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# ANISOTROPY OF THE EMISSION AND ABSORPTION BANDS OF SPINACH CHLOROPLASTS MEASURED BY FLUORESCENCE POLARIZATION AND POLARIZED EXCITATION SPECTRA AT LOW TEMPERATURE

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**Spectra of fluorescence polarization were measured between 4 and 120 K of spinach chloroplasts, oriented in a magnetic field. At least seven emission bands were observed. The well known bands near 685 nm ('F-685') and 735–740 nm ('F-735') and the band near 680 nm ('F-680') were strongly polarized parallel to the plane of the thylakoid membrane, whereas emission bands near 695 nm ('F-695'), 710, 730–735 and 760 nm showed perpendicular polarization. Assuming perfect orientation of the thylakoid membranes, we calculated orientation angles of 64, 47 and 66.5° for the emission dipoles of F-685, F-695 and F-735, respectively, with respect to the normal of the membrane. Excitation spectra of F-695 and F-735 in polarized light at 4 K provided information about the orientation of the absorption dipoles of chlorophylls *a* and *b*. The spectra thus obtained were in very good agreement with the linear dichroism spectrum. Moreover, they allowed us to distinguish between the pigments associated with Photosystems I and II, which is not possible from measurement of linear dichroism alone. The results indicate that a high degree of orientation is not confined to the long-wave absorbing bands, but also bands at shorter wavelength show a clear anisotropy. The calculated orientations were in quantitative agreement with the hypothesis that F-685 and F-735 are associated with chlorophylls absorbing at 676 and 710–715 nm, respectively.**

## Introduction

Information about the orientation of chlorophyll or bacteriochlorophyll in the photosynthetic membrane can be obtained by measurement of fluorescence polarization, of fluorescence in polarized excitation light and of linear dichroism (LD) [1–4].

At low temperature, at least four bands are observed in the emission spectrum of higher plant chloroplasts, which are called F-680, F-685, F-695 and F-735, respectively [5–7]. The first of these is due to emission from the light-harvesting Chl *a*/*b* complex [7], F-685 and F-695 come from PS II, and F-735 belongs to the PS I complex. Fluorescence polarization studies suggested that the corresponding emission dipoles are more or less oriented parallel to the membrane, and that the degree of orientation increases with increasing wavelength [1,8]. A similar conclusion with respect to the absorption dipoles was obtained from LD and fluorescence excitation spectra in polarized light [3,4,9]. Thus, both kinds of evidence suggested that a significant optical anisotropy only occurs for Chl *a* molecules absorbing and fluorescing at relatively long wavelengths.

In the present paper we report measurements of

Abbreviations: Chl, chlorophyll; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine.

fluorescence polarization of oriented chloroplasts at low tempertaures down to 4 K. In addition, we measured the ansiotropies of the absorption bands by recording polarized excitation spectra for F-695 and F-735. The results indicate that not only the long-wave, but also the short-wave $Q_y$ transition dipoles of chlorophyll show clear orientations with respect to the thylakoid membrane, and that the pigment-protein complexes in higher plants form a highly structured system of oriented chlorophylls.

## Material and Methods

Chloroplasts from spinach were isolated as described elsewhere [10]. The samples were suspended in a medium containing 50 mM Tricine, pH 7.8, 0.4 M sucrose, 10 mM KCl and 5 mM $MgCl_2$, and were mixed with glycerol (final concentration 55% (v/v)) in order to prevent crystallization upon cooling. Final chlorophyll concentration was 5 $\mu$g/ml.

Fluorescence polarization, LD and polarized excitation spectra were recorded with the apparatus described in Refs. 11 and 12, supplemented with two Glan-Thomson polarizing prisms, type MGT 3B14 (Karl Lambrecht). The chloroplast sample was contained in a square perspex vessel of 2 mm thickness. The chloroplasts were oriented by slowly cooling to 77 K in a magnetic field of 1.3 T and subsequently transferred to the apparatus. Fluorescence was detected at 90° to the incident beam. A correction for the polarization by the detection system was obtained by measuring the apparent polarization of an ethanolic solution of Chl *a*.

## Results and Interpretation

*Emission spectra of polarized fluorescence*

Fig. 1 shows low-temperature emission spectra of spinach chloroplasts oriented in a magnetic field. The samples were excited parallel to the direction of the magnetic field, i.e., perpendicular to the thylakoid membrane [8,13], and fluorescence was measured in the plane of the membrane, as indicated in the figure. The excitation beam, set at 660 nm, was vertically polarized, whereas the analyzing polarizer was in either vertical (↑) or horizontal position (→). The most prominent bands in the emission spectra are F-685, F-695 and F735; the spectra were measured at various temperatures between 120 and 4 K, and showed the usual temperature dependence for these bands [7]. The strong polarization of F-735 is in agreement with earlier measurements with maize and pea chloroplasts at 130 and 77 K, respectively [1,8,14], but polarization values of F-685 reported so far were considerably lower, and a negative polarization of F-695, as shown by our spectra, has not been observed earlier. Fig. 2 shows the corresponding values for the fluorescence polarization FP (the ratio of the intensities of the polarized beams) as a function of wavelength. Perhaps the most striking result is the large negative polarization of F-695, which can be best observed at 50 K, at which temperature F-695 is the most prominent band in the emission spectrum [7].

Because of the geometry used to obtain the spectra of Fig. 1, the polarization values obtained from these spectra reflect the orientation of the emission dipoles with respect to the membrane. The corresponding angles ($\phi$) with respect to the normal of the membrane can be calculated by means of the equation [2]:

$$p = (1 - 3\cos^2\phi)/(1 + \cos^2\phi)$$

where $p = (FP - 1)/(FP + 1)$, if perfect orientation of the membrane and perfect energy transfer are assumed. We found that the polarization of the fluorescence bands was not dependent on the wavelength of excitation or on the polarization of the exciting beam, which indicates that the latter condition was indeed fulfilled. As discussed by Garab et al. [8] there are at least three different factors that cause an imperfect orientation: (i) the thylakoid membranes and grana are not perfectly parallel to each other within the chloroplast, (ii) imperfect orientation of the chloroplasts themselves and (iii) contribution by the folded regions of the thylakoid.

If, nevertheless, perfect orientation was assumed, the calculated angles for F-685, F-695 and F-735 were found to be 64, 47 and 66.5°, respectively. These values deviate quite significantly from the 'magic angle' of 54.7°, indicating a high degree of order for the emitting chlorophylls. The calculations were based on the polarization values mea-

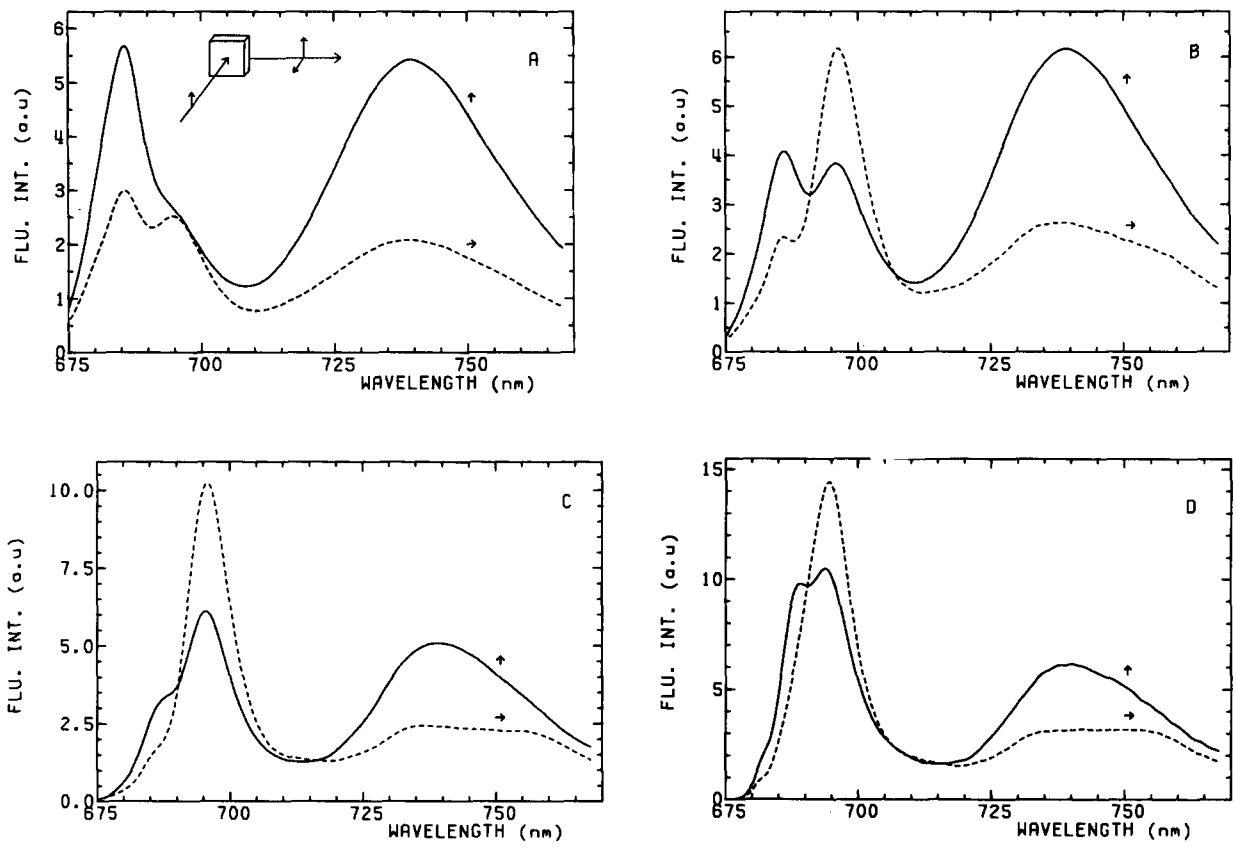


Fig. 1. Polarized fluorescence spectra of magnetically oriented spinach chloroplasts recorded at different temperatures. The chloroplasts were excited at 660 nm at right angles to the thylakoid membrane as indicated. The arrows indicate the direction of the polarizer in the detection beam, corresponding to fluorescence polarized either perpendicular (→) or parallel (↑) to the plane of the membrane. (A) 120 K, (B) 77 K, (C) 50 K, (D) 4 K. The spectra are plotted on the same relative scale. FLU.INT., fluorescence intensity; a.u., arbitrary units.

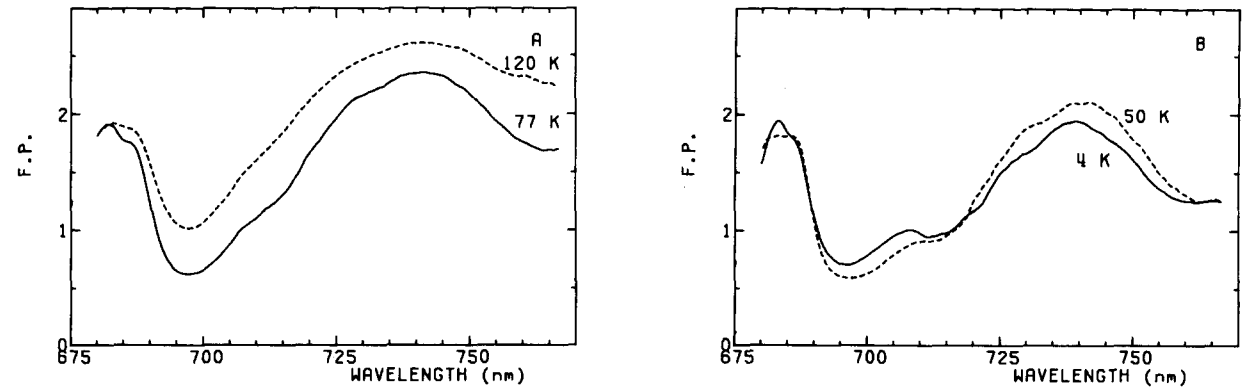


Fig. 2. Fluorescence polarization (F.P.) of the spectra given in Fig. 1.

sured at the temperatures most suitable for each band, i.e., 120 K for F-685, 50 K for F-695 and 120 K for F-735. The overlap between F-685 and F-695 is small at the temperatures indicated, and only a small correction was needed for the estimated effect of this overlap. The polarization of F-680, which originates from the light-harvesting Chl *a*/*b* complex [7] appears to be about the same as that of F-685, as indicated by the spectra measured at 4 K.

It should be noted that the degree of orientation in our samples must be higher than that calculated by Garab et al. [8] for maize chloroplasts oriented in a magnetic field. If we apply their 'order parameter', which comprises the three effects mentioned above, a value of $-0.27$ is obtained for $\cos^2\phi$ for F-735. This suggests that for any reasonable order parameter the true deviation from parallel and perpendicular orientation, respectively, is much less than indicated by the angles given above.

The emission and polarization spectra show the presence of at least three additional bands above 700 nm. The first one is a fairly weak band near 710 nm that is probably identical to that observed previously in maize chloroplasts [1,8]. In the fluorescence polarization spectrum (Fig. 2B) a distinct minimum can be seen at this wavelength at a value smaller than unity, indicating that the band is negatively polarized. Although the band at 710 nm is very small and hardly resolved in the fluorescence spectra (Fig. 1D), it was highly reproducible and appeared in all our 4 K spectra. The same appears to be true for the bands at 730–735 and near 760 nm, which are clearly visible only in the spectra of the perpendicularly polarized fluorescence spectra at 50 and 4 K, suggesting a negative polarization of these bands, which is confirmed by the fluorescence polarization spectra (Fig. 2B) showing a constant value in the region of 760 nm and a shoulder at 730–735 nm at lower values than the major band at 735–740 nm. The band at 760 nm shows a similar temperature dependence as F-695, and its excitation spectrum (not shown) indicated association with Photosystem II. This suggests that it may be a vibrational sub-band of F-695, as was earlier concluded for similar emission bands in red and blue-green algae [15].

*Anisotropy of the absorption dipoles detected by fluorescence*

The results presented above provide information about the orientation of the emission dipoles of Chl *a* with respect to the thylakoid membrane. Similar information about the absorption dipoles of Chl *a*, Chl *b* and other pigments is obtained by measurement of LD spectra [3,9,16], and by comparison of the bands in these spectra with those in the absorption spectrum measured in nonpolarized light. In a previous publication [11], we have shown that low-temperature excitation spectra of the various fluorescence bands allow a clear distinction between the light-absorbing pigments that contribute to PS I and to PS II emission, respectively. Therefore, one may expect that excitation spectra of oriented chloroplasts in polarized light should provide specific information about the orientation of the pigments associated with PS I and PS II, whereas the 'traditional' LD spectra do not differentiate in this way.

In Fig. 3 the excitation spectra, measured at 4 K, are given for the fluorescence detected at 698 and 735 nm, respectively. The excitation light was polarized either parallel or perpendicular to the plane of the membrane, while the fluorescence was detected at a right angle to the excitation light without polarizer. Most of the bands are clearly anisotropic. Fig. 4 gives the corresponding difference spectra; Fig. 5 shows the LD spectrum measured at 4 K. These spectra are in excellent agreement with each other; the LD spectrum shows the combined features of the difference spectra for PS I and PS II excitation, except that the 680-nm band of PS I merges with the 676-nm band of PS II as is also the case for the absorption spectrum [11]. The characteristic pattern of positive and negative bands around 650 nm is due to the light-harvesting Chl *a*/*b* protein [16–18]. In agreement with this, it is only present to a small extent in the PS I difference spectrum.

In an attempt to determine the anisotropy for the various absorption bands and to calculate the corresponding angles of orientation, we tried to fit the excitation spectra by means of Gaussian bands. This attempt was only partially successful. For PS II a reasonable fit for the two spectra was only obtained for the region 665–680 nm, which is dominated by the bands at 670 and 676 nm. For

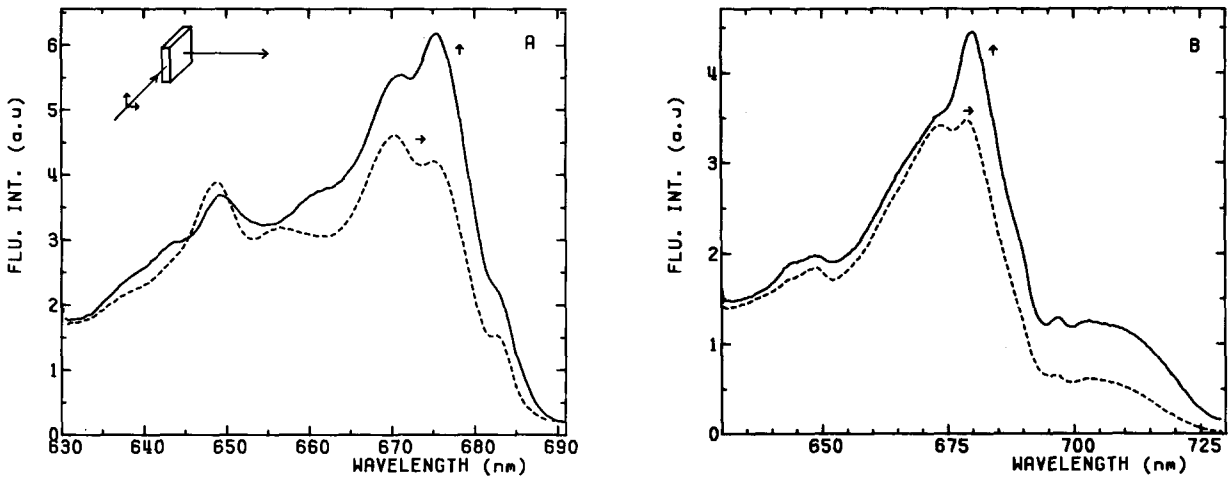


Fig. 3. Excitation spectra of the fluorescence of magnetically oriented chloroplasts excited with light polarized either perpendicular (→) or parallel (↑) to the plane of the thylakoid membranes. The excitation beam was parallel to the plane of the thylakoid membranes as indicated in the figure. Fluorescence was detected at right angles without polarizer at 698 nm (A) and 735 nm (B).

the PS I spectra a reasonable fit was obtained for the bands at 680 and 689 nm. Above 700 nm two bands are visible in the difference spectrum of Fig. 4, located near 705 and 710–715 nm. Especially the latter was strongly anisotropic. For the other bands only a qualitative assessment of the orientation was possible.

Table I summarizes the results thus obtained. The wavelengths of the maxima of the various bands were determined from the second and fourth derivatives of the excitation spectra. For those bands of which the amplitudes ($A_{\uparrow}$ and $A_{\rightarrow}$) could be determined with reasonable accuracy, the corresponding angles of orientation were calculated by means of the equation:

$$A_{\uparrow} - A_{\rightarrow} = A(1 - 3\cos^2\phi)/2$$

where $A = 2A_{\uparrow} + A_{\rightarrow}$, assuming perfect orientation of the membranes. In PS II, angles larger than the magic angle of 54.7° were obtained for the Chl *a* bands at 676, 670 and 661 nm. The band at 670

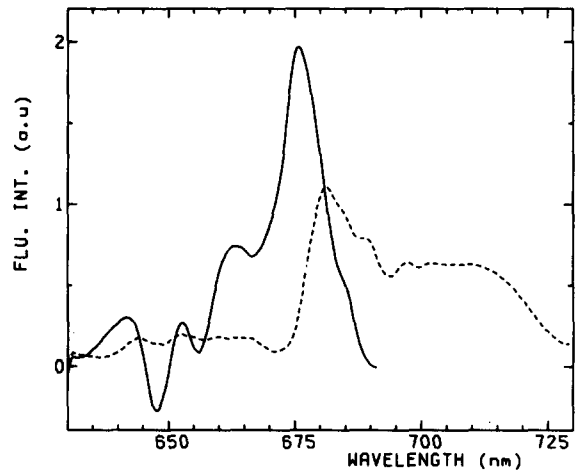


Fig. 4. Difference of the excitation spectra of Fig. 3. (— — —) Detection wavelength 735 nm, (———) detection at 698 nm.

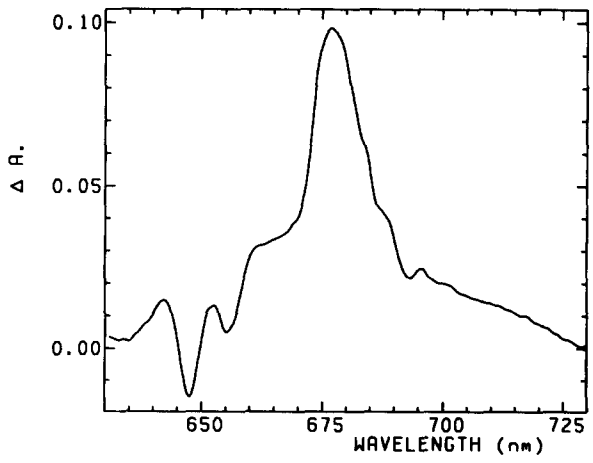


Fig. 5. LD spectrum ($\Delta A_{\uparrow} - \Delta A_{\rightarrow}$) of magnetically oriented chloroplasts at 4 K.

TABLE I

ANISOTROPIES OF CHLOROPHYLL ABSORPTION BANDS

The peak wavelengths and orientations of PS I and PS II chlorophylls were obtained from the polarized excitation spectra as described in the text; the orientations are given with respect to the normal of the thylakoid membrane. Short-wave bands in the PS I spectrum, which are mainly due to the light-harvesting Chl *a*/*b* protein, are omitted.

| PS I | | PS II | |
|---|---|---|---|
| Wavelength max. (nm) | Orientation angle (°) | Wavelength max. (nm) | Orientation angle (°) |
| 672 | < 54.7 | 643 | > 54.7 |
| 680 | 59 | 649 | < 54.7 |
| 688 | 61 | 656 | < 54.7 |
| 696 | > 54.7 | 661 | > 54.7 |
| 705 | 63 | 670 | 58 |
| 710–715 | 65 | 676 | 62 |
| | | 684 | < 54.7 |

nm was only weakly anisotropic. The bands at 684 and 656 nm reflected an orientation more or less perpendicular to the plane of the membranes. The band at 656 nm was not observed in the absorption spectrum [11]; it was clearly visible only in the spectrum obtained with perpendicularly polarized light, indicating a fairly pronounced orientation of the corresponding dipoles. The Chl *b* band at 643 nm showed predominantly parallel, the band at 649 nm predominantly perpendicular orientation.

Table I also lists the principal PS I bands and their orientations. Except for the band at 696 nm (see Fig. 4), the bands appear to be oriented increasingly parallel to the membrane with increasing wavelength, the calculated angle of the orientation of the band at 710–715 nm being 65°. The sharp drop in the difference spectrum (and also in the LD spectrum) below 680 nm is due to a perpendicular polarization of the 672 nm band. The slightly parallel polarization at still shorter wavelengths is probably due to vibrational subbands of the long-wave chlorophylls.

## Discussion

Various kinds of information can be obtained from measurements of fluorescence polarization and from excitation spectra of fluorescence in polarized light. For oriented thylakoid membranes where, certainly at low temperature, the effects of molecular motion of the pigments can be neglected, the information obtained concerns either energy transfer or the orientation of the transition dipoles with respect to the membrane and to each other.

Information about energy transfer can only be obtained if the much larger effects due to anisotropy are avoided, i.e., when the excitation beam and the direction of measurement of fluorescence are both perpendicular to the membrane [1,2,19]. In other cases the effects of anisotropy prevail, and information about the orientation of the absorption or emission dipoles is obtained depending on the type of measurement.

The fluorescence polarization spectra presented in this paper show contributions by at least seven different emission bands at low temperature. F-680, F-685 and F-735 have parallel polarization, corresponding to an angle of dipole orientation larger than the magic angle of 54.7°; F-695 and bands near 710, at 730–735 and near 760 nm are polarized perpendicular to the plane of the membrane. The band near 760 nm may be a vibrational band of F-695, as discussed above. The origin of the other bands is not known; the band near 710 nm may be identical to the band derived earlier from fluorescence and fluorescence polarization spectra [1,8, 20]. The existence of emission bands near 705, 715, 725, 735 and 755 nm in low-temperature emission spectra was deduced by Litvin et al. [21] from an analysis of the second derivatives.

Spectra of the fluorescence polarization of spinach, maize and pea chloroplasts at low temperature have been measured before by Garab and co-workers [1,8] and by Vasin and Verkhoturov [14]. All these spectra lack the negative polarization of the band at 695 nm, although they show a depression in the polarization around that wavelength. The spectra of Garab and co-workers were measured at 130 K, at which temperature the intensity of F-695 is low, especially in maize chloroplasts [7,12] and therefore gives only a small contribution to the polarization spectrum. Spectra of pea chloroplasts were measured by Vasin and Verkhoturov [14] at 77 K, but a very dense sample was used in these measurements.

On the basis of the earlier polarization spectra

it has been assumed that of the major emission bands only F-735 shows a significant anisotropy, whereas F-685 and especially F-695 were supposed to show only little orientation [1,8,14]. Our spectra, however, show large anisotropies for all three emission bands, and the calculated angles of orientation differ significantly from the magic angle. These calculations are based on the assumption of perfect orientation of the thylakoid membrane. More realistic assumptions of the degree of order would yield larger deviations from the magic angle.

The overall shape of the LD spectrum (Fig. 5) agrees with spectra obtained earlier with various orientation techniques [3,16–18]. However, our spectrum shows much more detail, especially above 680 nm, presumably because of the lower temperature applied. The same applies to the polarized excitation spectra for PS I and PS II emission. As far as we know, excitation spectra in polarized light or oriented chloroplasts were measured earlier at room temperature only [4,19,22]. Compared to the spectra of Breton [4], that were measured with the same geometry, the spectra measured at 4 K show a much higher resolution, and they indicate a highly organized structure for the PS I and PS II complexes and for the light-harvesting Chl *a*/*b* complex. They show that clear anisotropies are not confined to long-wave absorbing Chl *a* forms, as usually assumed. The main cause of the low LD values of oriented chloroplasts in the short-wave region, especially at room temperature, appears to be the overlap of positively and negatively polarized absorption bands in this region.

Recently, Biggins [23,24] reported that the effect of selective scattering on the LD spectrum may not be negligible as was previously reported [3,25]. By recording excitation spectra of the fluorescence, only the really absorbed quanta are considered and not, as is the case in an absorption spectrum, the apparently absorbed quanta. Polarized excitation spectra are therefore much less sensitive to selective scattering artefacts.

The angles of orientation shown in Table I for the absorption bands at 676 and 710–715 nm agree well with those for F-685 and F-735, respectively, which supports the hypothesis [11] that they belong to the same chlorophylls. For F-695 and the absorption band at 684 nm our results allow only a qualitative comparison: both bands are polarized perpendicular to the membrane.

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