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ELECTROCHROMIC ABSORBANCE CHANGES IN SPINACH CHLOROPLASTS INDUCED BY AN EXTERNAL ELECTRICAL FIELD

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Summary

Absorbance changes induced by electrical field pulses were studied in osmotically swollen spinach chloroplasts. The results and their interpretation on the basis of the geometry and electrical properties of the material may be summarized as follows:

1. The spherical vesicles, 'blebs', formed upon dilution of a chloroplast suspension consist of only a single membrane, while part of the thylakoid system remains concentrated in a few patches on its surface.

2. When an electrical field pulse is applied, an up to 3000-fold enhanced field is built up in the membrane, with a time constant of about 20 μs. From this the specific capacitance of the bleb wall was found to be 2 $\mu F \cdot cm^{-2}$.

3. The electrical field in the membrane causes several absorbance changes of the photosynthetic pigments with different dependencies on the direction of polarization of the measuring light. Some of these are due to field-induced changes in orientation, in particular of chlorophyll *a*, and have a relaxation time of less than 100 μs. Most of the absorbance changes directly reflect the kinetics of the membrane potential and can be ascribed to electrochromic shifts of photosynthetic pigments, mainly of carotenoids.

4. The carotenoid absorbance changes depend quadratically on the membrane potential; an apparent saturation at high applied field strengths is ascribed to dielectric breakdown at a membrane potential of about 1 V.

5. All carotenoids in the membrane contribute to the absorbance changes induced by an externally applied field, whereas the well-known light-induced electrochromic absorbance change at 518 nm is mainly caused by a minor

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Abbreviation: Tricine, *N*-tris(hydroxymethyl)methylglycine.

fraction of permanently polarized and spectrally red-shifted carotenoids. A computer simulation showed that this interpretation quantitatively explains the results and requires no unreasonable values of the various parameters involved.

Introduction

Light-induced charge transfer across the photosynthetic membrane generates an electrical membrane potential which may couple ATP synthesis to electron transport [1—3]. To distinguish effects of the membrane potential on the photosynthetic apparatus from other light-induced phenomena artifically created membrane potentials can be helpful. A transient membrane potential can be obtained by adding salt to a suspension of chromatophores [4] or chloroplasts [5], if the permeability of the membrane for the anion and the cation is different, e.g. in the presence of a specific ionophore. This technique has yielded important information, but it has some experimental restrictions imposed by the mixing procedure. Much better time resolution can be obtained by applying an external electrical field to the suspension. However, the membrane potential obtained in this way does not mimic the light-induced potential because in half of each membrane vesicles its sign is opposite.

With this technique delayed fluorescence was studied by Arnold and Azzi [6,7] and Ellenson and Sauer [8]. A 60-fold enhancement of the intensity of delayed fluorescence in osmotically swollen chloroplasts ('blebs') by an external field of $6 \cdot 10^2\ V \cdot cm^{-1}$ has been reported [6]. Witt et al. [9,10] showed that an external field of only $1.1 \cdot 10^3\ V \cdot cm^{-1}$ can induce ATP synthesis at a rate comparable to that of the light-induced ATP formation, whereas the light-induced field in the membrane was about $4 \cdot 10^5\ V \cdot cm^{-1}$. These large effects of an externally applied field can be explained by the enhancement of the field in the membrane predicted by electrostatic theory for the geometry and the electrical properties of the vesicles.

The large membrane potential generated by an external field should also lead to electrochromic absorbance changes of the pigments in the membrane. This should give new information about the pigments involved because, as stated above, the distribution of the field and hence the electrochromic changes are different from those induced by illumination. In this article we show that such electrochromic changes indeed occur.

A careful consideration of the geometry of the swollen chloroplasts allowed a quantitative interpretation in terms of the electrochromic theory. The results seem to justify the application of this theory to carotenoid shifts in the photosynthetic membrane and provide a tool to determine quantitatively the membrane potentials generated by external electrical pulses.

Materials and Methods

Spinach leaves, obtained from local shops, were ground in a cooled blender in a 50 mM Tricine buffer (pH 7.8) containing 0.4 M sucrose, 10 mM KCl and 2 mM $MgCl_2$. After filtration through nylon cloth the chloroplasts were sedi-

mented by brief centrifugation at 8000 × *g* and stored on ice. Just before measurement the pellet was diluted about 200 times in distilled water. The final chlorophyll concentration was about 50 μg/ml. Usually the ionic strength was somewhat increased by adding about 0.5 vol% of a 10 mM KCl, 2 mM $MgCl_2$ solution, in order to minimize field-induced scattering changes.

Absorbance changes were measured with a single-beam spectrophotometer with a linear polarizer (Oriel No. 2730) in the measuring beam. The photomultiplier was terminated with a suitable resistance and the signal was amplified (Tektronix 3A9 or 3A7) and stored in a signal averager (Datalab 102S). For better time resolution the kinetics shown in Fig. 7 were measured with a transient recorder (Datalab 905) between the amplifier and the averager.

The perspex cuvette had windows of 22 × 36 mm and an optical pathlength of 0.5 mm. Sandwiched between the windows were 38 parallel platinum wires of 0.5 mm diameter, alternatingly connected to the positive and the negative pole of a home-made power supply. Due to this design, the field in the sample was inhomogeneous. Since the observed phenomenon depends quadratically on the field, the effective field strength was taken to be $\langle F^2 \rangle$. The sample could be pumped through the channels between the wires by a flow system. The power supply gave pulses of up to 90 V between the wires if loaded with a typical cell resistance of 150 Ω. The specific conductivity of the suspension was measured with a 1000 Hz impedance bridge. It took 2 μs (90% completion) to put the pulse on or off or to invert its sign. Usually pulses of 25—100 μs duration were used, spaced at about 10 ms. Every 100 or 200 pulses the sample was renewed automatically in order to prevent a slight decrease in the amplitude of the absorbance changes observed after continued exposure to the electrical pulses. Between successive pulses (or during the pulse, Fig. 7) the sign of the power supply output was inverted to minimize absorbance changes due to movement of the chloroplasts and ions towards the electrodes.

Results

Size and shape of the blebs

When chloroplasts are suspended in a strongly hypotonic medium they form spherical vesicles of about 10 μm diameter, known as 'blebs'. Our blebs, formed by 200-fold dilution in distilled water of an isotonic suspension of spinach chloroplast had radii up to 12 μm, 4.5 μm on the average. From the microscopically determined size distribution (a typical example is given in Fig. 1, dashed line) we calculated an average surface area of 300 μm^2 and an average volume of 550 μm^3. The size distribution was not only dependent on the osmotic strength but, for a short time after isolation, also on the age of the chloroplast suspension used, and we confirmed the observation by Fowler and Kok [11] that large blebs tend to fall apart into vesicles with radii of 1—2 μm. In the phase-contrast image (Fig. 1) at least one, more often several dark patches are seen on the outer surface, which sometimes contain a cluster of very small vesicles. The amount of material in the patches seemed to decrease with the osmotic strength and was not very different when the chloroplasts had first been incubated in magnesium-free buffer to unstack the grana.

The dark patches are pigmented. A strongly heterogeneous pigment distri-

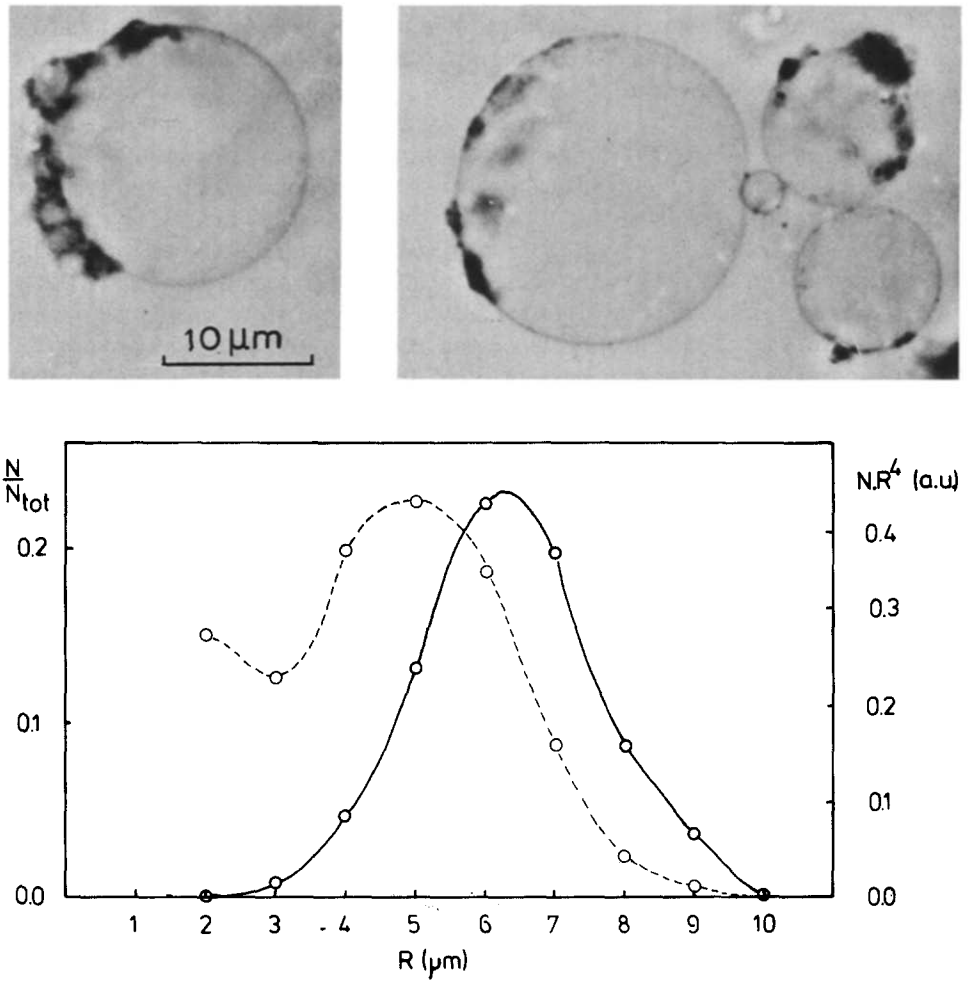

Fig. 1. Phase-contrast photomicrographs of the chloroplasts under the conditions used (blebs) and a typical size distribution (- - - - - -). The total number of vesicles (N_{tot}) was 1006. All vesicles with radii larger than 1.5 µm were counted. ———, the corresponding values of NR^4 indicating the relative contribution to the electrochromic absorbance changes.

bution over the bleb wall is also apparent from the extent to which the absorption spectrum of a bleb suspension is distorted by the particle-flattening effect [12]. The depression of the absorption peaks was only twice less than in a chloroplast suspension (determined as in Ref. 13). If the pigments were homogeneously distributed over the bleb wall the flattening would be equal to that in a suspension of homogeneously coloured spheres with the same radius and the same pigment content [14]. Such spheres would have a nine times lower average absorbance than the chloroplasts since their average radius was about three times larger. The influence of particle flattening at the 435 nm maximum would be about six times smaller than in the chloroplast suspension [12,13]. The factor of two actually observed suggests that about one third of the pigments were spread out over the bleb wall and that the observed flatten-

ing effect is nearly completely caused by the dark patches on the surface of the blebs.

Absorbance changes

Fig. 2 shows absorbance changes at 500 and 680 nm induced by an external electrical field of 120 μs duration in blebs. The electrical field is applied along a specific axis in space and the measured absorbance changes are different for light polarized in the direction of the field or perpendicular to it. The kinetics are clearly multiphasic at the two wavelengths. We tentatively resolved them into a rapid phenomenon with a rise and decay time of about 10 μs and other absorbance changes which proceeded almost linearly during the field pulse. The latter changes decayed biphasically in about 0.5 ms with an initial half-time of 30 μs in the blue-green region and 60 μs in the red region of the spectrum.

The slow changes were in part scattering changes. They had a rather flat spectrum. At 550 nm, where virtually no fast changes were detected, the same kinetics were observed with opposite sign if only scattered light was measured. For this measurement a lens was inserted between cuvette and photomultiplier

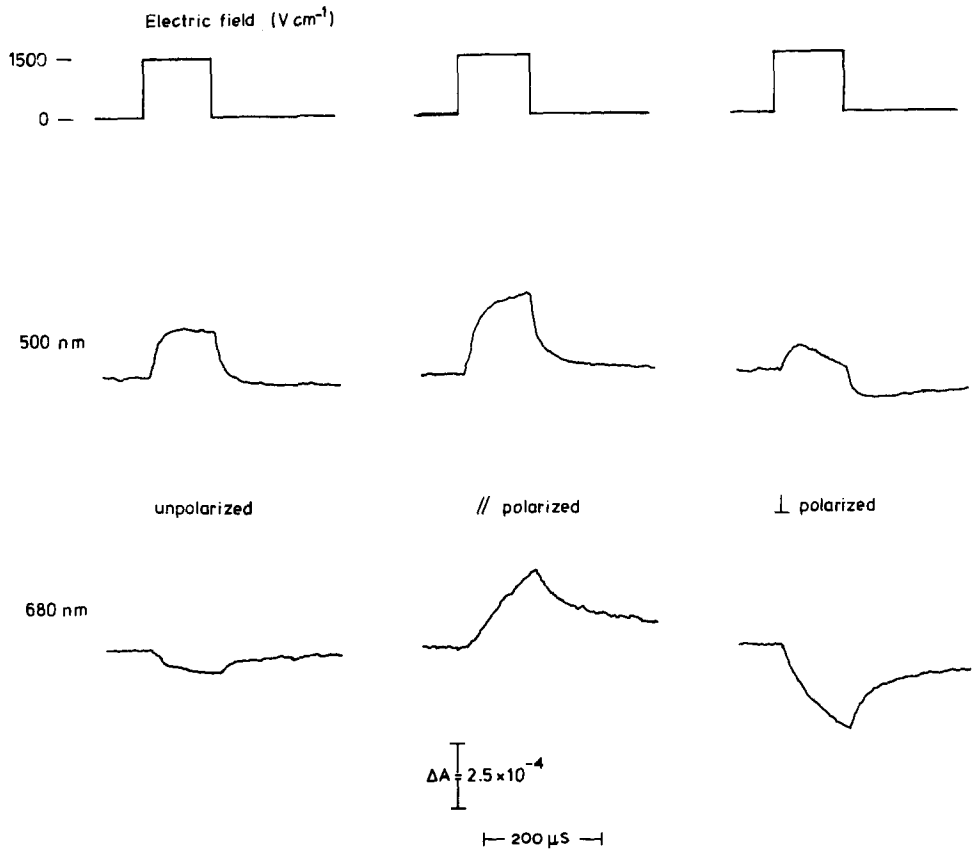

Fig. 2. Kinetics of the absorbance changes induced by external-field pulses (upper traces) at the indicated wavelengths and polarization direction of the measuring beam with respect to the applied field. Each trace represents the average of 1024 measurements.

to give a small image of the monochromator slit on a narrow black strip blocking also the light reflected by the platinum wires. The amplitude of the scattering changes could be reduced drastically by addition of salt. For this reason we usually added about 50 μM KCl and 10 μM $MgCl_2$ to give a final cell resistance of about 150 Ω. The remaining contribution by the slow changes could be corrected for by kinetic analysis or by measurement in the presence of gramicidin, to which only the scattering changes were insensitive.

The spectrum of the fast changes induced by 25-μs pulses is shown for both polarization directions in Fig. 3. The two spectra are not simply superimposable by a multiplication factor so the difference between them cannot completely be accounted for by a certain degree of orientation of the pigments which respond to the field. Probably part of the response measured is due to a field-induced change in orientation of pigments. This is particularly clear for the absorbance changes in the red region with a half-life of 60 μs (Fig. 4). A large band around 680 nm is observed, the sign of which depends on the polarization direction of the measuring beam. Fig. 4 suggests that the electrical field reversibly orients some chlorophylls in such a way that their transition moment turns towards the field direction. These absorbance changes were abolished by gramicidin. Apparently some pigment-protein complexes or individual pigment molecules are sufficiently mobile in the membrane to allow measurable effects with a relaxation time in the microsecond range. For comparison: the orientation of whole chloroplasts relaxes in several seconds [15].

The total absorption in three dimensions does not change by orientation. The two dimensions perpendicular to the field are equivalent. So by plotting $2\,\Delta A_{\perp} + \Delta A_{\parallel}$ orientation effects are eliminated. This procedure was followed in the subsequent figures. Fig. 5 shows the data of Fig. 3 corrected in this way

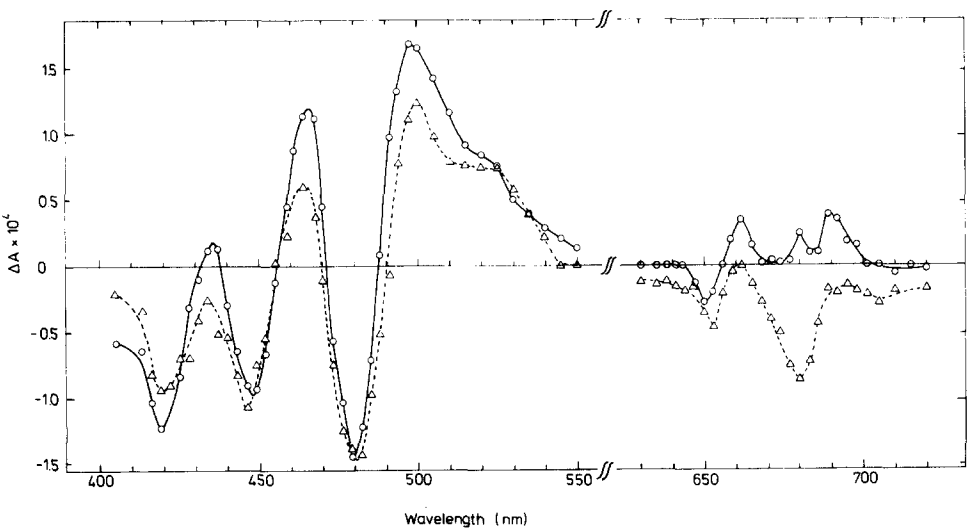

Fig. 3. Spectra of the rapidly reversible absorbance changes, isolated by extrapolation and subtraction of the slower phases, induced by external electrical field pulses (1500 V · cm^{-1}). The electrical vector of the measuring beam was polarized parallel (○———○) or perpendicular (△- - - - -△) to the direction of the applied field.

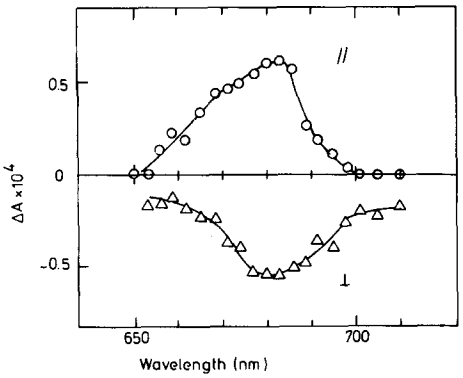

Fig. 4. Spectra of the field-induced absorbance changes decaying after the pulse with a half-time of 60 μs. Symbols are as defined in Fig. 3.

for orientation effects. The spectrum shows the characteristic waves of a carotenoid shift and some smaller contributions by chlorophylls. It will be shown in Discussion that these absorbance changes may be due to electrochromism. The spectrum was clearly different from that of light-induced electrochromic absorbance changes in the same suspension, which showed the well-known maximum at 518 nm and an 'isosbestic point' near 500 nm [16,17].

The amplitude of the absorbance changes shown in Fig. 5 was proportional to the square of the field strength applied, at least up to about 900 V · cm^{-1}. At still higher fields a saturation effect became apparent. This is illustrated by Fig. 6, where the amplitude of the fast component of absorbance changes at 500 nm, corrected for polarization effects, is plotted versus the square of the external field strength. The quadratic field dependence also distinguishes these absorbance changes from the light-induced 518 nm change, which depends linearly on the membrane potential [5,17,18].

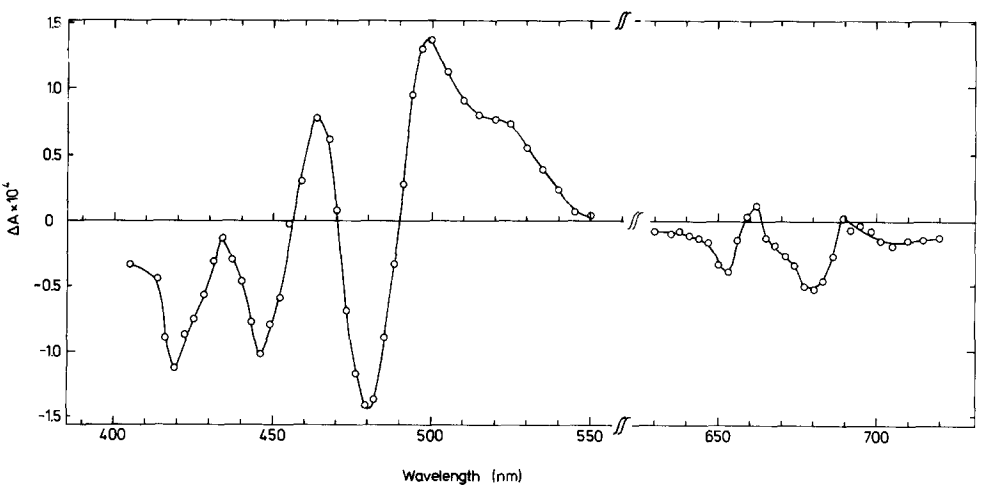

Fig. 5. Spectrum of the rapidly reversible absorbance changes corrected for field-induced orientation effects, obtained from Fig. 3 by plotting $\frac{1}{3}(\Delta A_{/\!/} + 2\,\Delta A_{\perp})$.

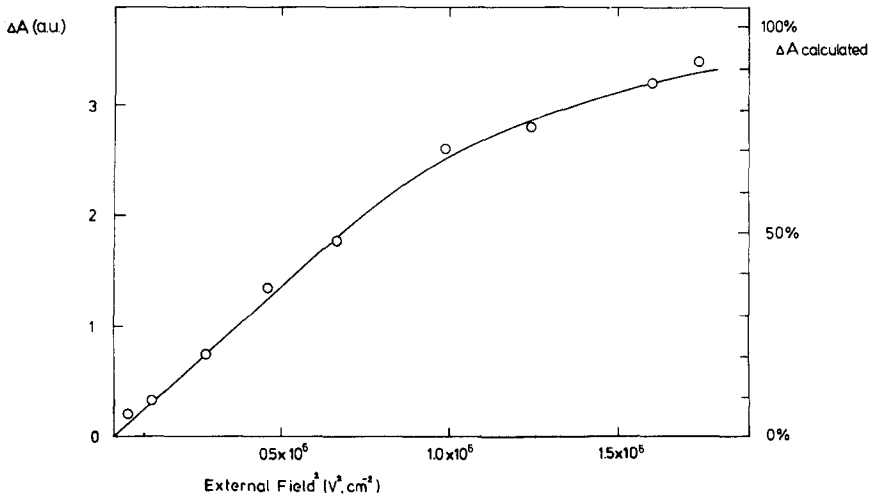

Fig. 6. Field dependence of the rapidly reversible absorbance changes corrected for orientation effects (○). ———, calculated on the basis of the assumptions outlined in Discussion.

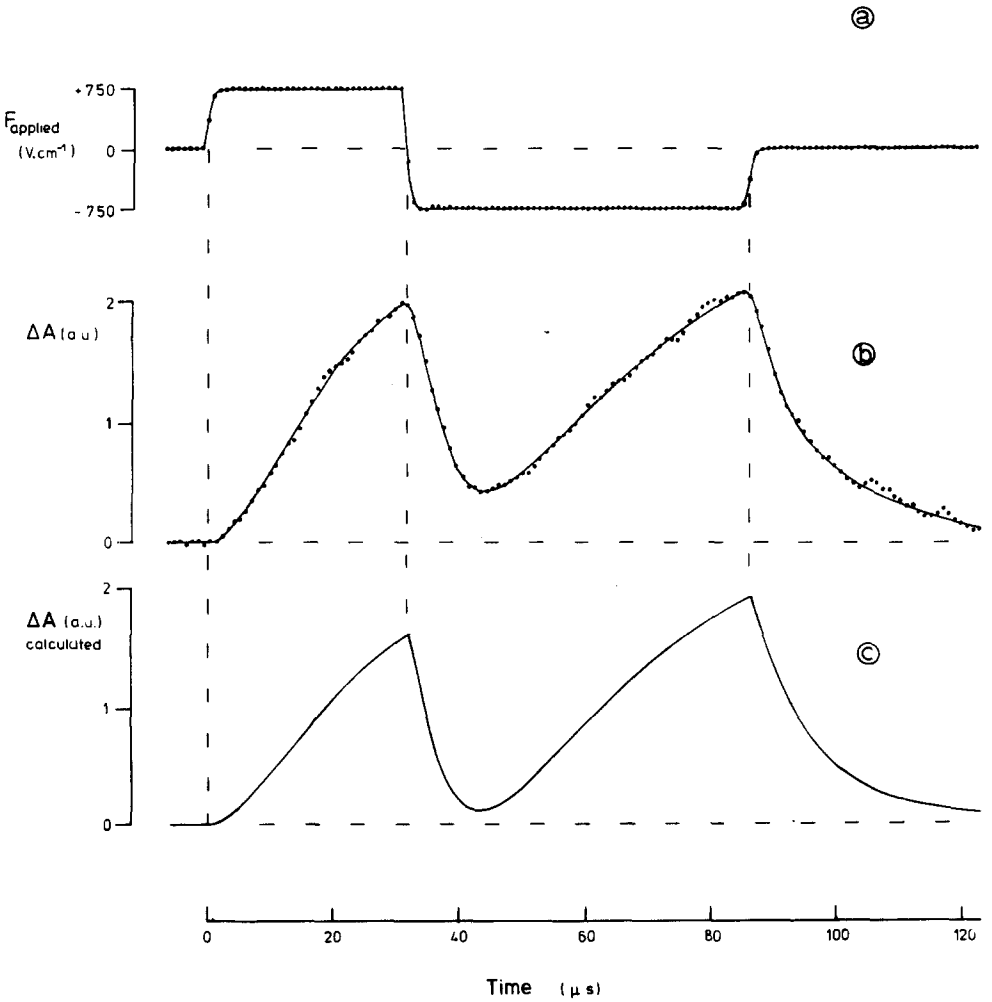

Fig. 7. Time-resolved kinetics of the absorbance changes at 500 nm induced by the electrical field pulse shown in trace a. The absorbance changes (trace b) were corrected for orientation effects ($\Delta A_{\parallel} + 2\,\Delta A_{\perp}$) and gramicidin-insensitive changes were subtracted. The trace is a result of 24 576 individual measurements. Curve c shows the calculated kinetics as explained in Discussion.

Fig. 7 shows the time-resolved kinetics of absorbance changes induced by an electrical field pulse of 750 $V \cdot cm^{-1}$ which is reversed after 32 μs. Upon the onset of the field the absorbances rises non-exponentially after an initial lag phase. Reversal of the field induces an initial dip followed by a slower rise. As will be shown in Discussion, the shape of the trace may be determined largely by the kinetics of the square of the local field strength sensed by the pigments.

Discussion

Bleb structure

The absorbance changes are induced by local electrical fields in the membranes and their interpretation requires knowledge of the electrical properties of the osmotically swollen chloroplasts (blebs).

In order to estimate these electrical properties the shape of the membrane system which constitutes the bleb must be considered. First of all, it seems very unlikely that the chloroplast envelope still surrounds the bleb, because its area would have to be stretched ten times and because osmotic shock releases the stroma contents into the medium. Therefore, the bleb structure must arise from the thylakoid membrane system. This system most likely consists of a single membrane-bounded entity [19,20]. The internal volume of the thylakoid system in intact spinach chloroplasts can be estimated at about 3 μm^3 [21—23]. The osmotic mechanism of bleb formation implies that this volume can increase at most to about 600 μm^3 by 200-fold dilution of the suspension, in agreement with the observed average bleb volume of about 550 μm^3. This interpretation implies that the bleb wall consists of a single membrane. Also the average surface area of the blebs, 300 μm^2, is not much less than the total membrane area of the thylakoid system, which may be estimated at 300—500 μm^2 (see e.g. Ref. 24).

The above considerations, and the observation that a considerable fraction of the thylakoid material remains concentrated in small patches on the bleb surface (Fig. 1), lead to the conclusion that the bleb wall cannot consist of more than one membrane.

Field-induced absorbance changes

The results reported here show three effects in the microsecond range induced by an electrical field applied to a suspension of blebs.

Firstly, a gradual change in light scattering during the field pulse was observed: it increases for light polarized parallel to the direction of the field and decreases for light polarized perpendicular to the field (Fig. 2). It persists in the presence of gramicidin.

Secondly, absorbance changes indicative of a field-induced orientation of pigments with a relaxation half-time of 60 μs were observed (Fig. 4) and such changes presumably occur on a shorter time scale as well (Fig. 3). Since the orientation effects were abolished by gramicidin, they were probably caused by the enhanced electrical field across the membrane. Movement of pigments, or of pigment-protein complexes with a restricted freedom of motion, might explain these rapid orientation effects.

The third effect causes the fast absorbance changes with the spectrum, field dependence and kinetics shown in Figs. 5—7, respectively. These results will be explained in the framework of the electrochromic theory [25—27].

It is well known that carotenoids, when exposed to an electrical field, show a marked electrochromism [28]. Interaction of the field with the polarizability of the molecules results in a small red shift of the absorption bands, proportional to the square of the field strength. The spectrum of the resulting absorbance changes is proportional to the first derivative of the absorption spectrum and its amplitude depends quadratically on the field strength applied.

In photosynthetic organisms carotenoid shifts are frequently observed [29—31] and ascribed to electrochromism caused by the light-induced membrane potential [32]. However, it has been shown in chromatophores of *Rhodopseudomonas sphaeroides* and in spinach chloroplasts that these absorbance changes are linearly dependent on the membrane potential [4,5,17,18]. Therefore it was postulated that the pigments concerned possess a permanent dipole moment induced by a permanent field imposed by their local environment [33,34]. This permanent field must have a preferential orientation in the direction of the light-induced field, which is from inside to outside of the membrane vesicles, in order to explain the observed red shift and it must be larger than the light-induced field in order to explain the linear field dependence. Furthermore, the light-induced carotenoid absorbance changes show a maximum at 518 nm, whereas the chloroplast absorption spectrum has an inflexion point at about 500 nm, mainly determined by the carotenoids. Therefore the light-induced changes may originate from a minor, spectrally red-shifted, fraction of the carotenoids. This may be understood when only these carotenoids are subjected to a permanent field sufficiently large compared to the light-induced field.

The spectrum of the absorbance changes induced by an electrical field (Fig. 5) resembles the first derivative of a carotenoid absorption spectrum and has a maximum at 500 nm, and the amplitude depends quadratically on the applied field strength. This suggests that the difference spectrum of the external field-induced changes is caused by all carotenoids. The fact that the difference spectrum is not dominated by the permanently polarized pigments may be understood if the geometry of the electrical field in the membrane vesicles is taken into account. The light-induced field always points outwards. Due to the low conductivity of the membrane as compared to the internal and external aqueous phases, the externally applied field also induces a field in the membrane perpendicular to the surface, but this is directed towards the inside for half of the vesicle and outwards for the other half. In this case, absorbance changes determined by the permanent field, which depend linearly on the field strength, will nearly be canceled: for each molecule which undergoes a red shift there will be a molecule located on the opposite side of the bleb which undergoes an almost equal blue shift. On the other hand the absorbance spectra of all carotenoids will be shifted towards longer wavelength irrespective of their location in the bleb wall as a consequence of quadratic electrochromism.

Quantitative evaluation

If the absorption spectrum of the pigments in the bleb wall and their

physical and geometrical parameters relevant for electrochromism are known, the size and form of the absorbance changes caused by a light-induced and an externally applied electrical field of known magnitude can be calculated. Conversely, from the measured electrochromic spectra induced by light and external field, the absorption spectra of the pigments can be obtained if the physical properties of the molecules and the magnitude of the fields are known. The condition that those spectra must fit in the total absorbance spectrum provides a quantitative test for the model given above.

We have carried out a computer simulation of the electrochromic difference spectra in the blue-green region induced by an external field and by illumination, based upon the simplifying assumption that only two different populations of pigments are important for electrochromism. The first population consists of pigments with a permanent dipole moment, which are largely responsible for the light-induced spectral changes. Between 500 and 550 nm the light-induced electrochromic difference spectrum of this pigment pool is mainly due to the special fraction of carotenoids with an induced permanent dipole moment. At shorter wavelengths contributions due to chlorophyll *a* and *b* are observed as well. Since these contributions are hard to express in terms of dipole moments and polarizabilities [35], we tentatively put them together with the 'polarized' carotenoids into one pool of pigments with uniform electrochromic behaviour. The form of the absorption spectrum of this pool was obtained by integration of the difference spectrum from Ref. 17, measured with chloroplasts, after correction for the particle-flattening effect [12]. Above 500 nm this spectrum is realistic; at shorter wavelength the spectrum is artificial but this did not seriously affect the outcome of the analysis.

An electrical field F perpendicular to the membrane will cause a frequency shift $\Delta\nu_1$ of the absorbance spectrum of these molecules equal to [28]:

$$\Delta\nu_1 = -\frac{\Delta\alpha_1}{2h}\langle\cos^2\phi\rangle_1(F^2 + 2F_pF) \tag{1}$$

where h is Planck's constant, ϕ is the angle subtended by the normal to the membrane and the molecular axis, $\Delta\alpha_1$ represents the difference between the polarizabilities in the excited and ground state (along the molecular axis) and F_p is the permanent field directed perpendicular to the membrane plane.

The second population consists of the major fraction of the carotenoids present in the bleb wall. Their absorption spectrum is red-shifted by an electrical field according to

$$\Delta\nu_2 = -\frac{\Delta\alpha_2}{2h}\langle\cos^2\phi\rangle_2F^2 \tag{2}$$

The values of $\langle\cos^2\phi\rangle$ can be obtained from linear dichroism data. If we assume that all polarized carotenoids have the same value of ϕ, the dichroism of the 518 nm change [36] suggests a value for $\langle\cos^2\phi\rangle_1$ of about 0.3. From the linear dichroism of the absorption spectrum of chloroplasts [37] we estimate that the value of $\langle\cos^2\phi\rangle_2$ for the bulk carotenoids is about 0.25.

The values of $\Delta\alpha_1$ and $\Delta\alpha_2$ were both taken to be $1.2 \cdot 10^{-33}$ J · cm² · V^{-2} [34,35]. The membrane potential induced by a single saturating light flash is taken to be 50 mV [38] corresponding to 10^5 V · cm^{-1} for a dielectric thick-

ness of the membrane of 5 nm [39]. The strength of the permanent field F_p should be an order of magnitude higher than that of the light-induced field [28,33], but probably less than 10^7 V · cm^{-1} [40].

The magnitude of the externally induced field in the membrane can be calculated if we take into account that the resistance of the membrane is much higher than that of the inner and outer media. For this system the field in the bleb membrane can be derived from Maxwell [41]:

$$F(\theta) = \frac{3r}{2d}(\cos\theta)F_{ex} \tag{3}$$

where F_{ex} is the externally applied field, r is the radius of the bleb, d is the effective dielectric thickness of the bleb wall and θ is the angle between the applied field and the normal to the membrane. The same formula has been derived by Zimmerman et al. [42]; the different formula given in Ref. 8 seems to be due to a miscalculation. As was discussed already, the bleb wall is believed to consist of a single membrane; therefore d is taken to be 5 nm [39]. The values of r were taken from the microscopically determined size distribution.

With these parameters we simulated the electrochromic difference spectra by applying Eqns. 1—3 and integrating over the surface of the bleb. The results shown in Figs. 8 and 9 were obtained with a permanent field of $4.8 \cdot 10^6$ V · cm^{-1}, which falls within the above-mentioned range. Furthermore from the observed particle-flattening effect we estimated that 35% of the pigments are located in the single-membrane bleb wall, whereas the microscopically observed patches contain the rest of the pigments, which only respond to the light-induced field. In Fig. 8a and b the experimental light- and external field-induced electrochromic spectra are indicated by circles. The solid curves show

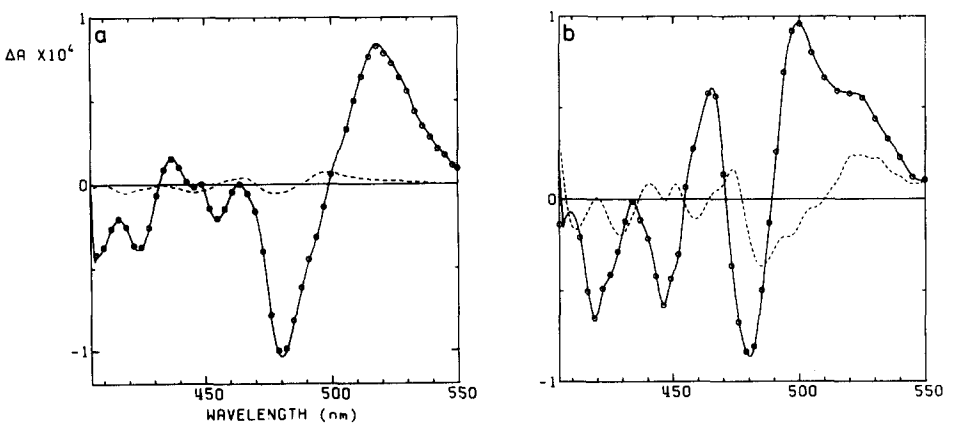

Fig. 8. Simulated electrochromic difference spectra induced by light (a) (———) and by an applied field of 900 V · cm^{-1} (b) (———). The experimental data (○) are shown at the amplitude corresponding to the field strengths used in the simulation. A small baseline shift (+9 · 10^{-6} ΔA) was applied to the data of Fig. 3 in order to obtain a positive integral in Fig. 9. A systematic error of this size would not be significant but is actually suggested by the spectral shape in the red region. - - - - - -, in (a) shows the contribution by the unpolarized pigments, and in (b) the contribution by the permanently polarized pigments.

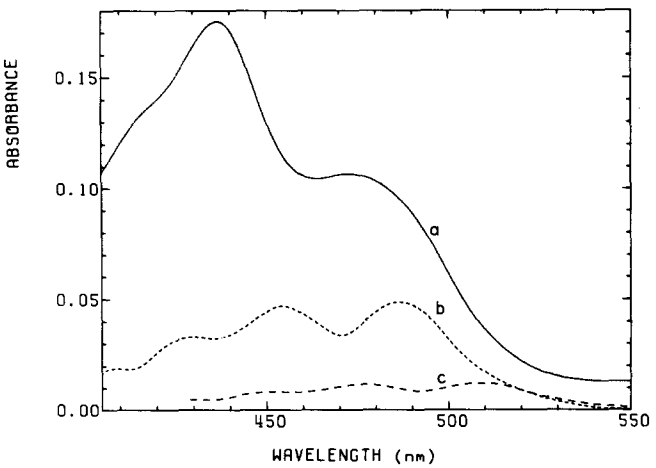

Fig. 9. (a) Absorption spectrum of the bleb suspension, corrected for particle flattening. (b) Calculated absorption spectrum of the unpolarized pigments. (c) Absorption spectrum of the permanently polarized carotenoids, shown at the calculated amplitude. The shape of this spectrum was taken to be identical to that of the unpolarized pigments, and corresponds at wavelengths above 500 nm to the spectrum used in the simulation.

the simulated difference spectra. These fits were optimized by iterative adjustment of the absorption spectra of the two pigment populations. The spectrum of the unpolarized pigments thus obtained is shown in Fig. 9, together with the estimated absorption spectrum of the permanently polarized carotenoids. At wavelengths above 500 nm the latter spectrum is identical to the spectrum used in the simulation. To indicate the absolute amplitude the measured absorption spectrum of the suspension, corrected for flattening, is also shown. As can be seen, the spectrum of the unpolarized pigments closely resembles that of carotenoids [43,44] and the absorption spectra of the two pigment pools account approximately for all carotenoid absorbance in the sample. The relative amount of polarized pigments is similar to that found in *Rps. sphaeroides* [45].

The simulated curve in Fig. 8a is mainly determined by the permanently polarized pigments; the contribution by the pigments without permanent field (dashed line) is small. The relation between the simulated absorbance change at 518 nm and the light-induced membrane potential was linear within 10% up to 350 mV. Similarly, the contribution of the polarized carotenoids to the external field-induced spectrum was small (dashed line in Fig. 8b). Therefore, the simplifying assumption that the polarized pigments all have the same electrochromic behaviour does not lead to a large distortion of the spectrum of the unpolarized pigments. Actually, taking more realistic values of the polarizabilities and dipole moments of chlorophylls into account, it can be argued that the contribution of chlorophylls to the field-induced electrochromic difference spectrum is even less than suggested here.

It should be noted that the simulation depends on many parameters, the amplitudes of which are only roughly known, but an equally good fit can be obtained by other sets of reasonable values of these parameters. We conclude

that the proposed interpretation of the spectral changes shown in Fig. 5 requires no unrealistic values of these parameters.

Saturation in the external field dependence

The computer simulation described above was carried out for an applied field strength of 900 $V \cdot cm^{-1}$, where a quadratic field dependence of the absorbance changes was observed (Fig. 6). The apparent saturation observed at higher field strengths may be due to the fact that the maximum membrane potential which can be generated by the electric field pulses is probably about 1 V, because of a sudden increase of the membrane conductance due to dielectric breakdown [46]. For an average bleb ($r = 4.5\ \mu m$) this value will be reached with an applied strength of 1.5 $kV \cdot cm^{-1}$. The expected field dependence was calculated on the basis of the microscopically determined size distribution of the blebs, the assumption of a maximum membrane potential of 1 V, and a quadratic dependence of the absorbance changes on the membrane potential. In each bleb the number of pigments responding to the field was taken to be proportional to the surface area of the bleb. The agreement of the calculated curve in Fig. 6 with the data (circles) suggests that dielectric breakdown indeed explains the apparent saturation at high applied field strengths. It should be noted that the curve is determined by the membrane potential, $F(\theta)d$, which can be calculated without knowledge of the membrane thickness (see Eqn. 3).

Kinetics

The kinetics of the field-induced changes (Fig. 7) may also be understood in terms of this model. When an external field is applied, the large enhancement of the field strength in the membrane, as depicted in Fig. 8B, requires charging of the membrane capacitance via the resistances of the internal and external medium. The resulting RC time, together with the quadratic field dependence, qualitatively explains the observed kinetics.

For a quantitative evaluation, we have to consider the electrodynamic properties of the material used. For this purpose we describe the membrane as a thin spherical shell insulator with a specific capacitance c_s and the internal and external aqueous phases as homogeneous material with a specific resistance ρ_s. For this system the kinetics are determined by the relation

$$\tau = \tfrac{3}{2}\rho_s c_s r \qquad (4)$$

where r is the radius of the bleb [47]. A preliminary study, in which the ionic strength of the suspension was varied and the size distribution of the blebs was determined indicates that τ indeed depends linearly on $\rho_s r$. The measured kinetics of the absorbance changes could be fitted with $\rho_s c_s = 2.0 \cdot 10^{-2}$ $s \cdot cm^{-1}$, taking into account the size distribution of the blebs in the suspension used. The specific conductivity ρ_s^{-1} was determined to be $1.5 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-1}$. Consequently the specific capacitance of the bleb wall is $2.0 \pm 0.2\ \mu F \cdot cm^{-2}$. The specific capacitance of thylakoids is not known but this value falls in the range of values reported for other biomembranes [48] and is in reasonable agreement with that reported for chromatophores ($1.1\ \mu F \cdot cm^{-1}$ [49]). The method used here is a relatively direct way to determine the membrane capaci-

tance and may be applicable to related systems.

We conclude that the shape of the difference spectrum, its amplitude, its field dependence and the kinetics of the absorbance changes are quantitatively accounted for by the electrochromic interpretation given above.

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