ELECTROCHROMIC ABSORPTION CHANGES OF A CHLOROPLAST SUSPENSION INDUCED BY AN EXTERNAL ELECTRIC FIELD

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1. Introduction

By applying external electric field pulses in the dark to a chloroplast suspension a stimulation of delayed fluorescence [1,2], ATP-synthesis [3] and conformational changes of the membrane-bound ATPase [4] can be induced. These results have been explained by the action of the electric potential difference across the photosynthetic membrane ($\Delta\phi$) induced by an external electric field, E_{ext}.

The external electric field method offers considerable advantages for a detailed investigation of ATP-synthesis. Certain information on the properties of the ATPase can be obtained only if phosphorylation is induced by an artificial transmembrane electric field instead of light (see [4] and section 4). Understanding of the special features of the external electric field method must, however, provide the basis for any such proposed investigations. The purpose of this work is, therefore, to analyse the transmembrane potential difference induced by an external electric field. This has been realized by measuring directly the electrochromic absorption changes (ΔA) of the membrane pigments which indicate the induced membrane potential. Such measurements can provide information on:

(i) An averaged value of the magnitude of the induced membrane potential;
(ii) The functional dependence of the membrane potential on the external electric field strength;
(iii) The time of energization of the membrane, i.e., on the kinetics of the rise and decay of the membrane potential;
(iv) The influence of ionophores, osmolarity, viscosity, pH and ionic strength on the amplitude as well as on the kinetics of the induced membrane potential.

Results obtained with respect to phosphorylation through the external electric field method, especially on the basis of the results of this work will be published in future (in preparation).

This work has been presented in part at the Biophysiktagung, Ulm, October 1–4, 1978

2. Materials and methods

2.1. *Chloroplast preparation*

Broken chloroplasts were prepared as in [5] from spinach grown either in a phytocell or obtained from the local market. After centrifugation the chloroplasts were resuspended in a medium containing 5×10^{-4} M $MgCl_2$ and 2×10^{-3} M tricine adjusted to pH 8 by NaOH. The chloroplasts were stored in an ice bath for ~30 min, then recentrifuged ($7000 \times g$, 0°C, 7 min) and resuspended in the same medium. Under these conditions the thylakoids can be observed by light microscopy to form spherical vesicles (blebs) [6,7]. Most experiments were performed with freshly prepared chloroplasts, but chloroplasts which were stored under liquid nitrogen until use have yielded similar results. The standard reaction medium contained: 5×10^{-4} M $MgCl_2$, 2×10^{-3} M tricine adjusted to pH 8, 2×10^{-4} M KCl and chloroplasts giving 10^{-5}–2×10^{-4} M chl. Deviations from this are given in the figure legends.

The chloroplast medium was suspended in an

electro-optic cell between two flat platinum electrodes which were parallel to the measuring light (distance of the electrodes, 13 mm; optical pathlength, 5 mm; quartz windows, 7 × 20 mm).

The monochromatic light beam (wavelengths band halfwidth ~5 nm) passed through the cell via an entrance slit (2 × 20 mm) at the center of one face of the cell between the electrodes. Transmitted light was collected on the cathode of the photomultiplier by a light guide which was placed directly onto the cell.

Optical changes were recorded by a repetitive pulse spectrometer [8]. In order to improve the signal/noise ratio up to 300 signals were digitized and averaged per measurement on a Nicolet NIC 1072. The rectangular voltage pulses were generated by a High Power Pulse Generator (Velonex V-2432) with the following specifications (a) output voltage 0–2000 V; (b) variable pulse polarity; (c) output current ⩽1.5 A; (d) rise time ~3 μs; (e) fall time ~10 μs; (f) pulse width 300 μs–20 ms; (g) pulse frequency ⩽10 Hz and single pulse. The time course of the external electric field pulse was measured with the same detection system using an attenuator.

The polarity of the voltage was changed after each pulse. The ion concentration of the solution was limited to such a level that the heating/500 μs pulse was <0.1°C.

The external electric field generates an electric potential difference ($\Delta\phi$) across a low-conducting membrane of vesicles suspended in a medium of higher conductivity. The magnitude of $\Delta\phi$ depends on the size, shape and orientation of the particles [9,10]. Under low osmotic pressure the thylakoids form 'blebs' [6,7].

The principle of the external electric field method is shown in fig.1: A $\Delta\phi$ of 200 mV is established over 2 μm if a homogeneous electric field of 1000 V/cm is applied to the solution (fig.1a). If a particle, whose conductivity (λ_i) is small compared to the conductivity of the solution (λ_a) is placed in the solution, the potential distribution around this particle is perturbed and $\Delta\phi$ = 300 mV is set up across a particle of 2 μm diam. This can be explained by the charge accumulation in the aqueous solution (fig.1b).

A vesicle consisting of a thin non-conducting membrane enclosing a droplet of the medium can be taken as a simplified model of the blebs (fig.1c). Under the assumption that the conductivity of the membrane (λ_M) is negligibly small compared to the conductivities of the internal and external media, the potential within the internal medium of the vesicle is constant and with an external field strength of 1000 V/cm a $\Delta\phi$ of 150 mV is set up across the membrane at the two horizontal poles. The arrows show the relative magnitude and the direction of the electric field generated in the membrane. If the membrane thickness is d, then the field in the membrane (E) is given by:

$$E = \frac{\Delta\phi}{d} = \frac{3}{2}\frac{R}{d}E_{ext}\cos\alpha = \frac{\Delta\phi_{max}}{d}\cos\alpha \qquad (1)$$

R, radius of the spherical vesicles; E_{ext}, external elec-

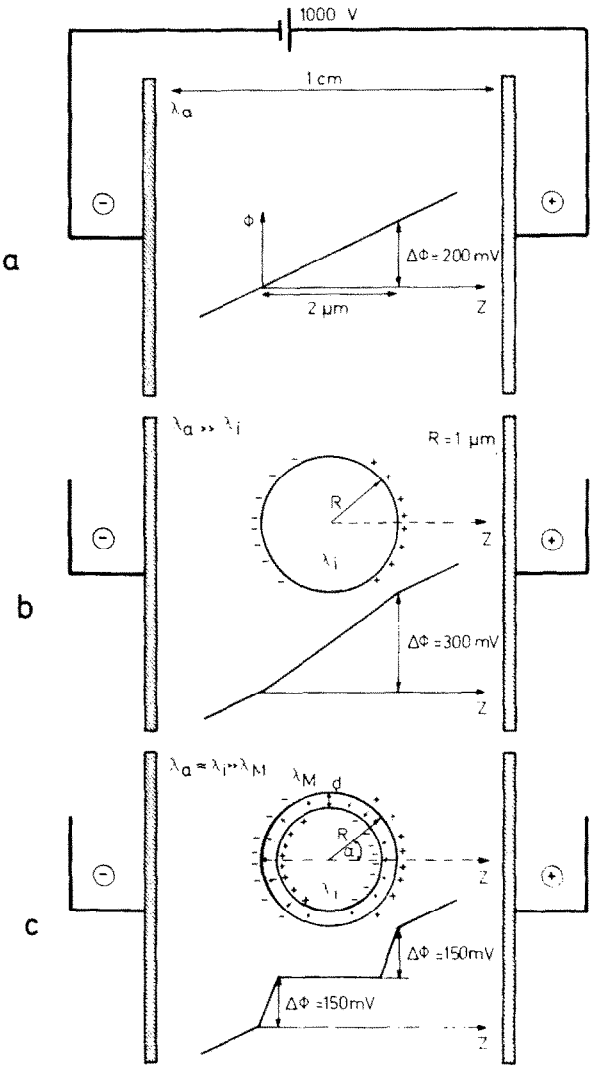

Fig.1. Principle of the external electric field method. See text for details.

tric field strength; α, angle between the direction of the external electric field and the normal to the membrane. The magnitude of the transmembrane electric field is larger than the applied field by the factor: $(3/2)\,(R/d)\cos\alpha$, with $R = 4\ \mu m$, $d = 30$ Å and $\cos\alpha = 1$, the induced electric field in the membrane is ~2000-times greater than the external electric field.

3. Results and discussion

3.1. *Absorption changes induced by external electric field pulses*

Fig.2b shows the time course of the ΔA_{520} generated by an external electric field pulse. Fig.2a shows the time course of the corresponding external electric field pulse. The ΔA_{520} (fig.2b) is a superposition of a fast and a slow component. The fast ΔA arise if the external electric field is switched on or off. The slow change of the transmitted light intensity occurs during the field pulse. The slow component depends only slightly on the wavelength and is observed also at 567 nm where no fast ΔA are detected (fig.2c). The slow changes are presumably caused by light-scattering changes due to orientation effects [11]. In order to separate the fast ΔA the slow component at 567 nm has been subtracted from the overall ΔA_{520} (fig.2d).

Taking into account that the external electric field induces a transmembrane electric field and considering the properties of the fast ΔA (see below) it can be concluded that the separated fast ΔA reflect the electrochromic response of chloroplast bulk pigments. 'Electrochromic ΔA' will be used for the separated fast absorption change.

The electrochromic $\Delta A_{410-550}$ is shown in fig.3. This spectrum was obtained either by difference measurements as outlined in fig.2 or by a linear extrapolation of the slow component to $t = 0$. Fig.4 shows

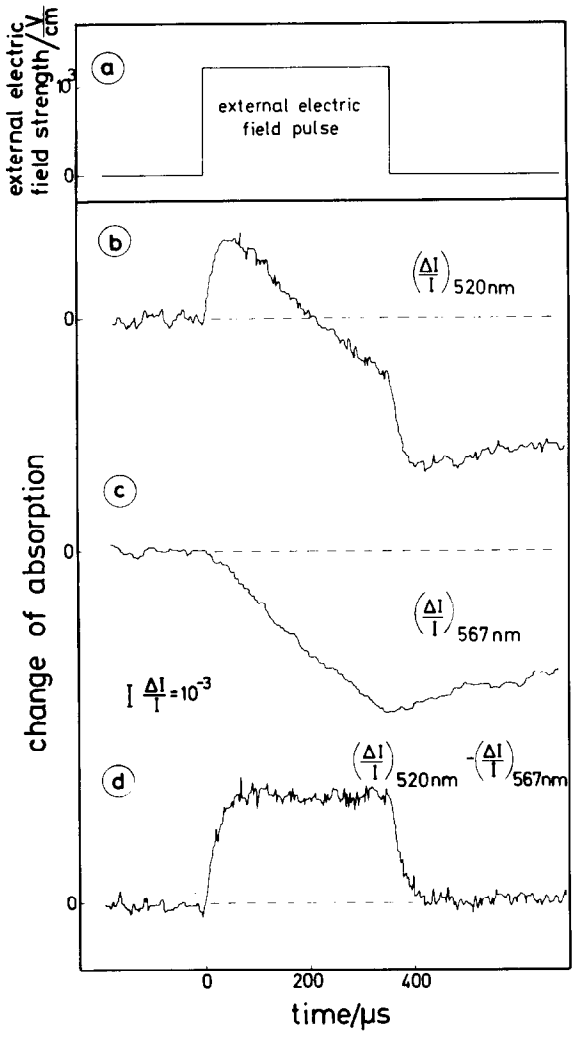

Fig.2. Time course of the ΔA_{520} (b) and ΔA_{567} (c) induced by external electric field pulses (a). The difference between the absorption changes (b–c) reflects the electrochromic ΔA_{520} (d) caused by the induced transmembrane electric field. Chloroplast suspension, 1.2×10^{-4} M chl; external electric field strength, 1200 V/cm; rise time of the external electric field pulse ~3 µs; decay time, ~10 µs.

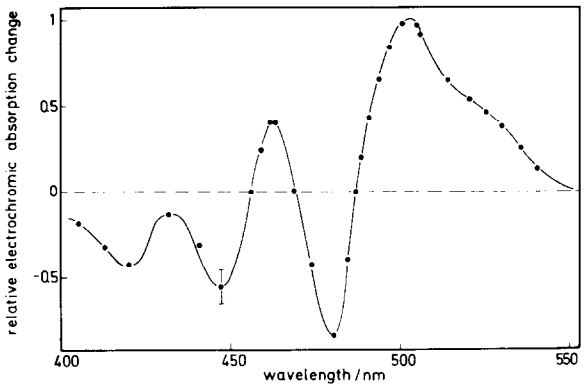

Fig.3. Spectrum of the electrochromic ΔA induced by external electric field pulses. The spectrum is normalized to the amplitude obtained at 504 nm with $(\Delta I/I) = 1.8 \times 10^{-3}$; optical pathlength, 5 mm; chloroplast suspension, 4×10^{-5} M chl; external electric field strength, 1000 V/cm.

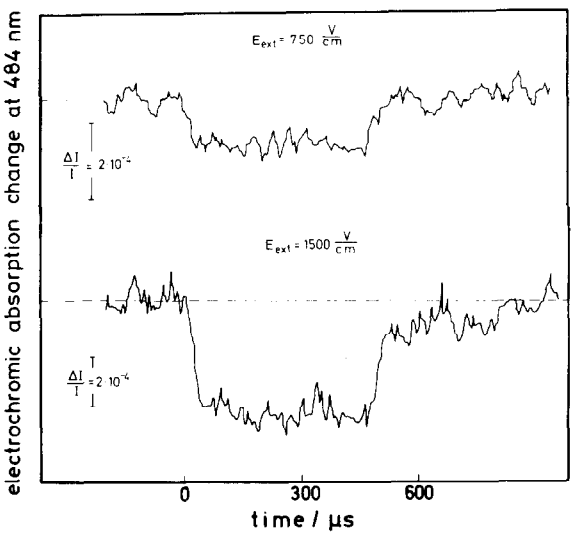

Fig.4. Time course of the electrochromic ΔA_{484} induced by external electric field pulses (determined by the difference between the ΔA_{484} and ΔA_{486}, $[(\Delta I/I)_{484\,nm} - (\Delta I/I)_{486\,nm}]$. External electric field strength 750 V/cm (top) and 1500 V/cm (bottom); chloroplast suspension, 10^{-5} M chl. Upper trace at expanded scale.

the time course of the electrochromic ΔA_{484} measured at two different external electric field strengths. The electrochromic ΔA have been separated by determining difference between ΔA_{484} and ΔA_{486}. At 486 nm fast ΔA do not occur (see fig.3). As the slow component depends slightly on the wavelength we have chosen the reference wavelength as close as possible to the measuring wavelength.

3.2. *Evidence for the electrochromic nature of the fast* ΔA *induced by an external electric field*

An electric field causes a shift of the absorption band of the pigments embedded in the membrane and thereby a change of the molar absorption coefficient ($\Delta\epsilon$) of [12–14]:

$$\Delta\epsilon = \frac{|\vec{\mu}_e - \vec{\mu}_g|}{hc}\cos\theta\, E\left(\frac{\delta\epsilon}{\delta\tilde{\nu}}\right)_E + \frac{1}{2}\frac{\alpha_e - \alpha_g}{hc}E^2\left(\frac{\delta\epsilon}{\delta\tilde{\nu}}\right)_E + \frac{1}{2}\frac{|\vec{\mu}_e - \vec{\mu}_g|^2}{h^2c^2}\cos^2\theta\, E^2\left(\frac{\delta^2\epsilon}{\delta\tilde{\nu}^2}\right)_E \qquad (2)$$

E, electric field strength; $\vec{\Delta\mu} = \vec{\mu}_e - \vec{\mu}_g$, permanent dipole moment difference between the excited and ground states of the dye molecule; θ, angle between $\vec{\Delta\mu}$ and $\vec{E}$; h, Planck's constant; c, velocity of light; $\tilde{\nu}$, wavenumber; $\Delta\alpha = \alpha_e - \alpha_g$, polarizability difference between the excited and ground states. The change of the transition dipole moment due to the electric field can be neglected in a first approximation [13,14].

3.2.1. Dependence of the amplitude of the fast ΔA on the external electric field strength

The amplitude of the electrochromic ΔA induced by saturating light [15] is linearly proportional to the electric field strength across the membrane at all wavelengths [16–20]. This linear dependence and the spectrum of the light-induced electrochromic ΔA [21] is due to the linear electrochromism (term 1 of eq. (2)) of the chlorophylls [22,23], which have a permanent dipole moment difference, and of those carotenoids, which are complexed with chlorophylls [24]. These carotenoids can be regarded as having a permanent dipole moment difference induced by the polarizing force of the chlorophylls. If the pigments have a permanent dipole moment difference with a preferential orientation relative to the field so that the average of $\cos\theta$ is not zero, the quadratic electrochromism (terms 2,3 of eq. (2)) is small compared to the linear effect at electric field strengths $\lesssim 2 \times 10^5$ V/cm corresponding to a saturating single turnover light flash [17]. For a more comprehensive discussion on the electric phenomena in the photosynthetic membrane see [25].

Whereas the light-induced electric field across the membrane is always directed from inside–out (positive inside/negative outside) [26], the transmembrane electric field induced by an external electric field is directed from the inside–out at one half of the vesicles and from the outside–in at the opposite half. The arrows in fig.1 (bottom) show the relative magnitude and direction of the electric field induced across the membrane. Therefore, electrochromic ΔA depending linearly on the electric field strength cancel out and the quadratic part of the electrochromic ΔA only should be observable by this method if the induced transmembrane electric field strength is high enough. Fig.5 shows the relative amplitude of the electrochromic ΔA as a function of the external electric field strength (double logarithmic plot) and thereby also in dependence on the transmembrane electric field strength because both are proportional to each other (see section 2). The slope of the line is 2, i.e., the amplitude depends quadratically on the induced electric field strength (data from fig.4 and corre-

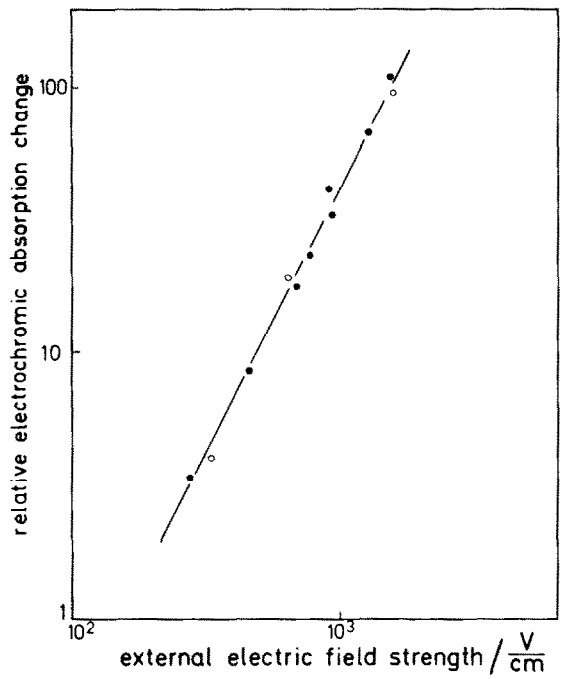

Fig.5. Relative amplitude of the electrochromic ΔA_{484} (○) (determined by the difference between the ΔA_{484} and ΔA_{486}) and ΔA_{520} (●) (determined by the difference between the ΔA_{520} and ΔA_{567} or in another set of experiments by linear extrapolation of the slow component to the time, $t = 0$) as a function of the external electric field strength (double logarithmic plot).

sponding measurements). The measurements have been made at 484 and 520 nm. The same slope at both wavelengths indicates the independence of the relation on the wavelength. The quadratic dependence on the field strength provides one piece of evidence that the fast ΔA in fig.2d and in the following figures are due to electrochromism.

3.2.2. Spectrum of the fast ΔA induced by an external electric field

Since under the conditions used in this work only the quadratic part of the electrochromic ΔA can be observed (see above) the spectrum of the electrochromic ΔA (fig.3) must be different from that of the linear electrochromic ΔA induced by light [21]. In principle the quadratic part of the spectrum should be due to a superposition of 3 effects:

(1) The main contribution should be caused by carotenoids which do not form an asymmetrical complex with a chlorophyll molecule (~70% [24, 27]). The ΔA of these carotenoids having no permanent dipole moment difference but only a polarizability difference must be proportional to the first derivative of their absorption spectrum (term 2 of eq. (2)) with respect to the wavenumber [28].

(2) A smaller contribution should be caused by carotenoids which form an asymmetrical complex with a chlorophyll molecule (~30% [24,27]). The quadratic electrochromism of these carotenoids having a dipole moment difference induced by the chlorophylls and a polarizability difference must be described by a superposition of parts proportional to the first and second derivative of their absorption spectrum (terms 2,3 of eq. (2)) [29].

(3) A third contribution is expected by the quadratic electrochromism of chl *a* and chl *b*. In the blue spectral region the quadratic electrochromism of chl *b* (minimum at 465 nm) coincides with the second derivative of its absorption, indicating that the quadratic electrochromism of chl *b* can be described mainly by a broadening of the blue absorption band (term 3 of eq. (2)) [22]. The quadratic electrochromism of chl *a* (maximum at 450 nm) is caused by a red shift of the blue absorption band (term 2 of eq. (2)) [23,30].

From these considerations and the spectrum shown in fig.3 the following conclusions can be made:

(1) The maxima and minima of the spectrum in fig.3 do not coincide with the maxima and minima of the quadratic electrochromism of chl *a* (maximum at 450 nm) and chl *b* (minimum at 465 nm). Obviously the contribution of the chlorophylls is small compared to the contribution of the carotenoids. This is expected according to a quantitative evaluation of eq. (2) and (3) [11].

(2) The spectrum (fig.3) looks qualitatively like the first derivative of a carotenoid absorption spectrum. According to term 2 of eq. (2) the spectrum can, therefore, be mainly described by the quadratic electrochromism of the unpolarized carotenoids (~70%). The maximum at ~505 nm coincides with the maximal slope ($\delta A/\delta\tilde{\nu}$) of the chloroplast absorption spectrum [31]. The decrease of the chloroplast absorbance above 500 nm is mainly determined by the long wavelength side of the carotenoid absorption band in vivo.

(3) The contribution of the carotenoids polarized by complexation with the chlorophylls is considered

to be small because the relative amount of these carotenoids has been estimated to be only ~30% of all carotenoids [24,27]. The complex formation is coupled with a shift to longer wavelengths (~20 nm) [24]. Therefore, a contribution to the difference spectrum can be expected over 510–550 nm. The shoulder on the long wavelength side in fig.3 may be caused in this way.

A detailed analysis of the spectrum of the quadratic electrochromism induced by an external electric field by comparison with electrochromic spectra of isolated pigments in vitro is in [11].

3.3. *Estimation of the magnitude of the transmembrane electric field induced by an external electric field*

The electrochromic ΔA_{515} induced by a saturating single turnover light flash [15] has about the same amplitude as the quadratic electrochromic ΔA around $\lambda = 504$ nm induced by an external electric field strength of 1000 V/cm. The field strength in the membrane induced by a saturating single turnover light flash is taken to be ${}^1E \sim 2 \times 10^5$ V/cm [17]. The magnitude of the transmembrane electric field induced by the external electric field can be roughly estimated as follows: The ΔA induced by the external electric field around $\lambda = 504$ nm is mainly due to the quadratic electrochromism of the unpolarized carotenoids (see above). In this case the ΔA can be calculated approximately by the equation:

$$\Delta A^{\lambda=504\,\mathrm{nm}} = 2.3\left(\frac{\Delta\epsilon}{\overline{E^2}}\right) \cdot \overline{E^2} \cdot C_{car} \cdot X \cdot 1 \qquad (3)$$

With $(\Delta\epsilon/E^2) = 4 \times 10^{-10}$ cm $\cdot$ $1/MV^2$, which is a mean value for the molar quadratic electrochromism of unpolarized carotenoids [28,30], $X = 0.7$, which is the estimated relative mole fraction of the unpolarized carotenoids [24,27], $C_{car} = 8 \times 10^{-6}$ M calculated from $C_{chl} = 4 \times 10^{-5}$ M and from the molar ratio in chloroplasts car:chl = 1:5 [32] and $l = 0.5$ cm the observed $\Delta A^{504\,\mathrm{nm}}_{quadr} = 1.8 \times 10^{-3}$ (see fig.3), can be explained if the mean square of the transmembrane electric field, $\overline{E^2}$, is assumed to be $\overline{E^2} = 7.3 \times 10^{11}$ V/cm^2. The mean square can be calculated by integrating over the sphere:

$$\overline{E^2} = \left(\frac{\Delta\phi_{max}}{d}\right)^2 \frac{\int_0^{\pi}\int_0^{2\pi} \cos^2\vartheta \sin\vartheta \,\mathrm{d}\vartheta\mathrm{d}\varphi}{\int_0^{\pi}\int_0^{2\pi} \sin\vartheta \,\mathrm{d}\vartheta\mathrm{d}\varphi} = \frac{1}{3}\left(\frac{\Delta\phi_{max}}{d}\right)^2$$

Taking into account that the thickness of the low-conducting part of the thylakoid membrane is $d \sim 30$ Å, the maximal transmembrane potential difference, $\Delta\phi_{max}$, generated by an external electric field of 1000 V/cm is $\Delta\phi_{max} \sim 450$ mV. In comparison with the transmembrane potential difference induced by a saturating single turnover light flash (~50 mV) [17] a roughly 10-times higher transmembrane potential difference can be induced by the external electric field. With eq. (1) from this value of $\Delta\phi_{max}$ an averaged value of the 'bleb radius' can be calculated to $R \sim 3$ μm. Regarding the simplifications of the calculation this value represents a rough estimation only. For the sake of simplicity a size distribution of the blebs has not been taken into account. Assuming a normal distribution (Gaussian) of the bleb radius with mean R_0 and variance σ^2 it can be shown that the mean square of the transmembrane electric potential difference $(\overline{\Delta\phi^2})$ is proportional to $E^2_{ext}\,(R_0^2 + \sigma^2)$ [11].

The maximal transmembrane potential difference which can be induced by an external electric field should be limited to a critical membrane potential difference of ~1.0 V, because the dielectric breakdown of biological membranes occurs at about this value [33].

3.4. *Kinetics of the transmembrane electric field induced by the external electric field*

The rise and decay time of the transmembrane potential difference reflects the charging and discharging of the membrane capacitance. Since the electrochromic response depends on the square of the transmembrane electric field strength, the kinetics of the rise is determined by:

$$\Delta A(t) = \Delta A_{max}\,(1 - e^{-t/\tau})^2 \qquad (4)$$

τ is the time constant for the rise of the transmembrane electric potential difference, i.e., the time constant for the charging of the membrane capacitance. For spheres the time constant τ is given by the relation [34]:

$$\tau = C_M R \frac{\lambda_i + 2\lambda_a}{2\lambda_i\lambda_a} \qquad (5)$$

C_M, capacitance of the membrane; R, radius of the blebs; λ_a, λ_i, specific conductivities of the external and internal medium. From eq. (5) it is expected that the rise time decreases with increasing specific conductivity of the suspending medium. Assuming that the internal conductivity of the blebs is ~3-times lower

than that of the external medium, which was found to be the case in a number of biological materials including swollen mitochondria [35] and red blood cells [36] it follows:

$$\tau = 3.5\, C_M R \frac{1}{\lambda_a} \qquad (6)$$

Fig.6 shows the rise of the electrochromic ΔA_{504} measured at two different specific conductivities of the suspending medium. The signal in the upper part of fig.6 shows the rise of the external electric field pulse. During the first few μs a negative electrically-induced artefact is superimposed on the ΔA (fig.6, center, bottom). Fitting the measured kinetics with the given function (eq. (4)) the time constant (τ) was determined to be 4.8 μs and 11 μs when the specific conductivity of the suspending medium (λ_a) was $4 \times 10^{-4}\ \Omega^{-1} \cdot cm^{-1}$ and $1.6 \times 10^{-4}\ \Omega^{-1} \cdot cm^{-1}$,

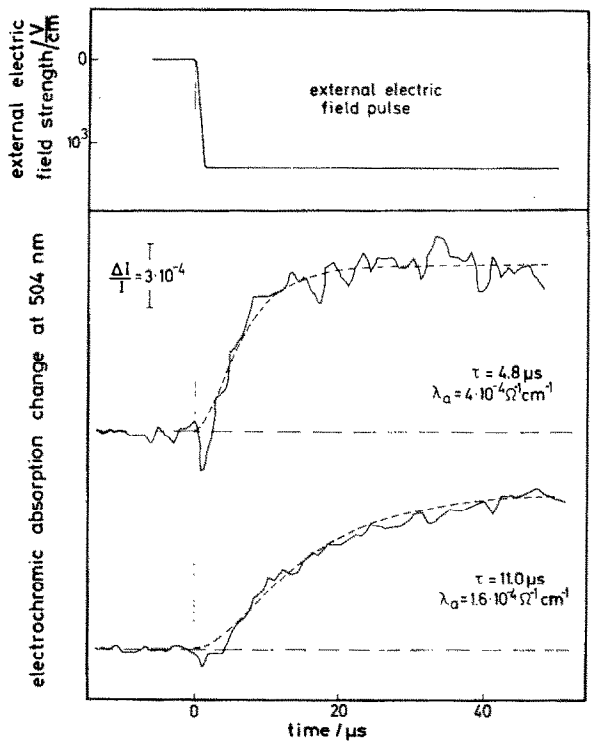

Fig.6. Rise time of the electrochromic ΔA_{504} induced by external electric field pulses (top). Specific conductivity of the suspending medium (adjusted by adding KCl): $\lambda_a = 4 \times 10^{-4}\ \Omega^{-1} \cdot cm^{-1}$ (center) and $\lambda_a = 1.6 \times 10^{-4}\ \Omega^{-1} \cdot cm^{-1}$ (bottom). Chloroplast suspension, 10^{-5} M chl; external electric field strength, 1300 V/cm.

respectively. According to eq. (6) the bleb radius can be calculated with $C_M = 10^{-6}\ F \cdot cm^{-2}$ [35,37] to $R \sim 5.2$ μm. Taking into account a size distribution of the bleb radius, e.g., a normal distribution, the kinetics of the rise can still be described approximately by an exponential function with a time constant (τ) which is somewhat greater than the time constant corresponding to vesicles with the mean radius [11]. Within the frame of the simplifications of the calculations the R-value agrees quite well with the estimation above.

3.5. *Influence of viscosity, osmolarity, pH and ionophores on the transmembrane electric field induced by the external electric field*

Increasing the viscosity of the solution by dextran-500 at constant specific conductivity the electrochromic ΔA do not change. On adding sucrose (2 M) to the solution, the electrochromic ΔA were abolished. This can be understood by assuming that a decrease of the particle size and thereby a decrease of the induced membrane potential is caused by increased osmotic strength. Possibly also a change of the bleb structure is induced by such a high osmolarity.

The transmembrane potential difference induced by an external electric field does not depend on the pH-value of the suspending medium between pH 5.0–9.0.

On adding the ionophore, valinomycin, at amounts up to 1 val/10 chl neither the amplitude of the electrochromic ΔA nor the kinetics are changed. This can be understood because the transmembrane electric potential difference generated by an external electric field depends essentially on the requirement that the conductivities of the internal and external medium are high compared to the conductivity of the membrane. Although valinomycin increases strongly the permeability of the membrane to K^+ [38,39], the specific conductivity of the membrane is still lower than the specific conductivity of the suspending medium. Therefore, only by adding an extremely high amount of valinomycin, the amplitude of the signal is expected to decrease. If the added amount of valinomycin is increased up to 1 val/1 chl, the amplitude of the signal is indeed significantly decreased (see fig.7). Although at such high concentrations additional effects of valinomycin on the thylakoid membrane have been observed [40], this decrease can probably be explained by the fall in electrical resistance of the membrane.

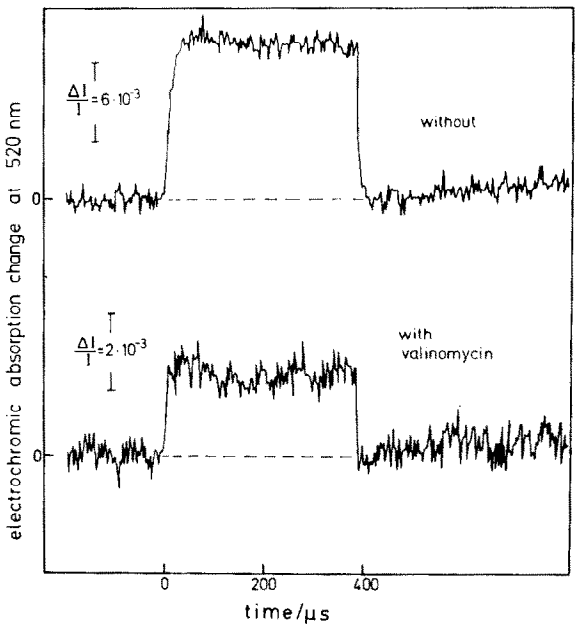

Fig.7. Electrochromic ΔA_{520} (determined by the difference between the ΔA_{520} and ΔA_{507}) induced by external electric field pulses without (top) and with valinomycin (bottom). Chloroplast suspension 2×10^{-4} M chl; external electric field strength, 1400 V/cm. See text for details.

3.6. *Consequences with regard to phosphorylation*

The special features of the external electric field method evaluated in this work offer the following advantages for the investigation of ATP-synthesis compared to conditions where the energization is induced by light:

(1) The functional dependence of ATP-synthesis and conformational changes of the ATPase on the membrane potential can be evaluated directly because the transmembrane potential difference is proportional to the external electric field strength. In the light always a membrane potential plus a pH-gradient is produced. Both components depend in a different way on the light intensity and neither the potential difference nor the pH-gradient depend linearly on the light intensity.

(2) The initial kinetics of ATP-synthesis and of conformational changes in the ATPase can be studied because the energization of the energy-transducing membrane is fast (~10 μs) compared to the turnover time of the ATPase [4,41].

(3) The dependence of ATP-synthesis on the time of energization (adjustable between 100 μs and 50 ms) can be obtained because the time of energization is identical with the duration of the external electric field pulse. The time course of the energization induced by light is not determined by the duration of the light pulse but depends in an uncontrolled way on the ion fluxes.

(4) The dependence of ATP-synthesis on pH ($pH_{out} = pH_{in}$) can be evaluated because the pH-gradient is zero and the membrane potential induced by the external electric field does not depend on the pH between pH 5.0–9.0. The energization by light is always coupled with the generation of a pH-gradient.

(5) The extent of the transmembrane electric potential difference can be increased up to the breakdown voltage.

The results obtained in regard of point (1) have already been published [4,41]. The results concerning the points (2)–(4) have been published in part [42, 43] and will be presented in future publications (in preparation).

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