number of ATP molecules synthesized on excitation with a short flash group in relation to the number of electrons transported in a single short flash; $\mathrm{Chl}/\Delta\mathrm{e^{-(1)}}$ = number of chlorophyll molecules per electron transported on excitation with a single short saturating flash $(t^{1/_2} \ll 10^{-4}\,\mathrm{s})$

$\Delta I/\Delta I^{(1)}$	$(\Delta\mathrm{H^+})^*/\Delta\mathrm{e^{-(1)}}$	$\Delta\mathrm{ATP}/\Delta\mathrm{e^{-(1)}}$	$(\Delta\mathrm{H^+})^*/\Delta\mathrm{ATP}$	$\mathrm{Chl}/\Delta\mathrm{e^{-(1)}}$
2.5	3.0	0.8	3.7	750
2.4	2.3	0.7	3.2	750
2.1	2.1	0.7	3.1	750
1.6	1.1	0.3	3.6	750
1.4	0.7	0.3	2.7	750
1.0	0.3	0.1	2.7	750

$$<(\Delta\mathrm{H^+})^*/\Delta\mathrm{ATP}> = 3.2$$

The ratios between the number of protons per electron transport chain and the number of ATP molecules are listed in the Table.

All these values, which have been calculated from the same experiments as those which are summarized in Fig.3, are centered around a value of three. In spite of the rather wide errors it is obvious that the $\mathrm{H^+}$/ATP-ratio is almost independent from the initial value of the electric potential difference. The same has been confirmed in a series of similar experiments.

Thus it can be concluded that under excitation with short flash groups the synthesis of one ATP molecule is coupled to the translocation of three $\mathrm{H^+}$-ions down their electrochemical potential gradient.

The constancy of the $\mathrm{H^+}$/ATP-ratio under variation of the initial value of the electric potential difference is meaningfull insofar as the ATPase coupled proton conductivity reveals a rather strong and peculiar dependence on the magnitude of the electric potential difference. The most important feature in this context is the existence of a "critical potential difference" below which practically no ATPase-coupled proton current can be observed (see Fig.2). The constant $\mathrm{H^+}$/ATP-ratio under variation of the electric potential difference thus yields restrictions for the properties of any direct squiggle phosphorylation, if such a direct phosphorylation does exist at all. The constant $\mathrm{H^+}$/ATP-ratio can be understood in terms of the modified chemical hypothesis only under two circumstances, *i.e.* either the squiggle phosphorylation rate reveals the same peculiar dependence on the electric potential difference as the ATPase-coupled proton flux, or the rate

of the squiggle phosphorylation is small compared with the rate of a chemiosmotic phosphorylation. These restrictions hold in the limits of the error in the results of the Table.

Thus it follows from our experiments that if a direct squiggle phosphorylation exists, it either behaves quite peculiarly or it is negligiable for excitation with short flash groups.

This H^+/ATP-ratio of about 3 is indeed very interesting since it includes only those protons which have interacted with the ATPase, while all the other protons, which have flowed across dissipative pathways are excluded. As a consequence, one has to expect that a determination of the H^+/ATP-ratio by a method which takes all the protons into account, would yield a value always greater than 3. This prediction of a lower limit of 3 for the overall H^+/ATP-ratio can be compared with the results of other authors.

Schwartz reported on an H^+/ATP-ratio of 2 [30]. His calculation has been based on the assumption that the ATPase coupled proton flux can be read out directly from pH changes in the outer medium. However, the idea has been put forward that the ATPase coupled proton flux alone is not detectable by pH changes in the outer medium because it is not counterbalanced by other ions [31]. As long as the interpretation of such measurements is subject to discussion, a calculation of H^+/ATP-ratios from measured e^-/ATP-ratios seems more valuable. This can be done taking into account the experimentally confirmed ratio of 2 protons translocated per electron transported [32,21,33]. Determinations of the lower limit of the overall e^-/ATP-ratio have revealed results of 1.55 [23], 2.0 [34] and 1.25 [35], corresponding to a lower limit of the overall H^+/ATP-ratio of 3.1, 4.0, and 2.5. Although these values come rather close to 3, no definitive conclusions can be drawn whilst uniform results are not obtained.

The "critical electric potential difference" has been regarded as an empirical fact confirming the necessity of an electric potential difference for phosphorylation. However, a mechanistic interpretation of this phenomenon has not been tried in this paper. A quantitative treatment of this subject is the aim of the following paper [36].

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