Biochimica et Biophysica Acta, 347 (1974) 443–463

BBA 46742

POLARIZED LIGHT SPECTROSCOPY OF PHOTOSYNTHETIC MEMBRANES IN MAGNETO-ORIENTED WHOLE CELLS AND CHLOROPLASTS

FLUORESCENCE AND DICHROISM

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(Received November 9th, 1973)
(Revised manuscript received February 18th, 1974)

SUMMARY

1. The wavelength dependence of the fluorescence polarization (FP) ratio and dichroism has been studied with magneto-oriented (10–13 kG) whole cells of *Chlorella pyrenoidosa, Scenedesmus obliquus, Euglena gracilis* and spinach chloroplasts suspended in their aqueous growth media (or Tris-buffered sucrose solution in the case of the chloroplasts) under physiological conditions. The FP ratio is defined as the fluorescence intensity polarized parallel divided by the intensity polarized perpendicular to the membrane planes.

2. The FP ratio is typically in the range of 1.2–1.9 in *Chlorella*, 1.20–1.25 in *Scenedesmus* and 1.4–1.5 in spinach chloroplasts at fluorescence wavelengths above 690 nm. Below 690 nm the FP ratio decreases steadily with decreasing wavelength and may be as low as approx. 1.05 at 660 nm. These results are interpreted in terms of the orientation of the Q_y transition moment vectors of the different spectroscopic forms of chlorophyll. For the chlorophyll *a* 680 form these vectors are inclined at angles of 30° or less (in *Chlorella*) with respect to the membrane planes, while the shorter wavelength chlorophyll *a* 670 forms appear to be not nearly as well oriented.

3. The *Euglena* fluorescence peak is red shifted to 714 nm (in the other algae and chloroplasts it is situated at 685 nm) and the FP ratio is approx. 1.20 in the 720–730 nm region and decreases with decreasing wavelength below 720 nm and is only 1.05 at 690 nm. This wavelength dependence is in good qualitative agreement with the fluorescence microscope studies of single chloroplasts of *Euglena* by Olson, R. A., Butler, W. H. and Jennings, W. H. ((1961) Biochim. Biophys. Acta, 54, 615–617).

4. By means of a model calculation it is shown that the high FP ratios observed with *Chlorella* are entirely consistent with the low values of the degree of polarization (0.01–0.06) determined by previous workers with unoriented cell suspensions.

5. The influence of reabsorption and the resulting distortion in the wavelength dependence of the FP ratio are described. The possibility that the fluorescence is

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polarized by scattering artifacts, rather than being a result of the intrinsic orientation of chlorophyll, is considered.

6. Linear dichroism studies with *Chlorella* and spinach chloroplasts confirm the orientation of the Q_y transition moment vectors deduced from the FP ratio. Furthermore, it appears that the porphyrin rings are tilted out of the membrane plane and that the carotenoid molecules tend to lie with their long axes in the lamellar plane.

7. In *Euglena*, dichroism studies indicate that chlorophyll *a* 680 is unoriented, while chlorophyll *a* 695 appears to be oriented similar to chlorophyll *a* 680 in *Chlorella* or spinach chloroplasts, a result which is also in accord with the measured FP ratio of *Euglena*.

8. The possibility that the magnetic field gives rise to the reorientation of individual chlorophyll molecules is shown to be highly unlikely.

INTRODUCTION

It has been shown recently that whole cells of *Chlorella*, *Scenedesmus*, *Euglena* and spinach chloroplasts can be oriented in magnetic fields of 10–20 kG [1]. This technique is useful for the study of the spectroscopic properties of a large number of oriented cells in suspensions under physiological conditions. By staining the lipids in *Chlorella* with the dye rhodamine B it was shown that the lamellae tend to be oriented with their planes perpendicular to the magnetic field [2]. The fluorescence emitted by magneto-oriented cells is strongly polarized and these suspensions exhibit dichroism and anisotropic light scattering as well. These effects are interpreted in terms of an intrinsic orientation of the chlorophyll *a* Q_y transition moment vectors in vivo.

The orientation of pigments and structural proteins in photosynthetic membranes has been studied most recently by Breton et al. [3] who employed a highly sensitive linear dichroism technique. The spinach chloroplasts used in their study were oriented by magnetic fields and by several mechanical methods. The previous [2] conclusion about the orientation of the lamellar planes in magnetic fields was confirmed. The dichroism exhibited by the chloroplasts was qualitatively independent of the method of orientation.

In this article, we report on the characteristics of the fluorescence and dichroism of oriented whole cells of *Chlorella*, *Scenedesmus*, *Euglena* and spinach chloroplasts. On the basis of a theoretical argument and various experimental evidence it is concluded that the observed effects are due solely to the reorientation of whole cells or spinach chloroplasts in the presence of the magnetic field and not to the reorientation of the individual chlorophyll molecules. The magneto-orientation technique is thus capable of revealing intrinsic orientation of pigment molecules in vivo.

The importance of reabsorption of fluorescence in determinations of the wavelength dependence of the polarized fluorescence intensities viewed parallel and perpendicular to the lamellar planes is described. Comparisons are made between fluorescence polarization and dichroism measurements performed by microspectrophotometry of single chloroplasts of *Euglena* [4, 5] and magneto-oriented suspensions of this organism.

The relationship between the fluorescence anisotropy displayed by magneto-

oriented photosynthetic membranes and the degree of polarization, P, which has been measured by many workers [7–12], is described in detail. The quantity P is defined by

$$P = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}} \tag{1}$$

where $F_{\parallel}$ and $F_{\perp}$ refer, as usual, to the fluorescence components polarized parallel and perpendicular respectively to the polarization of the exciting light beam $I_{\parallel}$, where $I_{\parallel}$ and the fluorescence viewing directions are at right angles to each other. P values have been measured with suspensions of unoriented cells [7–12] and are in the range of 0.01–0.06 and it is shown here that these low P values are entirely consistent with the highly anisotropic fluorescence which is observed when the cells are oriented in magnetic fields.

The possibility of a scattering artifact which could give rise to a fluorescence polarization even in the absence of a preferred orientation of the chromophores is considered. We show that the highly polarized fluorescence is due to an intrinsic orientation of the chlorophyll molecules, and not to scattering and a preferential polarization of the scattered light. The possible contribution of this effect is determined by measuring the depolarization of light incident on and scattered by *Chlorella* cells. Little preferential depolarization is found and it is concluded that this artifact cannot account for more than 10–20% of the overall fluorescence anisotropy, which is therefore indeed attributable to an intrinsic orientation of chlorophyll in vivo.

The linear dichroism spectra of suspensions of *Chlorella*, *Euglena* and spinach chloroplasts are subject to artifacts such as textural dichroism, scattering and the Duysens flattening effect [13]. These effects are considered and cannot reasonably account for the pronounced linear dichroism which is observed.

EXPERIMENTAL

The growth and sources of the algae used in this work, the method of preparation of the spinach chloroplasts, and the methods of measuring the polarized fluorescence emitted by whole cells in aqueous suspensions in the presence of magnetic fields, have been previously outlined [1]. A 750-W tungsten lamp and a blue Corning C.S. No. 4-72 broad band pass filter were employed for the excitation of the fluorescence. The exciting light and the fluorescence were coupled to the lamp and photomultiplier (RCA 7164R) respectively by means of 2-m long light guides, to avoid effects of stray magnetic fields. The fluorescence was viewed through HNB'P sheet polarizers (Polaroid Corp.), a Corning C.S. No. 2-64 640 nm red cut-off filter and a grating Monochromator (3.2 Å/nm). A light chopper in the exciting beam and a lock-in amplifier (Princeton Applied Research, H126) were used for enhancement of signal to noise ratio. The degree of polarization P was determined by means of a motor-driven polaroid sheet assembly which is described in detail elsewhere [14]. Both components of the fluorescence $F_{\perp}$ and $F_{\parallel}$ (see Eqn 1) were displayed simultaneously on a chart recorder (the lock-in amplifier was not used in these experiments) to measure the time dependence of P following removal of the magnetic field (Fig. 9).

The apparatus used in the linear dichroism measurements has also been described

in detail in a previous paper [1]. The thickness of the cuvettes used was either 1.5 or 10 mm. A slight modification was introduced in the apparatus by placing a Shibata [15] -type scatterer (a sheet of filter paper soaked in silicone oil) to enhance the collecting angle of the transmitted light and thus to minimize the distortions due to selective light scattering.

The apparatus used to measure the depolarization of the scattered light was similar to the one used for the measurement of the fluorescence, except that the blue broad band pass C.S. 4-72 filter in the excitation light path was replaced by a second monochromator. Both monochromators were at the same wavelength setting when the wavelength dependence of the depolarization of the scattered light was measured (Figs 6 and 7). For the experiment in Fig. 8, which gives the spectral composition of the scattered light for a given excitation wavelength (658 nm), the excitation monochromator was fixed, while the setting of the monochromator viewing the scattered light was varied. The direction of viewing of the scattered light was at 90° angles to the incoming light beam. However, both light beams were deliberately poorly collimated in order to determine the depolarization ratio for a wide range of scattering angles (about 90 ± 30 ° in these experiments).

In order to reduce the effects of the phototactic motion of *Euglena* in the fluorescence polarization and dichroism experiments, the cells were suspended in their usual growth medium to which Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), which is a high-molecular-weight water-soluble polymer, had been added. The viscosity of the suspension was 20 cP.

RESULTS AND DISCUSSION

The dependence of the fluorescence polarization on particle density

The fluorescence polarization (FP) ratio is defined here as the ratio

$$\mathrm{FP} = \frac{F(_{\parallel}M)}{F(_{\perp}M)} \tag{2}$$

where $F(_{\parallel}M)$ is the intensity of the fluorescence polarized parallel to the membrane plane, while $F(_{\perp}M)$ is the fluorescence intensity polarized perpendicular to the lamellar plane. Both are viewed along the direction of a vector lying in the membrane plane. The $F(_{\parallel}M)$ and $F(_{\perp}M)$ directions correspond to the analyzer direction being perpendicular $F(_{\perp}\vec{H})$ and parallel $F(_{\parallel}\vec{H})$ to the magnetic field respectively. In a previous paper [1] the FP ratio was defined as $F(_{\perp}\vec{H})/F(_{\parallel}\vec{H})$ and we deliberately introduce here a change in notation where $F(_{\parallel}M) \equiv F(_{\perp}\vec{H})$ and $F(_{\perp}M) \equiv F(_{\parallel}\vec{H})$. The relationship between the direction of the magnetic field, the orientation of the lamellar planes [1–3] and the mode and directions of observation of the fluorescence are depicted in Fig. 1(a).

The FP ratio which can be determined only when the cells are oriented, is always greater than unity, which reflects the preferred orientation of the fluorescence emitting chlorophyll *a* oscillators close to or within the planes of the membranes. It is basically independent of the geometry and wavelength of excitation.

Other quantities of interest are $F(_{\perp}\vec{H})/F_{(o)}$ and $F(_{\parallel}\vec{H})/F_{(o)}$ which represent the changes in fluorescence intensity viewed through analyzers oriented perpendicular

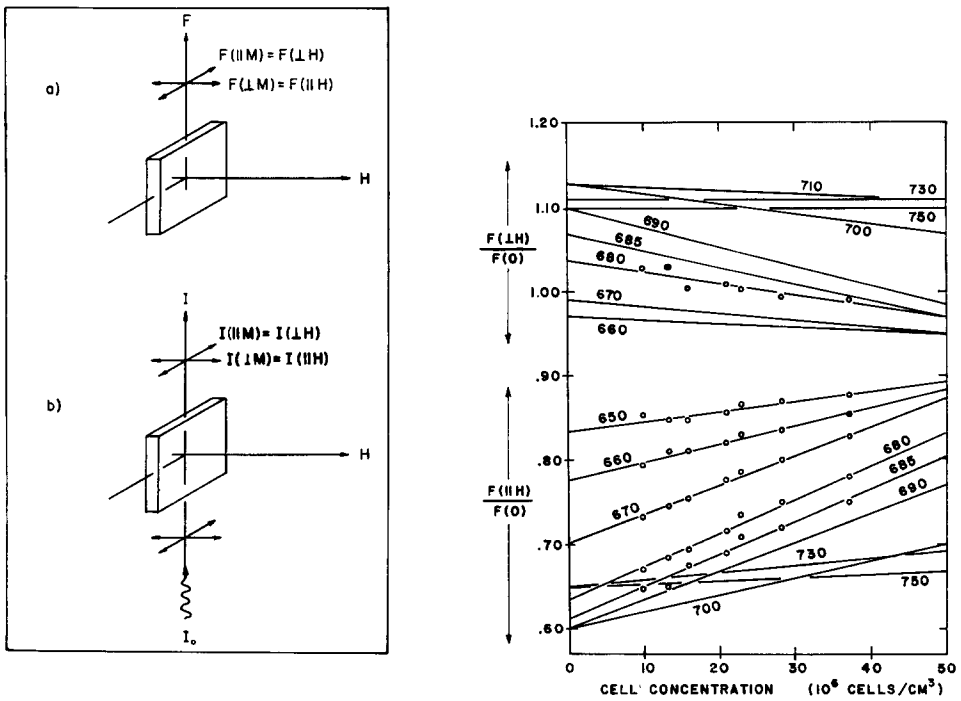


Fig. 1. (a) Orientation of lamellar planes in a magnetic field, the mode of observation of fluorescence and the notation. The fluorescence polarization ratio is defined as $FP = F(_{\parallel}M)/F(_{\perp}M) = F(_{\perp}\vec{H})/F(_{\parallel}\vec{H})$, where M is the membrane plane and $\vec{H}$ the magnetic field direction. (b) Mode of measurement of transmitted light intensities, the wavelength dependence of which is plotted in figure 3b.

Fig. 2. Polarized fluorescence intensity ratios referred to the fluorescence intensity in the absence of a magnetic field, $F_{(o)}$, as a function of *Chlorella* cell density. The field strength was 10.5 kG. (Many experimental points have been omitted for clarity.)

and parallel to $\vec{H}$ respectively and where $F_{(o)}$ is the fluorescence intensity in the absence of the magnetic field when the cells are unoriented and provides a convenient reference intensity level. These quantities allow additional insight into the anisotropic characteristics of membranes.

The intensity of the polarized fluorescence is influenced by scattering and reabsorption of the fluorescent light within each cell before it emerges from the particle, and also by scattering and reabsorption by other cells before the light enters the detector. The influence of inter-particle effects can be determined by carrying out experiments at different particle concentrations. The characteristics of single particles can be deduced by extrapolating the experimental plots to zero-particle densities.

The concentration dependence of the magnetic field induced fluorescence intensity changes of *Chlorella* suspensions is shown in Fig. 2. With the analyzer in the $F(_{\perp}\vec{H})$ orientation (Fig. 1(a)) there is generally an increase in the measured fluorescence intensity (although there is a decrease at some wavelengths), while there is a decrease in the $F(_{\parallel}\vec{H})$ orientation. The slopes of the lines in Fig. 2 are steepest at wavelengths

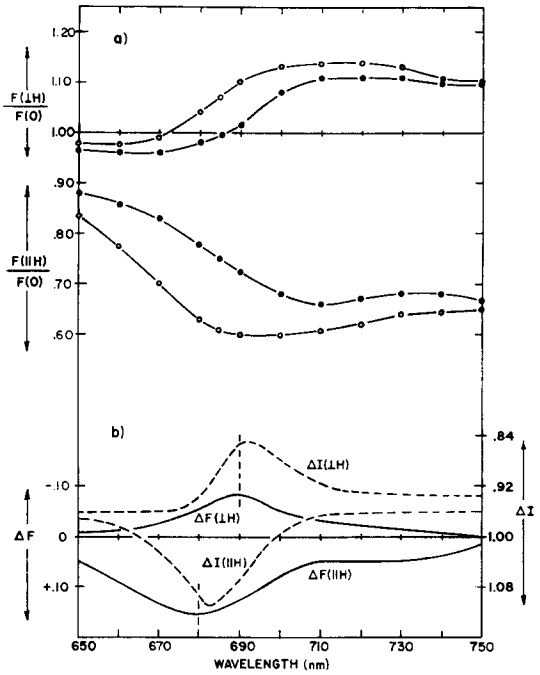


Fig. 3. (a) Fluorescence intensity ratios as a function of wavelength at two different particle densities taken from the data in Fig. 2: ○ · · · ○, "zero" particle density and ● - - - ●, $38 \cdot 10^6$ cells/cm^3. (b) ——— · · ·, the differences between the two sets of curves in (a). - - - - - -, $\Delta I = I(\boldsymbol{H})/I_0$, where $I(\boldsymbol{H})$ and I_0 are the intensities of light transmitted in the presence of a magnetic field (10.5 kG) and the absence of a field respectively; pathlength 1.5 mm, cell density $22 \cdot 10^6$/cm^3.

of strong absorption and light scattering [16] (670–690 nm). Van Nostrand [17] has shown that the slopes of the lines in Fig. 2 can be quantitatively related to the changes in optical density of *Chlorella* cell suspensions when the magnetic field is applied. This is a consequence of the inter-particle reabsorption and scattering effects which is demonstrated in Fig. 3. The $F(_{\perp}\vec{H})/F_{(o)}$ and $F(_{\parallel}\vec{H})/F_{(o)}$ values are plotted in Fig. 3(a) for two cases: (1) for a particle concentration of $38 \cdot 10^6$ cells/cm^3, and (2) extrapolated to "zero" particle density. The differences between (2) and (1) are plotted in Fig. 3(b). It is evident that with maxima at 680–690 nm, these two curves resemble the absorbance (or transmittance) of *Chlorella* suspensions. In order to appreciate this resemblance more fully, the ratios $I(_{\perp}\vec{H})/I_0$ and $I(_{\parallel}\vec{H})/I_0$ are also plotted in Fig. 3(b). The quantities $I(_{\perp}\vec{H})$ and $I(_{\parallel}\vec{H})$ are the intensities of a light beam which has passed through a 1.5 mm thick suspension of *Chlorella* cells ($22 \cdot 10^6$ cells/cm^3). I_0 represents the intensity of this beam when there is no magnetic field present. This date was taken from ref. 1, Fig. 3. Since the fluorescence light also must traverse a given thickness of the suspension before it emerges from the cuvette and is collected by the detector, there is indeed a resemblance between the $F(\vec{H})/F_{(o)}$ and $I(\vec{H})/I_0$ curves as is illustrated in Fig. 3b.

It should be noted that in the $F(_{\perp}\vec{H})$ orientation, i.e. light polarized parallel to the membrane planes, the maxima in both curves in Fig. 3(b) are shifted further

to the red (687–692 nm) than in the $F(\parallel \vec{H})$ orientation (680–684 nm). The actual absorption maximum is at 677 nm. These shifts are a consequence of enhanced reabsorption and increased flattening effect [14] for light propagating in a direction lying in the membrane plane and with a polarization parallel to this plane. Scattering also tends to shift these maxima to the red [16].

The wavelength dependence of the fluorescence polarization ratio

The FP ratio is an index of the orientational anisotropy of the chlorophyll *a* fluorescence emitting Q_y oscillators. The orientational anisotropy is due to an intrinsic preferred orientation of the Q_y transition moment vectors in the planes or close to the planes of the lamellae. This FP ratio, calculated for "zero"-particle density is plotted for *Chlorella* in Fig. 4(a). It is evident from Fig. 2 that the laborious procedure of determining the fluorescence polarization ratios at a number of particle densities and extrapolating to zero particle density is not necessary to obtain fairly accurate representations of this quantity for a single particle. It is sufficient to measure FP at two fairly low cell concentrations to ensure that there are no significant differences due to reabsorption. The results obtained with *Scenedesmus*, spinach chloroplasts and *Euglena* at fairly low cell densities are also shown in Fig. 4.

Reabsorption effects within a single cell or chloroplast can in principle give

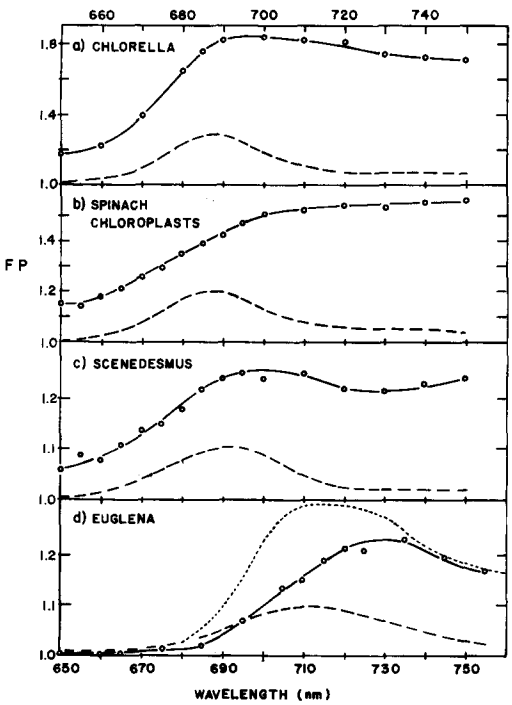


Fig. 4. ——···, fluorescence polarization ratio (FP) as function of wavelength; (a)–(c), *C. pyrenoidosa*, *S. obliquus* and spinach chloroplasts at 10.5 kG; (d) *E. gracilis*. ○- - -○, magnetooriented *Euglena* at 13.5 kG; - - - - - -, data taken from the microspectrofluorimetry study of Olson et al. [4]. – – –, fluorescence emission curves.

rise to a distortion of the wavelength dependence of FP. At the absorption maximum of 677 nm a single *Chlorella* cell is estimated to absorb 60% of incident unpolarized light [13]. For polarized light propagating along a membrane plane and with polarization parallel to this plane the absorption of light must be much higher than 60%. However, the absorption coefficient falls off rather strongly at the short and long wavelengths of the absorption band centered at 678 nm. At wavelengths above 700 nm the influence of reabsorption is negligible, and is not nearly as strong in the 650–660 nm region as it is in the 680–690 nm range. These conclusions are supported by the small slopes, (the steepness is a measure of the absorbance of a single cell), at 650–660 and above 700 nm in Fig. 2. In the long wavelength region FP tends to be large, while it is low at 650–660 nm. This large difference cannot be attributed to reabsorption effects.

Another possible artifact which can distort the FP curves is the wavelength dependent selective light scattering effect [18]. This effect is related to the anomalous dispersion in the region of the absorption maximum. The variations in the scattering intensity in the 650–670 nm region, are minor, while FP is dropping strongly with decreasing wavelength [18]. Furthermore, if FP were directly linked to some artifact which is a function of the complex refractive index, which varies strongly with wavelength, FP should exhibit a maximum at 690 nm and then drop precipitously in the 700–720 nm range, and also fall off sharply below 690 nm. While a small decrease above 700 nm is indeed observed in *Chlorella* and *Scenedesmus* which resembles in shape the scattering curve, such an effect is not observed with spinach chloroplasts. According to our own observations and those of others (Breton, J., personal communication), spinach chloroplasts are much less effective in scattering light than whole cells of *Chlorella*. Accordingly we attribute the small dip beyond 700 nm, but not the entire structure in FP, in *Chlorella* and *Scenedesmus* to an artifact which is probably related to the anomalous dispersion and/or selective light scattering (see below). The generally high FP ratios above 700 nm and the decrease in this quantity below 680 nm is attributed to the orientation of the Q_y oscillators of the different spectroscopic forms [19] of chlorophyll in vivo.

The short wavelength emitting spectroscopic forms (chlorophyll *a* 670) of chlorophyll appear to have a lower degree of orientation with respect to the lamellar planes than the long wavelength spectroscopic forms (chlorophyll *a* 680, 695). Most of the fluorescent pigments appear to be oriented in such a way that the Q_y transition moment vectors have a larger projection parallel to the membrane plane than along the normal to this plane. The experiments described here and elsewhere [1–3] are not capable of discerning any preferred orientation within this plane, even if it exists, since the magnetic field direction is the only experimental symmetry axis. It should be pointed out that Breton et al. [3] have also concluded on the basis of their linear dichroism studies that chlorophyll *a* 670 appears to be less oriented than the higher wavelength forms.

The fluorescence polarization ratio and the orientation of chlorophyll a

The highest FP ratio which we observed was about 1.9 with whole cells of *Chlorella pyrenoidosa*. However, a value of 1.5–1.6 is more typical and occasionally low values of approx. 1.20 are observed. This quantity can in principle be quantitatively related to the orientation of the transition moment vectors of chlorophyll *a* 680

which can be presumed to be the most important fluorescence emitting species in this alga. However, the measured FP ratio in our magneto-orientation experiments are minimum values. The chloroplast in *Chlorella* is cup-shaped and exhibits curvature. Since the lamellae tend to be perpendicular to the magnetic field [2], the "walls" of the cup-shaped chloroplast tend to be perpendicular to the field, while the "bottom" tends to be parallel.

Because of the non-perfectly planar shapes of the lamellae even in spinach chloroplasts, and because of their non-perfect orientation in the magnetic field (the lamellar planes are not all parallel to each other), the experimentally observed FP values are lower limits to the actual FP values for a single photosynthetic unit. The actual orientational anisotropy of chlorophyll should therefore be higher than the value calculated from the experimentally measured FP ratio.

Using the value of 1.9 for the FP ratio, a simple calculation shows that the Q_y oscillators of chlorophyll *a* 680 are inclined at angles greater than approx. 60° with respect to the normal to the lamellar planes. Since FP = 1.9 is a minimum value, the oscillators are probably oriented closer to the plane than the 30° indicated by this calculation. Calculations based on linear dichroism data by Breton et al. [3] indicate that the Q_y transition moments of chlorophyll *a* 680 are inclined at angles of 60–65 ° with respect to the normal to the membrane planes, which is in good agreement with the orientation calculated from the FP ratio.

A comparison between a microscope study and magneto-oriented suspension study of fluorescence polarization

The fluorescence of whole algal cells was examined with a polarized light microscope by Ruch [20] Goedheer [21, 22] and Olson et al. [4, 23]. Some evidence of orientation of chlorophyll in vivo was found, even though Ruch attributed part of the polarization to a textural effect (bifluorescence induced by the anisotropic optical properties of the lamellae). Olson et al. [4] examined the fluorescence of *Euglena* and *Mougeotia* single chloroplasts and found that $F(_{\parallel}M) > F(_{\perp}M)$ when the chloroplasts were viewed on edge. They expressed their results in terms of the quantity P' which was defined by

$$P' = \frac{F(_{\parallel}M) - F(_{\perp}M)}{F(_{\parallel}M) + F(_{\perp}M)} \tag{3}$$

P' was close to zero at 680 nm, increased to a maximum at approx. 716 nm and then decreased again for wavelengths above 720 nm.

In order to compare our FP results obtained with magneto-oriented *Euglena* suspensions with those of Olson et al., we have recalculated their P' data to give the FP ratio as defined by Eqn 2. Their results as well as ours are plotted in Fig. 4(d). The shape of the two curves are similar although the FP of the magneto-oriented suspension is somewhat lower and red-shifted. The lower FP of magneto-oriented *Euglena* suspensions is at least in part due to the fact that each cell has 4–5 chloroplasts which are probably not all perfectly aligned with respect to the magnetic field.

Even though the main absorption band in *Euglena* is at approx. 680 nm, as it is in *Chlorella*, *Scenedesmus* and spinach chloroplasts, it is well known that the fluorescence emission maximum can be considerably red shifted in *Euglena* [24]. In our samples of *Euglena* the fluorescence maximum was at 714 nm which was at

longer wavelengths than the maxima observed with the other algae and spinach chloroplasts which we have studied. Besides the main 680 nm absorption band in *Euglena*, there is an additional absorption band at 695 nm which appears as a shoulder on the long wavelength side of the main absorption maximum [25]. The low FP ratio at short wavelengths (680–700 nm) indicates that the orientation of the shorter wavelengths spectroscopic forms of chlorophyll in *Euglena* tends to be low. Indeed, dichroism studies with magneto-oriented *Euglena* cells show that the bulk pigments absorbing at approx. 680 nm are unoriented (no dichroism) while the 695 nm absorbing pigment forms (chlorophyll *a* 695) are oriented, i.e. exhibit dichroism (see below).

Orientational anisotropy (FP) and the degree of polarization (P)

In the past, the experimentally measured low values of P defined in Eqn 1 have been interpreted in terms of efficient energy transfer in the photosynthetic unit coupled with a low degree of orientation of chlorophyll molecules in vivo. In this section it is shown that the high FP ratios of up to 1.9 are consistent with the low values of the degree of polarization of $P = 0.01$–0.06 previously reported.

The previously reported values of P were obtained with suspensions of randomly oriented cells or chloroplasts. A possible exception may be Lavorel's work [8], in which the *Chlorella* cells may have been partially oriented in his flow system [10]. We have shown previously that measured values of P with oriented *Chlorella* cells depend strongly on the excitation and viewing directions and that much higher P values can be obtained with oriented cells than with unoriented cells [1]. These effects are due to the orientational anisotropy. When the fluorescence is viewed along the magnetic field direction, i.e. in a direction along the normal to the lamellae, the orientational effect is minimized and the true P value, with minimal interference from the orientational effect, can be estimated [14].

In order to show that low P values as defined by Eqn 1, and high FP values as defined by Eqn. 2 are mutually consistent, a simple model calculation can be performed. The chlorophyll molecules in vivo in the photosynthetic lamellae, for the purposes of this calculation, are replaced by a simple three dimensional oscillator model with orthogonal vectors $\vec{a}_1$, $\vec{a}_2$ and $\vec{a}_3$ which specify the emitting and absorbing transition moment vectors of chlorophyll. Since experimentally, no preferred direction within the lamellar planes is detectable, the latter shall be defined by the orthogonal vectors $\vec{a}_1$ and $\vec{a}_2$ with $\vec{a}_1 = \vec{a}_2 \neq \vec{a}_3$; the normal to this plane is specified by $\vec{a}_3$. The fluorescence intensities are proportional to the squares of the vectors ${a_1}^2$, ${a_2}^2$ and ${a_3}^2$ and we set $F(_{\parallel}M) = {a_1}^2 = {a_2}^2$ and $F(_{\perp}M) = {a_3}^2$. The relationship between the experimentally observed FP ratio and the model vector system is thus

$$\mathrm{FP} \rightarrow a_1^2/a_3^2 = a_2^2/a_3^2 \tag{4}$$

It is assumed that light is absorbed and emitted by all three oscillators and that energy absorbed by any one oscillator is redistributed (by energy transfer) among all three oscillators and the probability of emission of each oscillator is proportional to ${a_i}^2$, where a_i is the length of each vector. The relationship between the model and the experiment is that $a_1 = a_2 > a_3$ since FP > 1.0.

Similar calculations of P have been performed by Hemenger and Pearlstein [26] who have considered various cases of energy transfer in relation to the dimensionality and ordering of chlorophyll in the photosynthetic unit.

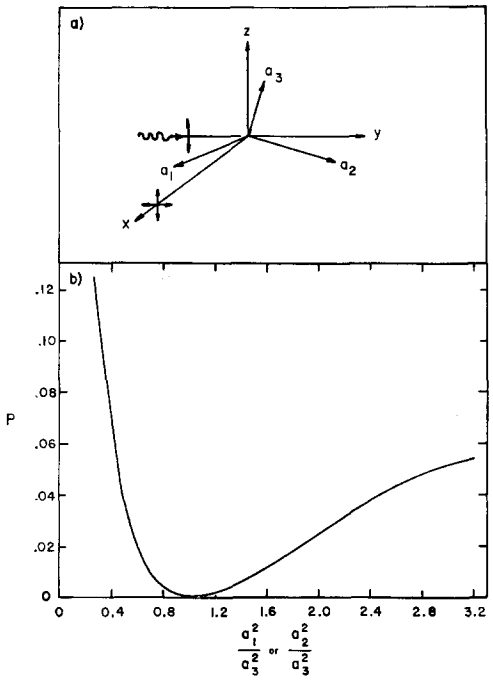


Fig. 5. Model calculation of the degree of polarization (P) as a function of the orientational anisotropy parameter a_1^2/a_3^2, where the latter is assumed to represent the fluorescence polarization ratio (FP). (a) The model, and (b) the result of the calculation using Eqns 1, 7 and 8.

It is now assumed that the orthogonal system $\vec{a}_1$, $\vec{a}_2$, $\vec{a}_3$ is randomly oriented with respect to the laboratory axes $\vec{x}$, $\vec{y}$, $\vec{z}$ in which the excitation and fluorescence viewing directions are defined (Fig. 5(a)).

The two fluorescence components defined in Eqn 1 are given by

$$F_{\parallel} = F_z = \{\sum_{i=1}^{3} a_i^2 \cos^2(a_i, z)\}\{\sum_{i=1}^{3} a_i^2 \cos^2(a_i, z)\} \tag{5}$$

$$F_{\perp} = F_y = \{\sum_{i=1}^{3} a_i^2 \cos^2(a_i, z)\}\{\sum_{i=1}^{3} a_i^2 \cos^2(a_i, y)\} \tag{6}$$

The first term enclosed in the brackets specifies the absorption of light by each of the three vectors which is a function of the direction cosines of the angles between $\vec{a}_i$ and $\vec{z}$, and the second term between brackets gives the contribution of each oscillator to the $\vec{z}$ or $\vec{y}$ polarized fluorescence intensities.

After averaging over all possible orientations [27] of the orthogonal system of oscillators and setting $a_1 = a_2$ the following expressions are obtained:

$$F_{\parallel} = 3(2a_1^4 + a_3^4) + 2(a_1^4 + 2a_1^2 a_3^2) \tag{7}$$

and

$$F_{\perp} = 2a_1^4 + a_3^4 + 4(a_1^4 + 2a_1^2 a_3^2) \tag{8}$$

Substituting Eqns 7 and 8 into Eqn 1, the degree of polarization P can be

calculated as a function of the anisotropy parmeter a_1^2/a_3^2. The results are plotted in Fig. 5(b). In the limiting cases, the following well known results are obtained: (1) Three-dimensional oscillator system with $a_1^2 = a_2^2 = a_3^2$: $P = 0$; (2) two-dimensional oscillator with $a_1^2 = a_2^2$ and $a_3^2 = 0$: $P = 1/7$; (3) one-dimensional oscillator with $a_3^2 \neq 0$ and $a_1^2 = a_2^2 = 0$: $P = 1/2$.

In the model calculation shown in Fig. 5(b), if we set a_1^2/a_3^2 equal to the maximum observed experimental FP ratio of 1.9, a P value of approx. 0.02 is obtained which appears to be in reasonable agreement with recent measurements of P in *Chlorella* [9]. However, the previous experimental determinations of P were carried out with exciting light of wavelengths below 650 nm. Since the fluorescence emitting Q_y oscillators absorbing in the 670–680 nm region were not excited directly, these P values tend to be low. Furthermore, recent experiments [14, 28] indicate that P is higher upon excitation within the red band (670–680 nm) than below these wavelengths. Any quantitative correlation between experimental values of P and FP ratios and comparisons with Fig. 5(b), thus require that the P values refer to red band excitation.

In any case, the calculation indicates that the intrinsic orientation of chlorophyll can contribute significantly to P values measured with randomly oriented cells or chloroplasts. A more detailed discussion of this subject has been published elsewhere [14].

Light scattering and the fluorescence polarization ratio

In this section we consider the possibility that a fluorescence photon emerging from a given chloroplast or cell can be scattered and depolarized to such an extent that artificially high values of FP ratios are produced, assuming the chlorophyll molecules are randomly oriented.

In principle, scattering can be reduced by matching the refractive indices of the two layers at the boundary of which the scattering occurs. Increasing the refractive index of the solution in which the *Chlorella* cells were suspended by adding sucrose (0–0.4 M) had no measurable effect on the FP ratio within the experimental reproducibility of $\pm 5\%$. However, it is likely that the refractive indices in the interior of the lamellar structures are not sufficiently modified to give rise to any changes in the light scattering.

We attempted to estimate the contribution of scattering of fluorescence to the experimentally determined FP ratios by measuring the extent of depolarization of incident light scattered at angles of 90 ± 30 °. In these experiments *Chlorella* cells were used because they are more efficient scatterers of light than spinach chloroplasts. Incident light is scattered selectively [18] exhibiting a wavelength dependence which is related to the anomalous dispersion characteristics [21] of the chloroplasts. For example, the scattering intensity exhibits a peak and a valley on the long and short wavelength sides respectively of the 678 nm absorption band in *Chlorella* [18].

In our own scattering depolarization experiments we assume that incident light and fluorescent light are scattered and depolarized by the lamellar chloroplast structures in a similar manner. Any selective polarization of the fluorescence by scattering should in this case be paralleled by a selective depolarization of the scattered light.

The schematic experimental arrangement is shown in Figs 6(a) and 7(a). The *Chlorella* cells were oriented in a magnetic field of 13 kG. The polarized

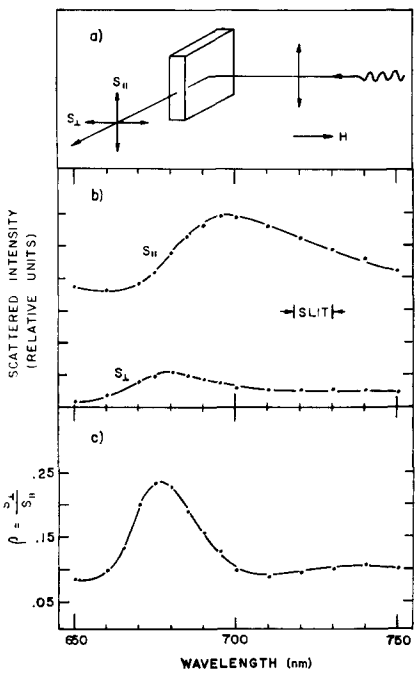


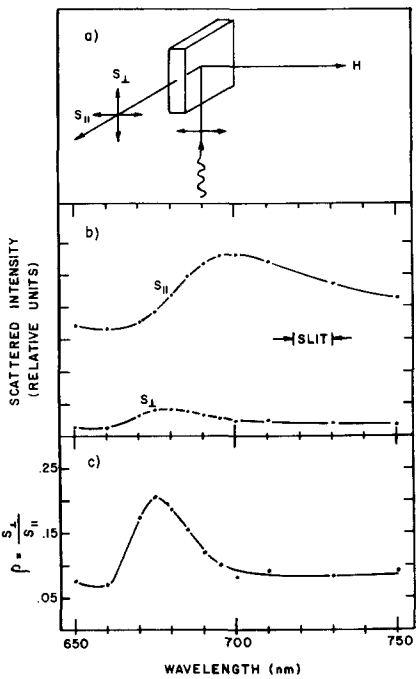
Fig. 6. Depolarization of scattered light as a function of wavelength. (a) Experimental configuration; actually the detector collected light scattered at angles of $90 \pm 30°$. (b) The wavelength dependence of the polarized ($S_{\parallel}$) and the depolarized components ($S_{\perp}$). (c) The ratio of the two components. *Chlorella* oriented with a field of 13.5 kG, cell density $2 \cdot 10^7$ cm^{-3}.

scattering component is termed $S_{\parallel}$ and the depolarized component $S_{\perp}$. In order to account for the high FP ratios observed by scattering, and if our assumptions are correct, $S_{\perp}$ would have to be quite large and should tend to be largest for light scattered and polarized parallel to the membrane planes. The depolarization of the scattering light characterized by the ratio

$$\rho = \frac{S_{\perp}}{S_{\parallel}} \tag{9}$$

should vary with the geometric configuration. ρ is a measure of the anisotropy of the scattering system. This quantity varied from about 4–25% depending mainly on the wavelength. Furthermore ρ changed by up to 20% when the magnetic field was removed and the cells were allowed to randomize. This effect was wavelength dependent and is a further indication of the intrinsic anisotropic properties of the membranes. However, a more detailed discussion of this point is beyond the scope of this article.

The wavelength dependence of the intensity of the two components $S_{\parallel}$, $S_{\perp}$ and their ratios ρ are plotted in Fig. 6(b,c) and 7(b,c). The $S_{\parallel}$ curves, which represent the polarized component of the scattered light, are similar to the curve published by Latimer and Rabinowitch [18]. The $S_{\perp}$ curves exhibit a maximum at 678 nm which



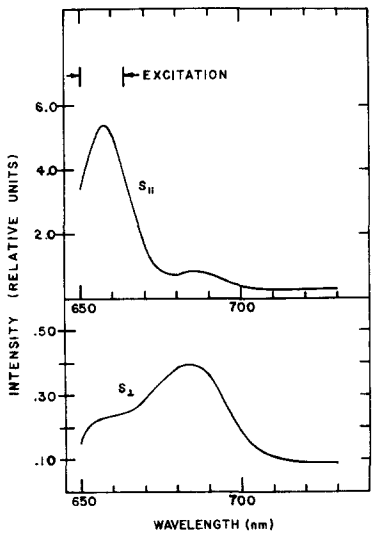


Fig. 7. Same as in Fig. 6, but different direction of incident light beam.

Fig. 8. Spectral composition of $S_{\parallel}$ and $S_{\perp}$. The excitation wavelength was fixed at 658 nm, and the viewing monochromator wavelength was varied. Experimental configuration as in Fig. 6.

coincides with the absorption maximum; there is a corresponding peak in ρ in the same wavelength region.

The peak in ρ at 678 nm can be shown to be due to fluorescence. Since the intensity of the $S_{\perp}$ component is low, the fluorescence contributes a sizeable amount to the total $S_{\perp}$ signal in wavelength regions where the absorbance, and thus the probability of absorption of light, are relatively high, i.e. at the maximum of the absorption band. The contribution of the fluorescence to the scattered light is most easily seen by keeping the excitation wavelength fixed at 658 nm and by varying the wavelength of the viewing monochromator and thus examining the spectral purity of the scattered light. The spectral composition of $S_{\parallel}$ and $S_{\perp}$ with 658 nm excitation is shown in Fig. 8. In the $S_{\parallel}$ spectrum the main scattering intensity peak occurs at the 658 nm wavelength, but a small peak at 685 nm which is due to fluorescence, can also be seen. The latter is much more prominent in the $S_{\perp}$ curve.

Similar experiments at wavelengths above 700 nm have shown that the contribution of fluorescence to ρ in this wavelength region is negligible. At shorter wavelengths fluorescence contributes significantly to the $S_{\perp}$ component and ρ is therefore not a reliable measure of depolarization by scattering. Therefore only the results above 700 nm may be considered to give reliable values of ρ. This quantity was found to vary between 4 and 12%, depending on the particular configuration of the incident and scattered light directions with respect to the lamellae, and on the particular sample of *Chlorella*.

Using these values of ρ (for wavelength above 700 nm only), it is estimated that the contribution of scattering to the FP ratio above 700 nm cannot be more than 10–20%. Because of difficulties in measuring fluorescence-free ρ ratios below 700 nm, no definite conclusions can be reached for shorter wavelengths. However, the FP ratio for *Chlorella* in Fig. 4(a) does appear to have a peak at approx. 690–710 nm and then a slight decrease at longer wavelengths. Because the shape of this curve is somewhat reminiscent of the selective light scattering curve of Latimer and Rabinowitch [18], it is possible that this wavelength dependence of FP is due to scattering as discussed here. However, if scattering would account for the entire wavelength dependence of FP, the decreases in FP should be equally sharp on both the short and long wavelength sides of the scattering peak at 695 nm. This is not the case, since below 690 nm the decrease in FP is very sharp, while above 710 nm the decrease in FP is weak and appears to set in about 15 nm beyond the 695 nm scattering intensity maximum. Furthermore, in spinach chloroplasts, which are known to be much less effective than *Chlorella* cells in the scattering of light, the FP ratio is constant for wavelengths above 700 nm (Fig. 4(c)), while the short wavelength decrease is still present.

On the basis of these considerations we conclude that scattering may contribute no more than one-fifth of the overall fluorescence polarization ratio of 1.9 in *Chlorella*. However, this effect is smaller in spinach chloroplasts, and in general cannot account for the large FP ratios and cannot explain the sharp decrease in the FP ratios below 680 nm. The FP ratio, therefore, appears to be a good measure of the intrinsic orientation of the chlorophyll Q_y transition moment vectors in vivo.

On the possibility of the reorientation of pigment molecules by the magnetic field

In a previous paper [1] the possibility was raised that the observed fluorescence polarization and dichroism effects are due to a reorientation of individual chlorophyll molecules (or chlorophyll–protein complexes) by the magnetic field. If this were the case, it would not be possible to reach any conclusions about the intrinsic orientation of chlorophyll in vivo. This possibility (reorientation of individual molecules by the magnetic field) can be eliminated on both theoretical and experimental grounds.

We have previously demonstrated experimentally that chlorophyll molecules in benzene solution cannot be oriented by magnetic fields of up to 145 000 G [29]. If magnetic orientation of a particle of volume V and diamagnetic susceptibility anisotropy $\Delta\chi$ is to occur, the magnetic potential energy U should be at least ten times larger than the thermal energy kT [1]. The potential energy is given by

$$U = -\tfrac{1}{2}\Delta\chi V\boldsymbol{H}^2 \tag{10}$$

where $\boldsymbol{H}$ is the magnetic field strength. We assume that V is approx. 10^{-20} cm^3 (which is larger than the volume of a chlorophyll molecule) and $\Delta\chi$ is approx. $-5 \cdot 10^{-4}$ which is a reasonable value for a chlorophyll molecule [2]. Since U/kT is approx. 10, the corresponding field strength must be greater than 600 000 G in order to bring about an orientation of such a particle. This is a much larger field than is used in these experiments (about 10 000 G), and indeed much larger than the maximum magnetic field presently available in any laboratory.

There is no doubt that entire particles or cells are oriented by the magnetic field, since the relaxation to thermal equilibrium after the orienting field is removed

is a function of the external viscosity [1]. If reorientation of pigments inside the cells were to occur also, and if this reorientation effect were to account for the magnetic field induced dichroism and fluorescence polarization observed in our experiments, it should also manifest itself in an enhanced degree of polarization P (defined by Eqn 1) in the presence of the magnetic field. The very fact that we observe a decrease in P when the particles are magnetically oriented and when the fluorescence is viewed along the normal to the membrane planes [14], is an argument against this possibility. Furthermore, it is shown in Fig. 9 that the relaxation of P after the field is removed is a function of the external viscosity. While the results shown in Fig. 9 were obtained with spinach chloroplasts, identical results are obtained with whole cells of *Chlorella*. The parallel and perpendicular fluorescence components $F_{\parallel}$ and $F_{\perp}$ (Eqn 1) are also plotted in Fig. 9 and it is evident that all three quantities P, $F_{\perp}$ and $F_{\parallel}$ increase or decrease at the same rate, within experimental error.

The overall decrease in the fluorescence intensity in Fig. 9 when the magnetic field is removed is due to the excitation and viewing geometry [1], while the increase in P after the magnetic field has been removed is due to the orientational anisotropy, which is discussed above.

Finally, Breton et al. [3] have reported that the linear dichroism spectra obtained with spinach chloroplasts is independent of the method of orientation. In

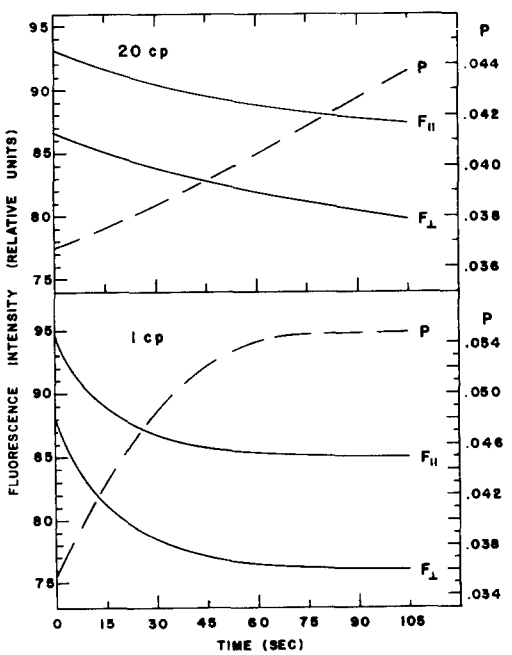


Fig. 9. Relaxation of the degree of polarization P and the fluorescence components $F_{\parallel}$ and $F_{\perp}$ (all defined by Eqn 1) after removal of the magnetic field (13.5 kG) at $t = 0$, at two different viscosities. (In the upper figure the relaxation is incomplete at $t = 105$ s). Spinach chloroplasts, Ficoll added to the suspensions to change the viscosity. Excitation with a Corning CS2-64 (640 nm cut-off) and 670 nm (FWHM 10 nm) interference filter. Viewing 720 nm (FWHM 10 nm) and Oriel 700 nm cut-off filter. Stray light was less than 1 % and a small correction [14] for the intrinsic polarization of the apparatus was made. FWHM, full width at half maximum.

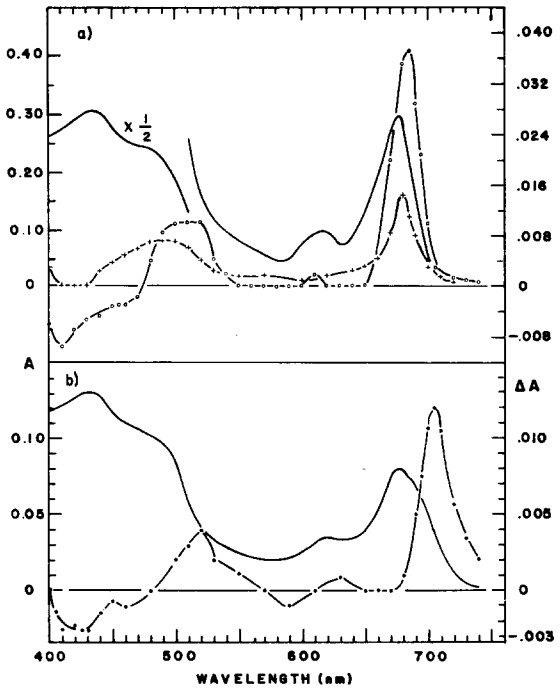


Fig. 10. Linear dichroism (ΔA) and absorbance (A) spectra. (a) ○–○, *Chlorella*, $\Delta A/A$ (683 nm) approx. 0.12, $4.5 \cdot 10^8$ cells/cm^3, 1.5-mm cuvette, the A curve is that of the *Chlorella* suspension. + – +, spinach chloroplasts (10-mm cuvette), $\Delta A/A$ (680 nm) = 0.31. (b) *Euglena* (10 mm cuvette), $\Delta A/A$ (705 nm) = 0.15, $2 \cdot 10^5$ cells/m^3. ΔA determined at 13 kG.

addition to the magnetic field, they have used a variety of mechanical orientation methods, including the flow technique. Based on all of these considerations, we conclude that the reorientation of individual chlorophyll molecules is highly unlikely, and certainly does not occur to a measurable extent.

Linear dichroism

The linear dichroism of suspensions of cells is given by

$$\Delta A = A_{\parallel} - A_{\perp} \tag{11}$$

where A denotes the absorbance of the suspensions. Furthermore,

$$A_{\parallel} = \log_{10} \frac{I_0(_{\parallel}M)}{I(_{\parallel}M)} \text{ and } A_{\perp} = \log_{10} \frac{I_0(_{\perp}M)}{I(_{\perp}M)} \tag{12}$$

The directions of polarization of the incident (I_0) and transmitted (I) light beams with respect to the membrane planes are defined in Fig. 1(b). Thus ΔA is positive if the transition moment vectors of the chromophores are oriented close to or in the plane of the lamella, and is negative if they tend to be oriented closer to, or along the normal to the plane.

Linear dichroism spectra of suspensions of *Chlorella*, *Euglena* and spinach

chloroplasts are shown in Fig. 10. The absorbance (A) of suspensions of unoriented cells of *Euglena* and *Chlorella* are also shown (the absorbance of spinach chloroplast suspensions is similar to the spectrum shown for *Chlorella*). The following features are noteworthy: (1) In the region of the Q_y absorption maximum, ΔA generally has the shape of the absorption spectrum A but is red shifted ($\Delta A_{max} = 683$ nm for *Chlorella*, 680 nm for spinach chloroplasts and 705 nm for *Euglena*). (2) In the region of the Soret band (400–460 nm), ΔA is negative in *Chlorella* and *Euglena* and exhibits a dip (to about zero) in spinach chloroplasts. For spinach, the qualitative aspects of the ΔA spectrum is similar to the one obtained by Breton et al. [3] who used a sensitive optical rotation technique and a different method of orientation of the chloroplasts. The major difference is that in our case the $\Delta A = 0$ baseline appears to be shifted in a positive sense (as compared to Fig. 2, ref. 3). Furthermore, probably because of the higher sensitivity of their optical rotation method, there is more structure in the ΔA spectrum of Breton et al. [3]. (3) In all cases ΔA is positive in the carotenoid absorption region (approx. 480–540 nm) indicating that the carotenoid molecules tend to be aligned with their long axes in the plane or close to the lamellar plane. This conclusion is also in agreement with the results of Breton et al. [3]. (4) In *Euglena* the maximum in ΔA is shifted considerably further to the red than in either *Chlorella* or spinach. The linear dichroism in the region of the main absorption maximum is quite small. These results show that the bulk pigments in *Euglena* absorbing at approx. 680 nm are unoriented (or have a low degree of orientation) while the 695-nm absorbing pigment forms (chlorophyll *a* 695) are oriented, with their transition moment vectors lying in the plane or close to the plane of the lamellae [5]. This result is in reasonable agreement with Kreutz's analysis [26] of the data of Olson et al. [25] who studied single chloroplasts of *Euglena* using a polarizing microscope.

Linear dichroism measurements are subject to several artifacts which can cause distortions in the ΔA spectra. Textural dichroism, or dichroism of shape, can give rise to non-zero values of ΔA even if the pigments are unoriented [3, 21]. Recently, these effects were discussed in detail by Breton et al. [3], who concluded that the contribution of these artifacts to their ΔA spectra were not sufficiently large to account for the large values of the linear dichroism. Their arguments apply equally to our measurements and we will discuss here only some aspects of these effects:

(1) Textural dichroism. For a given unoriented pigment absorbing in different wavelength regions ΔA should have the same sign and should also be proportional to the absorbance A. In all cases shown in Fig. 10, ΔA does not follow A, particularly in the Soret absorption band region where ΔA is either zero or negative, while it is strongly positive in the 680 region. In *Euglena*, the linear dichroism is weak in the region of the 680 nm band and the peak in ΔA appears at 705 nm where the absorption is weak. We thus conclude that the contribution of this effect is negligible in our measurements.

(2) Selective light scattering. We have shown elsewhere [16] that scattering can give rise to a red shift in the dichroism maxima. This effect may well account for the slight shift of the ΔA peaks with respect to the maxima in A in *Chlorella* and spinach chloroplasts. It should be emphasized that in the results reported in Fig. 10, scattering effects were minimized by using the Shibata technique [15].

(3) The Duysens flattening effect [13] can in principle give rise to a distortion of the ΔA spectra. The absorbance of a suspension of particles, neglecting scattering

effects [30] is described by

$$A = \frac{Np}{2.303}(1-10^{-\sigma}) \tag{13}$$

where N is the number of particles in the illuminated suspensions and the factor Np is equal to the total area of all particles in the light beam projected on a plane perpendicular to the light beam, divided by the area of this beam, $10^{-\sigma}$ is the average transmittance of a single particle in the suspension, and σ is the average absorbance of each particle. In terms of Eqn 13 the linear dichroism is

$$\Delta A = \frac{Np}{2.303}[10^{-\sigma_\perp}-10^{-\sigma_\parallel}] \tag{14}$$

This equation predicts that ΔA should be proportional to the particle density, a condition which we have verified experimentally. Of interest are the quantities $\sigma_\parallel$ and $\sigma_\perp$ from which the relative orientation of the pigment molecules can be deduced, assuming that Beer's law is applicable to these particles individually. If Eqn 14 is divided by Eqn 13, one obtains

$$\frac{\Delta A}{A} = \frac{10^{-\sigma_\perp}-10^{-\sigma_\parallel}}{1-10^{-\sigma}} \tag{15}$$

$\sigma_\perp$ and $\sigma_\parallel$ can be determined if the quantity $1-10^{-\sigma}$ is known independently, since $\Delta A/A$ can be measured experimentally.

For small values of σ, Eqn 15 reduces to:

$$\frac{\Delta A'}{A'} = \frac{\sigma_\parallel-\sigma_\perp}{\sigma} \tag{16}$$

which is essentially the expression used by Breton et al. in the analysis of their results [3]. Differences in the quantities ΔA as defined according to Eqns 15 and 16 thus are due to the flattening effect. Both of these functions are plotted in Fig. 11 for two values of the dichroic ratio $\sigma_\parallel/\sigma_\perp$ as a function of the average absorbance per particle σ. The latter is given by

$$\sigma = \frac{\sigma_\parallel+\sigma_\perp}{2} \tag{17}$$

If the flattening effect is operative, we note that for values of $\sigma < 0.45$, ΔA increases more rapidly with increasing σ than $\Delta A'$. However, ΔA exhibits a maximum at $\sigma \simeq 0.45$ and decreases steadily for $\sigma > 0.45$, while $\Delta A'$ continues to increase. According to Fig. 11, neglect of the flattening effect for $\sigma \lesssim 0.45$ does not introduce too much of an error, while for $\sigma > 0.45$ this error can be quite substantial.

The average absorbance of a single *Chlorella* cell has been estimated by Duysens and σ is approx. 0.4 (or $1-10^{-\sigma} = 0.4$) at 680 nm [13]. We note that at this wavelength and for this value of σ, it is possible to estimate the ratio of $\sigma_\parallel/\sigma_\perp$ by using curves such as in Fig. 11 and experimental values of $\Delta A/A$. With *Chlorella*, this ratio varies from sample to sample, which is similar to the behavior displayed by the FP ratio. For the sample in Fig. 10, $\Delta A/A$ is approx. 0.12, and typical values

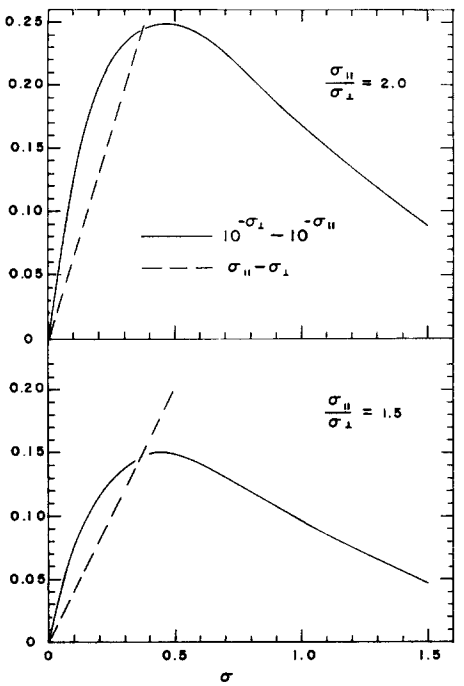


Fig. 11. The effect of flattening on linear dichroism spectra. Plots of the functions $\Delta A = 10^{-\sigma_{\perp}} - 10^{-\sigma_{\parallel}}$ and $\Delta A' = \sigma_{\parallel} - \sigma_{\perp}$ (in units of $N_p/2.3$) as a function of the average absorbance σ per particle.

with other samples vary between 0.10–0.30 (at 683 nm). These values yield a ratio of $\sigma_{\parallel}/\sigma_{\perp}$ is approx. 1.3–2.5. Since this ratio, to a first approximation, should be comparable to the FP ratio, this result is in good agreement with the FP ratios of 1.2–1.9 which are observed with *Chlorella*.

Similar conclusions apply to spinach chloroplasts and *Euglena*. In the latter case however, both dichroism and the fluorescence polarization indicate that only the long wavelength forms of chlorophyll have a measurable degree of orientation, while chlorophyll *a* 680 appears to be unoriented.

ACKNOWLEDGEMENTS

We would like to thank J. Breton for stimulating discussions and F. Trentacosti for her assistance with some of the dichroism measurements.

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