MEMBRANE-BOUND ATP SYNTHESIS GENERATED BY AN EXTERNAL ELECTRICAL FIELD*

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1. Introduction

In the primary act of photosynthesis an electric potential difference, $\Delta\varphi$, is generated across the energy coupling membrane by a light-induced vectorial electron transfer [1,2]. In a consecutive step protolytic reactions with the charges at the outer and the inner membrane surface lead to the formation of a pH gradient, ΔpH [3]. Through measurements of the relaxation of $\Delta\varphi$ simultaneously with the formation of ATP quantitative relationships were obtained between both events in respect to the extent, rate and functional unit [4]. This indicates that phosphorylation is coupled with the discharging of the electrically energized membrane. A coupling of ATP formation with the relaxation of ΔpH was first demonstrated by Jagendorf and Uribe [5].

Regarding the cooperation of $\Delta\varphi$ *and* ΔpH quantitative relations were elaborated in respect to the kinetics of ATP synthesis [6]. In respect to the energetics there is accumulating evidence that the free energy, ΔG, stored in $\Delta\varphi \approx 100$ mV [7] and $\Delta pH \approx 3$ [8,9] is with $H^+/ATP \approx 2.5$ [6,10] in agreement with data of ΔG [11] necessary for ATP synthesis. These and other results support the electrochemical hypothesis of Mitchell [12]. Under natural conditions electron transfer, field generation and ΔpH formation are always coupled with each other. Therefore, with respect to the mechanism of photophosphorylation it is of interest if phosphorylation is also possible with an electrochemical potential difference formed artificially and if phosphorylation can be induced under these conditions by ΔpH only or by $\Delta\varphi$ only. It was demonstrated by Jagendorf [5] that an artificial proton gradient ($\Delta pH \gtrsim 3$) set up across the functional membrane is sufficient for ATP formation. ATP formation by an artificial electrical potential difference set up with a K^+-gradient across the membrane gave reasonable yields only when this gradient was superimposed on a preexisting pH gradient of $\Delta pH \gtrsim 1$–3 [13,14]. At $\Delta pH = 0$ the generated amount of ATP is extremely small [15] (see Discussion).

The purpose of the present work is to prove if phosphorylation can be induced by an *external* electrical field. In respect to the role of $\Delta\varphi$ in phosphorylation this method offers additionally the following advantages compared with conditions where the potential is induced by light or K^+-gradients:

(a) The external field generates a field at the membranes of the suspended vesicles by polarisation. Therefore, a formation of a pH gradient across the membrane does not take place.

(b) The induced field can be 'switched' on and off in times which depend on the membrane capacity and conductivity of the solution, i.e., in times short in comparison with the duration of the pulse (30 ms).

(c) The magnitude and duration of the induced voltage across the membrane can be varied in a wide range. This voltage can be kept constant during the pulse.

(d) The sensitivity of the ATP measurement can be improved by repetitive excitation.

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Abrreviations: BV, benzyl viologen; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TBT, chlorotri-*n*-butyltin.

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2. Materials and methods

Isolated spinach chloroplasts were suspended in a cuvette between two platinum electrodes with a distance of 2 mm and an area of 5 cm^2. Voltage pulses of $\Delta\varphi_{ex}$ = 220 V were applied to the electrodes which correspond to an external electrical field strength of $F_{ex} = 1.1 \cdot 10^3$ V/cm (fig.1).

It was necessary to avoid that the external electrical pulse deactivates the sensitive enzyme system of the chloroplasts. Deactivation is possible (a) by heating of the suspension through the input of electric energy and (b) by the reactions of the chloroplasts with radicals produced at the electrodes. To prevent these effects the whole system was cooled (5°C). Furthermore, the duration of each applied voltage pulse was restricted to 30 ms. The polarity of the voltage was changed after each pulse. The ion concentration used for phosphorylation was limited to such a level that in 30 ms the current (~ 3.5 A in 1 ml solution) produced a heating of no more than 6°C per pulse. The time between the pulses was so long (15 s) that the heating was reversed. The reduced ion concentration decreases the optimal light-induced phosphorylation only slightly. To prevent chemical deactivations, the electrodes were adjusted to each other in such a distance (2 mm) that the reactive zone at the surface of the electrodes was small in comparison with the total layer of the chloroplasts. This was proved by measuring the light-induced phosphorylation after the suspension was treated with voltage pulses at varying electrode distances d.

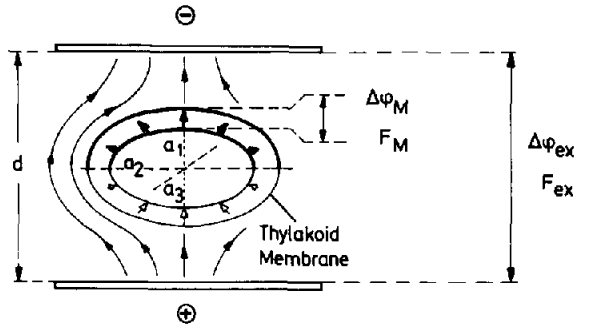

Fig.1. Scheme of a vesicle (thylakoid assembly) in an external electrical field F_{ex} and electrical potential difference $\Delta\varphi_{ex}$, respectively. The distance of the electrodes is d. F_M and $\Delta\varphi_M$ are the electrical field and potential difference respectively which are set up across the thylakoid membrane by the external voltage (details see text).

During the voltage pulse besides other ions H^+- and OH^--ions are discharged at the electrodes. A depletion of these ions might change the pH value at the electrodes. This could lead to a rise of a pH gradient across the membranes of those thylakoids which are located in the interface between the electrodes and the solution.

To exclude this possibility we used additionally a more complicated cuvette. The platinum electrodes have a distance of 22 mm. The layer of the chloroplasts located in the middle of the cuvette has again a thickness of 2.0 mm. The distance between the electrodes and the chloroplasts (10 mm) was bridged by a 1 M KCl-solution. The KCl-solution and the chloroplasts were separated by a membrane filter. In this way on one hand the voltage is practically applied in full across the 2.0 mm chloroplast layer. On the other hand, the distance of 10 mm excludes any contact between the reactants at the electrodes and the chloroplast.

An external electrical field, F_{ex}, induces at suspended vesicles with low-conducting shells a local field, F_M, across the shell which is much larger than the external field, F_{ex}. On the other hand, the local voltage, $\Delta\varphi_M$, across the shell is much smaller than the external voltage, $\Delta\varphi_{ex}$. In respect to chloroplasts Arnold and Azzi [16] and later Ellerson and Sauer [17] showed that with external fields delayed light emission is stimulated.

The value of F_M and $\Delta\varphi_M$ depends on the conductivity in the inner and outer space of the vesicle and in the shell itself. Furthermore, it depends on the size, shape and orientation of the vesicle. F_M and $\Delta\varphi_M$ may be calculated by solving Laplace's equation. A solution for ellipsoids was obtained using the following approximations [18]: (a) The thickness of the shell is small compared to the smallest semi-principal axis of the ellipsoids. (b) The conductivity of the shell is negligible compared to the conductivity of the inner and outer space. The potential difference generated under these conditions by an external static and homogeneous electric field is

$$\Delta\varphi_M = \frac{2 \cdot a_j F_{ex}}{2 - a_1 a_2 a_3 \int_0^\infty d\lambda / (\lambda + a_j^2)\,(\Pi_{n=1}^3 (\lambda + a_n^2))^{1/2}} \quad (1)$$

where a_n (n = 1–3) denote the semi-principal axis and a_j the semi-principal axis parallel to the external field, F_{ex}. λ is an ellipsoidal coordinate.

We approximate the subunits of the broken chloroplasts in the average as ellipsoids consisting of several thylakoids with interconnections between each other (fig.1). This is a reasonable assumption regarding microscopy studies. With $a_1 = 1 \cdot 10^{-5}$ cm, $a_2 = a_3 = 8 \cdot 10^{-5}$ cm and an external field $F_{ex} = 1.1 \cdot 10^3$ V/cm the potential difference, $\Delta\varphi_M$, at the position of their maximum value was calculated. If a_1 is parallel to the external field it results $\Delta\varphi_M = 65$ mV; if a_2 or a_3 are parallel to F_{ex} it follows $\Delta\varphi_M = 100$ mV. Because the thylakoids are nearly randomly distributed during the voltage pulse, the effective potential difference may be between $\Delta\varphi_M = 65$–100 mV. Taking into account that the thickness of the low-conducting part of the thylakoid membrane is $d \approx 40$ Å (bilayer of lipids) the electrical field F_M set up across the membrane has a value of $F_M = 1.6$–$2.5 \cdot 10^5$ V/cm.

We have shown elsewhere [20] that the light-induced field which drives phosphorylation points from the inner membrane surface (positive) to the outer surface (negative). With an external field this can be realized for one half thylakoid (upper one in fig.1) because at the other half the field is directed in the opposite direction in respect to the center. At the 'right' half the induced maximal voltage is localized only in the area of the poles because the voltage declines to zero at the equator (arrows in fig.1). Consequently an external voltage can simulate the light-induced one in an area which is only a part of the total thylakoid.

Chloroplasts were prepared as described elsewhere [21] from spinach grown either in a phytocell or obtained from the local market. Additionally 10 mM ascorbate was added during grinding. The chloroplasts were stored with suspension medium (0.4 M sucrose; $5 \cdot 10^{-3}$ M tricine adjusted to pH 8 with NaOH; $5 \cdot 10^{-4}$ M $MgCl_2$) in an ice bath and used within 2 h after preparation. The reaction medium contained 10^{-2} M tricine adjusted to pH 7 or pH 8 with NaOH; $5 \cdot 10^{-4}$ M $MgCl_2$; $5 \cdot 10^{-4}$ M K_2HPO_4; 1 μCi ^{32}P per ml; 10^{-4} M KCl; $5 \cdot 10^{-2}$ M sucrose; $3 \cdot 10^{-4}$ M ADP and chloroplasts giving an average chlorophyll concentration of $4 \cdot 10^{-4}$ M.

The measurements were performed in the dark at about 5°C. Phosphorylation was measured by the ^{32}P-method [22]. The voltage pulse generator provides square wave pulses with the following specifications: (a) output voltage: 0 to 220 V; (b) variable pulse polarity; (c) current: 0 to 12 A; (d) rise and fall times: ~ 1 μs; (e) pulse duration: 1–30 ms; (f) pulse frequency: up to 10 Hz and single pulses.

3. Results and discussion

Fig.2 shows the amount of ATP per chlorophyll generated by external voltage pulses of $\Delta\varphi_{ex} = 220$ V with a duration of 30 ms each. The yield increases linearly with increasing number of pulses. This indicates (a) that the ATP generating system starts in full with the first pulse and (b) that at least up to ten pulses the system is not deactivated. The amount of ATP generated by ten pulses is $3.8 \cdot 10^{-3}$ Mol ATP/Mol Chl. This is the average value of 20 different measurements. (The deviation by different preparations is ± 30%.) If we regard that the voltage in the 'right' direction is applied only to one half of each vesicle (see above) the yield is $7.6 \cdot 10^{-3}$ Mol ATP/Mol Chl. In respect to the duration of the applied field this corresponds to a

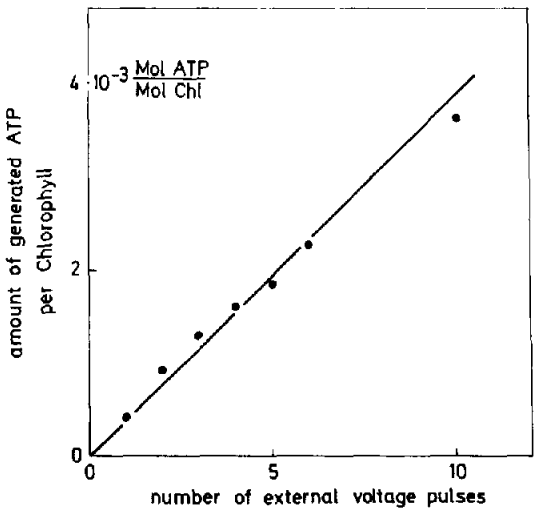

Fig.2. Amount of ATP per chlorophyll in dependence of the number of external voltage pulses. Pulse duration: 30 ms (details see text).

rate of $2.5 \cdot 10^{-2}$ (Mol ATP)/(Mol Chl·s). Considering that on the 'right' half of the vesicles the voltage declines to zero at the equator (see fig.1), the yield and rate in respect to the effective area is even higher. The yield obtained with an imposed diffusion potential (see Introduction) which maintains probably one second across the membrane is at $\Delta pH = 0$ about $6 \cdot 10^{-4}$ Mol ATP/Mol Chl [15].

If we assume that there exists one ATPase per 860 chlorophyll molecules [19] at least 6.5 ATP molecules were synthesized per ATPase by the external electrical pulses. This means that the yield is produced by several turnovers of the ATPase.

In order to prove that the measured ATP is not the result of artefacts the following experiments were carried out (see table 1). (1) In the presence of substances which inhibit the reaction of the ATPase as Dio-9, Phlorizine and TBT practically no ATP formation is observed when the electrically induced voltage is set up across the membrane. (2) In the presence of DCMU which inhibits the light-induced linear electron flow and thereby also the electrical potential and phosphorylation, ATP is generated in full when the artificially induced voltage is applied. (3) The omission of the electron acceptor BV (benzyl viologen) which also inhibits the light-induced linear electron flow and phosphorylation has also no influence on the electrically induced phosphorylation. (4) The synthesis of ATP is drastically reduced (< 10%) by the omission of ADP or P_i. The remaining ATP is probably due to endogeneous reactants. (5) At constant voltage external the ATP yield is independent of the magnitude of the external current through the cuvette (varied by the salt concentration). (6) Also the slight increase of the temperature (6°C) of the suspension during the voltage pulse has no influence on the yield. (7) Any contribution by processes at the electrodes were excluded by controlling the results with the refined cuvette consisting of separated electrode compartments (see Materials and methods).

Table 1
Amount of ATP generated by applying ten external voltage pulses of 30 ms each at varying additions (details see text)

Additions	Amount of ATP
Control	$3.8 \cdot 10^{-3}$ Mol ATP / Mol Chl
+ DCMU ($3 \cdot 10^{-5}$ M)	85% of control
± BV (10^{-4} M)	100% of control
+ Phlorizin (10^{-2} M)	0% of control
+ Dio 9 (80 μg/ml)	20% of control
+ TBT ($5 \cdot 10^{-6}$ M)	0% of control

To compare the electrical induced ATP yield with light-induced phosphorylation the chloroplasts were excited with saturating light pulses of the same duration (30 ms). The light pulses were produced by flash groups (15 flashes of 20 μs duration per group). The darktime between the groups was 20 s. The amount of generated ATP with ten flash groups was $14 \cdot 10^{-3}$ Mol ATP/Mol Chl, i.e., the electrically induced ATP yield is about the same as the light-induced one. The saturating light pulse induces an intrinsic electric potential difference of $\Delta\varphi_M \approx 175$ mV measured by the field indicating absorption changes at 515 nm [1] and a pH gradient of $\Delta pH \approx 1.0$ measured by the amino-acridine method [23]. This means that in toto an electrochemical potential of maximal 235 mV may be built up in the light pulse. Because the ATP yields are similar, it is supposed that the artificially induced voltage at the membrane might be of similar magnitude. This value is 2.6 times higher than the maximal value calculated with eq. (1) assuming subunits of the size as outlined above. The difference may be due to an over-simplification of the chosen size of the subunits, and due to the neglection of their heterogeneity.

In comparison with phosphorylation in continuous light, the electrical induced rate of about $2.5 \cdot 10^{-2}$ Mol ATP/(Mol Chl · s) (see above) is nearly as high as the rates which are obtained in saturating continuous light at pH 8 ($4 \cdot 10^{-2}$ Mol ATP/(Mol Chl · s)). The here described results show that an artificial electrical potential difference alone is sufficient to generate phosphorylation with yields comparable with those induced by light under natural conditions. Under natural conditions the electrical potential is always accompanied by a pH gradient. (In one single turnover flash the gradient may be relatively small but a gradient does exist [24].) Therefore, assumptions that besides the field a small pH gradient or accumulation of protons are a necessary pre-requisite for phosphorylation could not be excluded. However, the here demonstrated 'phosphorylation with a

battery' shows that ATP synthesis is possible with an electrical potential difference only. This can be concluded because the here induced transmembrane field is based only on polarisation which is not accompanied by pH gradient (see Introduction). The protons driven by this field may be 'taken' not from the inner aqueous phase but from the membrane near the inner surface. The result with the external field also shows that phosphorylation is independent of the origin of the transmembrane electrical potential difference.

It should be pointed out that the action of the electric field on the ATPase and the proton current through the ATPase, of course, subsequently may induce individual reactions within the ATPase between H^+, P, H_2O, the nucleotides, etc., which additionally may be coupled with conformational changes [25–27] before ATP is synthesized and released into the solution.

Further results on the basis of the here described method will be published in a forthcoming paper.

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