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EFFECT OF ELECTRIC FIELDS ON THE ABSORPTION SPECTRUM OF DYE MOLECULES IN LIPID LAYERS*

V. REFINED ANALYSIS OF THE FIELD-INDICATING ABSORPTION CHANGES IN PHOTOSYNTHETIC MEMBRANES BY COMPARISON WITH ELECTROCHROMIC MEASUREMENTS IN VITRO

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SUMMARY

The comparison of light-induced absorption changes in photosynthesis with electrochromic spectra of the isolated pigments in vitro is renewed more thoroughly and described in detail, involving new measurements of the linear electrochromism of oriented chlorophyll *b* [6].

1. The coincidence of the maxima and minima in the in vivo spectrum with those in the in vitro superposition is better than in previous studies [4].

2. The molar ratio of the pigments now used for the superposition of the in vitro spectra is the same as that in vivo.

3. From this and from surface-pressure/area diagrams of the chlorophylls on a water surface, conclusions are drawn concerning the preferential orientations of the dipole moment differences of the red and blue absorption bands of the bulk chlorophylls in the membrane.

4. From the comparison of the electrochromism of the carotenoids with the absorption change at 520 nm in vivo, it is concluded that the bulk of the carotenoids are oriented at a rather flat angle in the membrane ($\approx 16°$).

INTRODUCTION

It was pointed out by Junge and Witt that specific light-induced absorption changes of a chloroplast suspension are due to the generation of an electric field across the thylakoid membranes [1, 2]. This has been shown by kinetic [1, 2], electric [3], and spectroscopic [4] experiments. In the latter case, the whole spectrum of these "field-indicating absorption changes" as measured by Emrich et al. [5] was compared

* As to numbers I–IV of this series, see refs. 10, 13, 6, 9.

with electrochromic spectra of the isolated pigments in capacitors [4]. The main features of the in vivo spectrum could be reproduced as a superposition of the electrochromic spectra. However, the molar ratio of the pigments used for the superposition was not equal to the ratio in the chloroplasts, and the absorption change at $\lambda =$ 478 nm, which was attributed to chlorophyll *b*, did not exactly coincide with the electrochromic absorption change of chlorophyll *b* in capacitors.

This coincidence could be greatly improved by new electrochromic measurements on asymmetrically oriented chorophyll *b* layers [6]. Under these conditions, the main part of the absorption change was found to be a linear function of the electric field strength ("linear electrochromism") and to be approximately proportional to the first derivative of the absorption with respect to the wavenumber.

In earlier experiments, the chlorophyll *b* molecules had been distributed symmetrically in relation to the capacitor plane [7]. In that case, only a small linear electrochromism was found, and its curve shape did not differ very much from the spectrum of the quadratic electrochromism, as expected theoretically [8–10]. From the new experiments the light-induced electric field strength F across the thylakoid membranes could be calculated [6] by comparing the linear electrochromism of chlorophyll *b* with the light-induced absorption change of chloroplasts at $\lambda = 478$ nm [5]. The resulting value of $F \cong 10^5$ V/cm agrees well with other calculations [19, 27]. No additional permanent field acting on the chlorophyll *b* molecules need be postulated to obtain this agreement.

After this progress, it is of interest to compare the new linear electrochromic spectrum of chlorophyll *b* (together with the spectra of chlorophyll *a* and the carotenoids) with the spectrum of the field-indicating absorption changes in vivo [5]. For this comparison, the spectrum of the linear electrochromism of chlorophyll *a* determined by Kleuser and Bücher [11] will be used. The evaluation of the electrochromic spectrum of the carotenoids is described in the following section.

RESULTS

The electrochromic spectrum of the carotenoids

This can be calculated from the absorption spectra, because the electrochromism of long conjugated polyene chains is proportional to the first derivative of their absorption [4, 12–14]. A symmetrical carotenoid has no dipole moment and shows primarily no linear, but only a quadratic electrochromism [9]. According to Eqn. 17 from ref. 13, this is given by:

$$\frac{\overline{\Delta\varepsilon_q}}{F^2} = \frac{\overline{\cos^2\vartheta}}{2hc}\Delta\alpha_{||}\frac{d\tilde{\varepsilon}}{d\tilde{\nu}} \qquad (1)$$

where $\overline{\Delta\varepsilon_q}$ is the change of the molar absorption coefficient induced by the electric field strength F. (The dashes indicate averaging over the molecules of different orientation.) $\Delta\alpha_{||}$ is the difference between the polarizabilities of the ground state and the excited state parallel to the long axis of the carotenoid molecule. ϑ is the angle between the long axis and the electric field. $\tilde{\nu}$ is the wavenumber, h the Planck constant, and c the velocity of light.

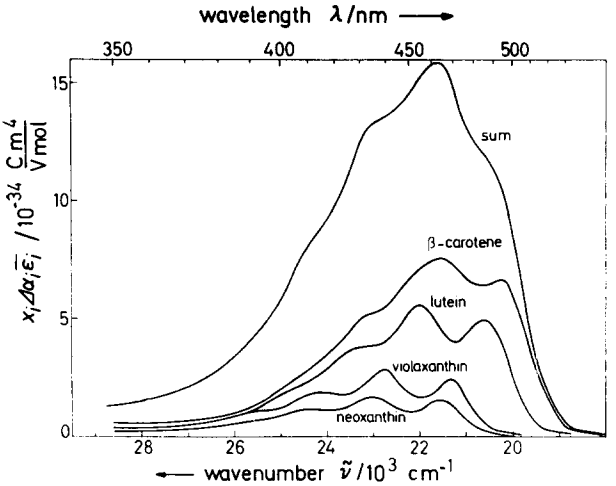

Fig. 1. Absorption spectra of the carotenoids (from refs. 16 and 17), weighted according to their polarizability differences $\Delta\alpha_i$ and to their mole fractions x_i in chloroplasts.

In chloroplasts, the mean molar quadratic electrochromism of the summed carotenoids i is given by

$$\left(\overline{\frac{\Delta\varepsilon_q}{F^2}}\right)_{car} = \sum_i x_i \left(\overline{\frac{\Delta\varepsilon_q}{F^2}}\right)_i \tag{2}$$

The relative mole fractions x_i of the four main carotenoids in chloroplasts are [15]: $x(\beta\text{-carotene}) = 0.312$; $x(\text{lutein}) = 0.333$; $x(\text{violaxanthin}) = 0.215$; $x(\text{neoxanthin}) = 0.14$; $\Sigma x_i = 1$. Assuming the mean value $\overline{\cos^2\vartheta}$ to be the same for all carotenoids, we may write the combination of Eqns. 1 and 2 as:

$$\left(\overline{\frac{\Delta\varepsilon_q}{F^2}}\right)_{car} = \frac{\overline{\cos^2\vartheta}}{2hc} \frac{d}{d\tilde{\nu}} \left(\sum x_i \Delta\alpha_i \bar{\varepsilon}_i\right) \tag{3}$$

The spectra $\bar{\varepsilon}_i(\tilde{\nu})$ of the four carotenoids, multiplied by the different factors x_i and $\Delta\alpha_i$, are shown in Fig. 1.

The spectra of β-carotene and lutein in chloroform solution from ref. 16 and of violaxanthin and neoxanthin from ref. 17 were all normalized on the same maximum molar absorption coefficient ($\varepsilon_{max} = 1.4 \cdot 10^5$ l/mol · cm), in view of the strongly differing data of the literature.

For the polarizability differences $\Delta\alpha_i$ parallel to the long axis of the molecules, the following values in $C \cdot m^2 \cdot V^{-1}$ were inserted: β-carotene: $1.73 \cdot 10^{-37}$; lutein: $1.19 \cdot 10^{-37}$; violaxanthin and neoxanthin: $8.67 \cdot 10^{-38}$.

The value for lutein (which has 10 conjugated double bonds) was calculated from electrochromic spectra in ref. 16 by means of Eqn. 16 in ref. 13. The values for β-carotene (11 conjugated double bonds) and for violaxanthin and neoxanthin (9 conjugated double bonds each) are obtained from Labhart's electrochromic measurements [12] on bixindimethylester and crocetindimethylester with 11 and 9 con-

jugated double bonds, respectively, if the electric field F acting on the dye molecule is set equal to the external field F_a. This seems to be a better approximation for the carotenoids than a correction of the field with the Lorentz formula $F = F_a\,(\varepsilon_r+2)/3$ (with the relative permittivity $\varepsilon_r = 1.88$ for hexane), which holds only for spherical molecules [9].

The first derivative of the sum of the curves in Fig. 1, according to Eqn. 3, yields the mean molar quadratic electrochromism of the carotenoids in the chloroplasts.

However, the experimental field-indicating absorption changes of chloroplasts are not a quadratic, but a linear function of the light-induced electric field. This was explained by assuming that the carotenoids are exposed to a high permanent field due to unsymmetrical complex formation with polar molecules [4, 9, 13, 14]. A mean effective permanent field F_p parallel to the external field F_a produces a secondary linear electrochromism from the quadratic one [9], which is given by:

$$\overline{\Delta\varepsilon_l} = 2\left(\frac{\overline{\Delta\varepsilon_q}}{F^2}\right)_{car} F_p F_a \tag{4}$$

If F_a is much smaller than F_p, the quadratic part of the absorption change can be neglected compared to the linear part. Then the contribution of the carotenoids to the absorption change of the chloroplast suspension is approximately obtained by multiplying 2.3 $\overline{\Delta\varepsilon_l}$ with the concentration c_{car} of the carotenoids in the suspension and with the length l of the cuvette:

$$\Delta A_{car} = 2,3\,\Delta\bar{\varepsilon}_l c_{car} l \tag{5}$$

Inserting Eqns. 4 and 3 into Eqn. 5, we get:

$$\Delta A_{car} = \frac{2,3}{hc}\,\frac{d}{d\tilde{\nu}}\left(\sum x_i \Delta\alpha_i \bar{\varepsilon}_i\right)\overline{\cos^2\vartheta}\, F_p F_a c_{car} l \tag{6}$$

The spectrum of ΔA_{car}, obtained by differentiation of the sum curve in Fig. 1 according to Eqn. 6, is given by the dashed curve c in Fig. 4 (bottom).

Quantitatively, the following values were inserted into Eqn. 6: $l = 2$ cm (the value of $l = 1$ cm quoted in ref. 5 is a misprint; H. M. Emrich, personal communication); $c_{car} = 2 \cdot 10^{-6}$ mol/l (calculated from $c_{chl} = 10^{-5}$ mol/l as quoted in ref. 5, and from the molar ratio in chloroplasts car : chl = 1 : 5 as quoted in ref. 18); $F_a = 2 \cdot 10^5$ V/cm (which was calculated for the light-induced electric field across the thylakoid membranes [6, 19]); $F_p = 2 \cdot 10^6$ V/cm (which is sufficiently greater than F_a to allow for neglecting the quadratic term in Eqn. 5 within the limits of experimental error, but still not unattainable by asymmetrical complex formation [14]).

The only remaining variable is the angle ϑ between the electric field and the long axis of the molecules. Agreement between the absorption changes in vivo and in vitro at $\lambda = 520$ nm (Fig. 4, top and bottom) is obtained on putting $\vartheta = 74°$. As the electric field is perpendicular to the plane of the membrane [3], the inclination angle of the molecules in the membrane is $90°-\vartheta$. A value of $\vartheta = 74°$ means that the carotenoid molecules on the average lie rather flat in the membrane, which agrees with the results of Breton et al. [20].

Complex formation with polar molecules will not only cause a linear electrochromism, but also a shift of the absorption spectrum to longer wavelengths (solvatochromism). Another reason for such a shift is the exciton interaction of two equal molecules. In this case, the shift according to McRae and Kasha [21] is given by

$$\Delta\tilde{\nu} = \frac{\text{const}}{r^3}(1-3\cos^2\beta) \tag{7}$$

where r is the distance and β the angle between the two parallel transition dipole moments and the connecting line of their centres. This equation describes not only shifts to lower but also to higher wavenumbers, depending on whether β is $< 55°$ or $> 55°$. This explains our observation that the absorption spectrum of lutein in multilayers on slides (compared to the spectrum in chloroform solution) is not only shifted to the red side (by about 800 cm^{-1}), but also broadened [16].

On the other hand, the spectra of carotenoids in vivo show sharp single bands, which are not broadened (compare Fig. 7 in ref. 13). Therefore, for the present comparison with the in vivo electrochromism, we have not used the spectra of lutein in multilayers (as we did in former studies), but only the absorption spectra of carotenoids in liquid solution. However, the first derivative of the summation curve in Fig. 1 was shifted by 800 cm^{-1} to lower wavenumbers (corresponding to a wavelength shift of $\Delta\lambda = 20.8$ nm at $\lambda = 500$ nm), before inserting it into Eqn. 6.

Orientation of the chlorophylls in vitro and in vivo

Before we can compare the electrochromic spectra of the chlorophylls in capacitors with the absorption change spectrum of chloroplasts, we need some information about the directions of the dipole moment differences for the blue and the red bands. This is because these directions, relative to the electric field, determine the sign of the linear electrochromic absorption changes. Some hints concerning the orientation in vitro can be gained from the surface-pressure/area diagrams of the chlorophylls on the water surface (Fig. 2).

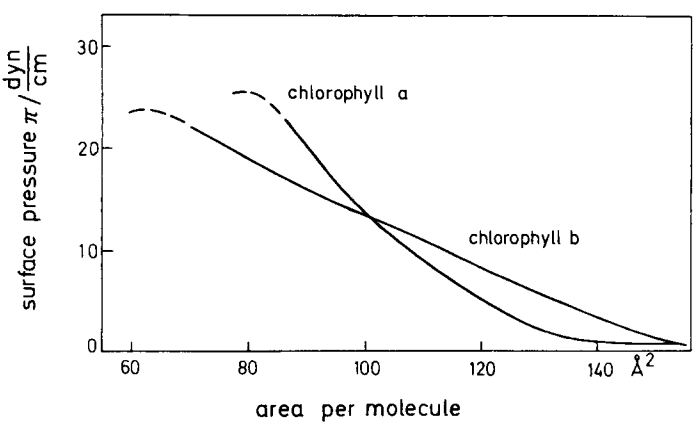

Fig. 2. Surface-pressure/area diagrams of monomolecular films of chlorophyll *a* and chlorophyll *b* on a water surface (pH = 7.2–7.5), measured by the Wilhelmy plate method (using a Rosano Surface Tensiometer, Federal Pacific Electric Co.), [16].

If we start from small surface pressures, the porphyrin rings of the chlorophyll molecules will lie flat on the water surface. In this case, an area of 195 $Å^2$ per molecule would result from the calotte model, assuming that the phytol chains are perpendicular to the water surface [22]. With increasing surface pressure, the area of chlorophyll *a* decreases at first much faster than the area of chlorophyll *b* (Fig. 2). This difference can easily be explained from the different molecular structure: chlorophyll *b* has an additional polar C=O-group at the corner of the porphyrin ring, opposite the phytol chain. Thus, the porphyrin ring of chlorophyll *b* is more strongly anchored in the water surface than the porphyrin ring of chlorophyll *a* and therefore cannot so easily be set upright with increasing surface-pressure.

For chlorophyll *a*, on pushing the surface film together, the edge of the porphyrin ring opposite to the phytol chain will rise from the water surface, so that the plane of the ring forms an acute angle with the phytol chain. In the limiting case of a high surface pressure, the ring plane should stand perpendicularly on the water surface, like the phytol chain. Then, from the calotte model, an area per chlorophyll *a* molecule of approx. 93 $Å^2$ would result (small side of the porphyrin ring plus cross section of the phytol chain) [22]. This agrees with the area at the collapse point of the chlorophyll *a* film (Fig. 2) within the limits of experimental error.

For chlorophyll *b*, on the other hand, on pushing the surface film together, the reduction of the film area will be achieved by lifting that edge of the porphyrin ring which bears the phytol chain. In this case, even the polar ester group of the phytol will rise from the water surface, so that the plane of the ring forms an obtuse angle with the phytol chain. Thus the area of chlorophyll *b* at the collapse point of the film is much smaller than the area of chlorophyll *a* (Fig. 2).

As far as the orientation of the molecules in the monolayer remains preserved during the process of pulling out the slide from the water surface, the orientations of chlorophyll *b* and chlorophyll *a* in the capacitor are depicted in Fig. 3 (left).

On the right-hand side of Fig. 3, the orientation of the bulk chlorophylls in the thylakoid membrane is depicted. The porphyrin ring of chlorophyll *b* is oriented with the corner bearing the phytol chain* towards the inside of the thylakoid [6].** For chlorophyll *a*, a similar orientation is assumed. These pictures seem to be the only possibility to get a qualitative agreement between the electrochromic absorption changes in vitro and in vivo (see next section).

The vectors in the porphyrin squares in Fig. 3 indicate the directions of the transition dipole moments of the main blue and red absorption bands, as they result from molecular orbital calculations [24–26]. The directions of the dipole moment differences of the blue and red absorption bands are not available in the literature. However, by estimating the influence of the polar groups on the chlorin skeleton molecular orbitals, which are involved in the blue and red main transition, it can be expected that at least for chlorophyll *b* the dipole moment differences have approximately the same directions as the corresponding transition dipole moments. (For

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* This corner is indicated by the point of the blue vector. The phytol chain itself is not shown here, because it is not known whether it forms an obtuse or an acute angle with the porphyrin plane.

** The evidence in ref. 6 is based on the supposition that the light-induced absorption change in chloroplasts around $\lambda = 478$ nm is mainly due to chlorophyll *b* (which follows from the comparison with experiments on chlorophyll *b*-deficient mutants, cf. e.g. ref. 23), and on the finding that the sign of this absorption-change in vivo is opposite to that of chlorophyll *b* in vitro at this wavelength.

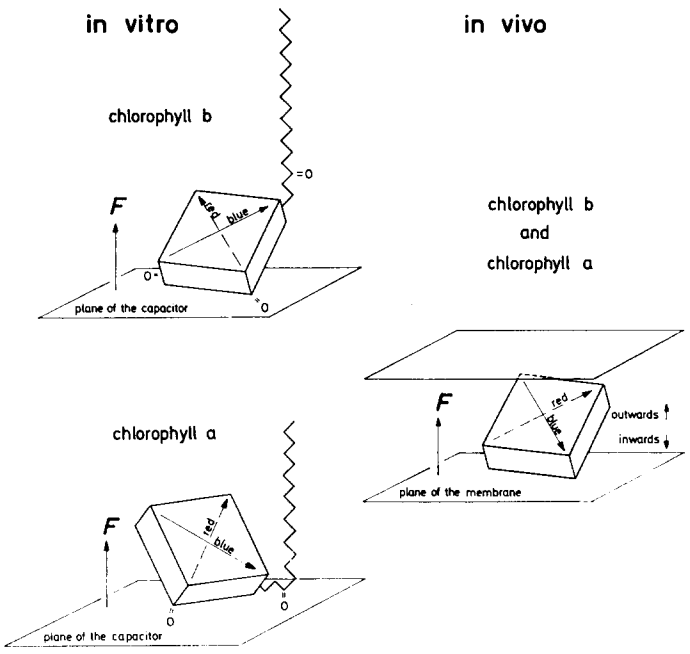

Fig. 3. Orientation of chlorophyll *b* and chlorophyll *a* relative to the electric field *F* in vitro and in vivo. The porphyrin rings are symbolized by square blocks. In vitro, the corners which bear hydrophilic groups are marked by O-atoms, and the phytol chains are symbolized by zigzag lines. In vivo, the direction of the phytol chains is not known; so the orientation is indicated only by the red and the blue vector. In vitro, the electric field direction away from the slide is defined to be positive. In vivo, the light-induced electric field across the membrane is directed from inside to outside of the thylakoid.

chlorophyll *a* it might also be that both dipole moment differences have approximately the same direction as the red vector and opposite sign to each other.)

What consequences can be drawn from Fig. 3 for the comparison of the electrochromic spectra in vitro and in vivo? For chlorophyll *b*, the blue vector of the dipole moment difference has in vitro a component in the direction of the electric field, but in vivo a component opposite to the electric field. Therefore, the sign of the linear electrochromism of chlorophyll *b* in the blue spectral region must be changed, if the in vivo spectrum is to agree with the in vitro spectra. However, the sign in the red spectral region must remain unchanged, as the red vector has a component in the direction of the field in vitro as well as in vivo.

For chlorophyll *a* the directions of the blue and red vectors relative to the electric field are in vivo qualitatively the same as in vitro. Thus the spectrum of the linear electrochromism of chlorophyll *a* may be used qualitatively unchanged.

Superposition of the electrochromic spectra

In Fig. 4, the spectrum of the "light-induced field-indicating absorption changes" of a chloroplast suspension from ref. 5 is compared to the linear electrochromism of chlorophyll *a*, chlorophyll *b* and the carotenoids (curves a, b, and c, respectively, Fig. 4, bottom). The curve for chlorophyll *b* is taken from ref. 6 (Fig. 1), except for the altered sign in the blue spectral region (compare last section) and

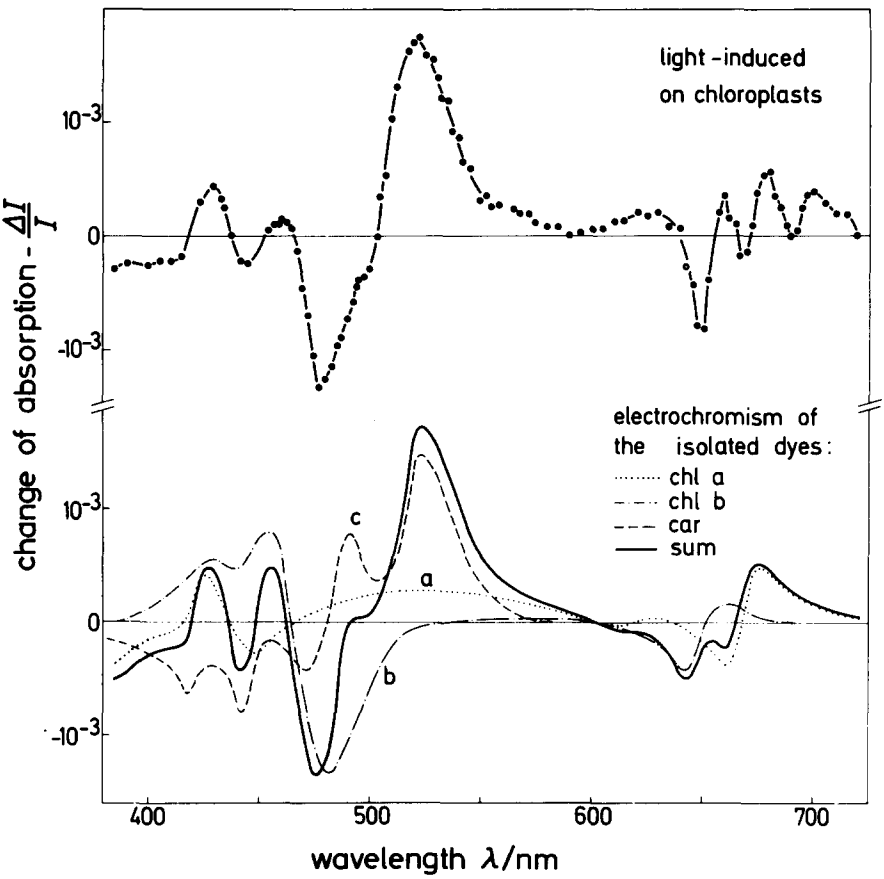

Fig. 4. Top: spectrum of the light-induced field-indicating absorption changes of a chloroplast suspension from ref. 5. Bottom: linear electrochromic absorption changes of the isolated dyes, converted to the concentrations in the chloroplast suspension.

transferred to a linear wavelength scale. The absorption-change of chlorophyll *b* at 482 nm in Fig. 4 (bottom) is normalized to the absorption change of the in vivo spectrum at 477 nm in Fig. 4 (top). From this identification, a light-induced electric field in the thylakoid membranes of $F \cong 10^5$ V/cm can be calculated [6], which is in good agreement with other calculations [19, 27]. The curve for chlorophyll *a* in Fig. 4 (bottom) is taken from ref. 11 (Fig. 3 middle, solid line; the opposite sign of the ordinates is due to an opposite field direction in ref. 11). The ordinates of this curve were conformed to the curve of chlorophyll *b* using a molar ratio of chlorophyll *a* : chlorophyll *b* = 2.3 : 1. (The natural molar ratio varies from 1.3 to 3.6 [28].) For details, see the appendix.

A natural molar ratio for chl : car = 5 : 1 [18] was also used in the curve of the carotenoids in Fig. 4 (bottom) (cf. text following Eqn. 6). The resulting summation curve (solid line in Fig. 4, bottom) has the same number of maxima and minima at nearly the same wavelengths as the in vivo spectrum (Fig. 4, top). Even the characteristic shoulder at 495 nm is reproduced at the same wavelength. Only the maximum at

700 nm is excluded, which seems to be due to a reaction of special chlorophyll *a* aggregates.*

On the whole, the agreement is now rather convincing. This gives further evidence for the electrochromic nature of the specified absorption changes in vivo and for the molecular models outlined in Fig. 3. By means of Fig. 4, the origin of the "field-indicating absorption changes" and the contributions of the different dyes to the different maxima and minima can be seen directly.

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APPENDIX

The electrochromic spectrum of chlorophyll *a* from [11] was conformed to the scale in Fig. 4 by the formula

$$\Delta A_{\text{chl } a} = (\overline{\Delta\varepsilon_l}/F_a)_{\text{chl } a} \cdot \left(\frac{\Delta A}{\overline{\Delta\varepsilon_l}/F_a}\right)_{\text{chl } b} \cdot \frac{c_{\text{chl } a}}{c_{\text{chl } b}} \tag{8}$$

where ΔA and $\overline{\Delta\varepsilon_l}/F_a$ were referred to $\tilde{\nu} = 425$ nm for chl *a* and to $\tilde{\nu} = 482$ nm for chl *b*. Eqn. 8 follows from an application of Eqn. 5 on chlorophyll *a* at 425 nm and on chlorophyll *b* at 482 nm. $\overline{\Delta\varepsilon_l}/F_a$ for chlorophyll *a* is calculated from the quotations in ref. 11: $\Delta A_l/F_a = 4.27 \cdot 10^{-12}$ cm/V at 425 nm; the surface concentration in the diluted monolayer is one molecule per 182 Å^2, and the amount of chlorophyll *a* per area of the light beam cross section is magnified by the factor $1/\cos\beta$, with the refracting angle $\beta = 27.8°$ [13].

Thus

$(\overline{\Delta\varepsilon_l}/F_a)_{\text{chl } a\,(425\text{ nm})} = 1.8 \cdot 10^{-2}\ \text{cm}^3 \cdot \text{V}^{-1} \cdot \text{mol}^{-1}$

From ref. 6 (Fig. 1), we read: $(\overline{\Delta\varepsilon_l}/F_a)_{\text{chl } b\,(482\text{nm})} = 13.5 \cdot 10^{-2}\ \text{cm}^3 \cdot \text{V}^{-1} \cdot \text{mol}^{-1}$

From Fig. 4, we read: $\Delta A_{\text{chl } b\,(482\text{nm})} = -1.34 \cdot 10^{-3}$. The molar ratio in chloroplasts is $c_{\text{chl } a}/c_{\text{chl } b} \cong 2.3$. Inserting these values into Eqn. 8 yields:

$\Delta A_{\text{chl } a\,(425\text{ nm})} = 4.1 \cdot 10^{-4}$

which was used as reference for drawing curve a in Fig. 4 from the curve in ref. 11, Fig. 3 (middle).

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* The absorption change at 700 nm cannot be caused by a simple band shift or band broadening effect, as its integral is not zero. Further experiments are in progress (K. -U. Sewe and R. Reich, in preparation).

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