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ORIENTATION OF PIGMENTS AND STRUCTURAL PROTEINS IN THE PHOTOSYNTHETIC MEMBRANE OF SPINACH CHLOROPLASTS: A LINEAR DICHROISM STUDY*

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SUMMARY

1. The rotation of the plane of polarization, when monochromatic light traverses an anisotropic sample, is proportional to the linear dichroism (ΔA) of this sample. A sensitive technique of measurement of this rotation (sensitivity corresponding to $2 \cdot 10^{-5}$ absorbance unit for ΔA) has been applied to mechanically or magnetically oriented chloroplasts (800–185 nm). The contribution of artifacts encountered in linear dichroism measurements (textural dichroism, selective polarized scattering, selective polarized reflection) is discussed and is shown to be negligible.

2. A high degree of orientation of the pigments with respect to the normal to the plane of the lamellae has been detected. By comparing linear dichroism and absorption data, this orientation is analyzed in terms of the value of the angle φ between the normal and the directions of the transition dipole moments.

3. The directions of y polarized transitions of Ca-680 and longer wavelength forms of chlorophyll *a* lie close to, or in the lamellar plane ($\varphi > 60$–$65°$), Ca-670 is less oriented or oriented with φ slightly $> 55°$.

4. The negative dichroism in the Soret band of chlorophyll *a* implies that the directions of x polarized transitions are tilted out of the membrane plane ($\varphi \simeq 48°$)

5. Chlorophyll *b* molecules are oriented in a similar way.

6. Carotenoid molecules lie nearly parallel to the lamellar plane.

7. For structural proteins in intact thylakoid membranes an orientation of tryptophan residues is detected and the 220–185-nm signals are tentatively assigned to an orientation of α-helical regions parallel to the membrane plane.

INTRODUCTION

The results of the past studies on the degree of orientation of pigments in the photosynthetic membranes are often conflicting. The first experimental results by Menke[1,2], Frey-Wyssling and Steinmann[3], and Ruch[4] involving polarized light microscopy on unicellular algae, were interpreted as an effect of the stacking of lamellae in the grana (textural dichroism) rather than as an orientation of pigments.

* For one of us (J. Breton), this work represents partial fulfillment of the requirements for the Thèse de Doctorat ès Sciences, Université de Paris (registered C.N.R.S. A.O. 7. 768).

On the other hand, Goedheer[5,6], using the same technique with monochromatic light, found a small orientation of chlorophyll *a* molecules and suggested that carotenoids were also orientated. Later Olson *et al.*[7–10], by means of linear dichroism and polarized fluorescence measurements on unicellular algae, detected only a particular form of chlorophyll *a* absorbing near 695 nm (Ca-695) which was highly orientated. At the same time Sauer and Calvin[12] and Sauer[13] using spinach chloroplast fragments orientated by electric field or by velocity gradient, presented a linear dichroism spectrum in the visible spectral range and confirmed the orientation of Ca-695 only. Kreutz[14] suggested a model, on the basis of these results, in which all the chlorophyll *a* molecules were positioned in the membrane. Thomas *et al.*[15], working in the red part of the spectrum with mechanically orientated spinach chloroplasts, described Ca-680 as slightly orientated but did not reveal any particular orientation of Ca-695.

In a preliminary paper[16] we reported a linear dichroism spectrum of orientated spinach chloroplasts from 750 to 350 nm; this spectrum was interpreted in terms of an orientation of chlorophylls and carotenoids with respect to the normal to the plane of the chloroplast lamellae. At the same time, linear dichroism measurements on a photosynthetic bacterium by Morita and Miyazaki[17] showed an orientation of bacteriochlorophyll molecules quite similar to the one we described for chlorophyll *a* molecules in chloroplasts. Also, having observed the effect of a static magnetic field on the fluorescence of *Chlorella*[18], Geacintov *et al.*[19,20] described a magnetically induced orientation of photosynthetic systems, as revealed by linear dichroism and polarized fluorescence experiments on the red band of chlorophyll *a*.

Linear dichroism can give information on structure and energy transfer in the photosynthetic apparatus. A very sensitive technique of linear dichroism measurement has been applied to chloroplasts oriented either mechanically or magnetically. The theory of this measurement and the treatment of linear dichroism data are given. Artifacts linked to linear dichroism of orientated membranes are described and evaluated. The orientation of the different forms of chlorophyll *a*, of the chlorophyll *b*, of carotenoids and of chromophores belonging to structural proteins in the chloroplast, are elucidated.

METHODS

Material

Spinach chloroplasts were extracted at 4 °C in 0.4 M sucrose–0.02 M Tris–HCl (pH 7.8) using a Waring blendor. After filtering through cheesecloth, the crude juice was centrifuged at $500 \times g$ for 2 min. The supernatant was centrifuged again at $2000 \times g$ for 10 min to obtain the chloroplast pellet.

Chloroplast lamellae were prepared by ultrasonic treatment (Bronwill Biosonik III. 20 kHz. 80% full power output. 5×5 s) at 4–6 °C, of osmotically shocked chloroplasts suspended in 5 mM sodium ascorbate (pH 7). Unbroken grana were eliminated by a centrifugation $20000 \times g$ for 20 min leaving a clear dark green supernatant (λ_{max} 678 nm). For measurements in the ultraviolet, chloroplast lamellae free of soluble proteins were obtained in the same conditions from EDTA-washed grana[21]. Some chloroplast lamellae could be pelleted by a 60-min centrifugation at $50000 \times g$ when needed.

For linear dichroism measurements we used either a Fica or a Cary 60 spectropolarimeter. In some instances linear dichroism spectra have been obtained with a dual-beam spectrophotometer (Perkin–Elmer 356) with the two beams set at the same wavelength and orthogonally polarized (Polaroid HN 32).

Absorption spectra were recorded with a Cary 15 or a Perkin–Elmer 356 spectrophotometer.

A conduction-type cryostat with the sample in vacuum was used for low-temperature measurements.

Orientation of chloroplasts and lamellae

Electron micrographs show that spinach chloroplasts are usually ellipsoidal. They possess a major axis parallel to the plane of the chloroplast lamellae. By orientating those axes in parallel directions it should be possible to detect orientated forms of pigments, if there are any.

This operation could be achieved by picking up chloroplasts out of a centrifugation pellet with a small hair paintbrush and spreading them over an optically polished quartz plate (diameter 22 mm). Good orientation was easily obtained by a repetitive slow motion of the brush until hardening of the preparation occurred. A slight admixture of polyvinyl alcohol (Touzart et Matignon, 25%, w/v) in 10 mM phosphate buffer (pH 7) to the chloroplast pellet made the spreading easier and improved the degree of orientation. The same process was used for isolated lamellae. Since they are flat vesicles they tended to orientate with their planes parallel to the spreading direction.

Another way of orientating chloroplast lamellae was to make a "dry film" by air-drying a drop of a suspension of isolated lamellae on a quartz plate. X-ray diffraction studies of such orientated preparations have shown that the degree of orientation of the lamellae parallel to the quartz plate is high (Breton, J. and Sadler, D. M., unpublished).

We also used an electromagnet that fits in the sample compartment of the Fica spectropolarimeter. A magnetic field up to 10.5 kG could be applied to a suspension of chloroplasts in a quartz cell (air gap of the electromagnet: 10 mm). The magnetic field is perpendicular to the light beam. As described in ref. 20 the chloroplasts tended to orientate with the plane of their lamellae perpendicular to the applied field.

Linear dichroism measurement with a spectropolarimeter

When plane polarized light traverses a sample, the light usually becomes elliptically polarized and the plane of polarization is rotated. Here we are not interested in the ellipticity of the light (linked to sample birefringence and circular dichroism) but only in the rotation of the polarization plane, whose origins are:

(1) ORD: rotation of the plane of polarization induced by the optically active molecules.

(2) Linear dichroism when the sample is anisotropic:

Let us consider an anisotropic sample in the U–V plane with the special direction along U (Fig. 1). Absorbance of incident light, propagated along W, linearly polarized with the electric vector parallel to U, is defined as $A_{\parallel}$, and the electric vector parallel to V as $A_{\perp}$ (Fig. 1a). Linear dichroism (ΔA) with respect to the direction U is defined as

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

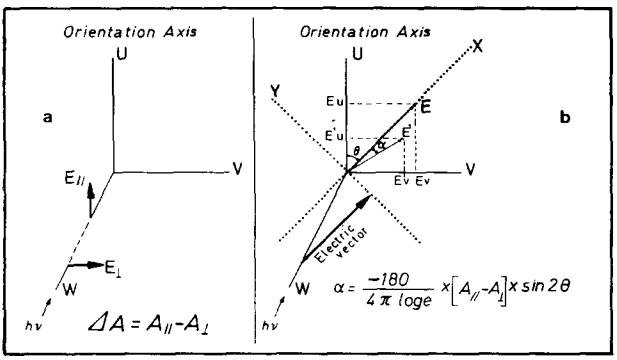

Fig. 1. Linear dichroism measurements on an anisotropic sample in the U–V plane. (a) Definition of linear dichroism. (b) The optical rotation α is linked to the linear dichroism.

Now if we send, on the same sample, plane polarized light with the electric vector E making an angle θ with the special direction U (Fig. 1b), E is not equally absorbed along U and along V. After traversing the sample, E becomes E' whose projections on U and V axes are:

$$E'_u = E_u \cdot e^{a}\| = E\cos\theta e^{a}\| \text{ where } a_{\|} = -\frac{A_{\|}}{2\log e}$$

$$E'_v = E_v \cdot e^{a}\perp = -E\sin\theta e^{a}\perp \text{ where } a_{\perp} = -\frac{A_{\perp}}{2\log e}$$

The projections of E'_u and E'_v on X and Y axes are:

$$E'_{ux} = E\cos^2\theta e^{a}\| \qquad E'_{vx} = E\sin^2\theta e^{a}\perp$$

$$E'_{uy} = E\cos\theta\sin\theta e^{a}\| \qquad E'_{vy} = -E\sin\theta\cos\theta e^{a}\perp$$

The projections of E' on X and Y axes are:

$$E'_x = E'_{ux} + E'_{vx} = E(\cos^2\theta e^{a}\| + \sin^2\theta e^{a}\perp)$$

$$E'_y = E'_{uy} + E'_{vy} = E\sin\theta\cos\theta(e^{a}\| - e^{a}\perp)$$

If $(a_{\|} - a_{\perp})$ is small compared to unity (we will see later that this condition is fulfilled in this study):

$$e^{(a\| - a\perp)} \simeq 1 + (a_{\|} - a_{\perp})$$

$$E'_x = E e^{a}\perp[\sin^2\theta + \cos^2\theta e^{(a\| - a\perp)}] \simeq E e^{a}\perp[1 + (a_{\|} - a_{\perp})\cos^2\theta]$$

$$E'_y = E e^{a}\perp\sin\theta\cos\theta[e^{(a\| - a\perp)} - 1] \simeq E e^{a}\perp(a_{\|} - a_{\perp})\sin\theta\cos\theta$$

if α is the angle between E and E':

$$\text{tang}\,\alpha = \frac{E'_y}{E'_x} = \frac{(a_{\|} - a_{\perp})\sin\theta\cos\theta}{1 + (a_{\|} - a_{\perp})\cos^2\theta}$$

α being small and $a_{\parallel} - a_{\perp} \ll 1$:

$$\alpha(\text{rad}) \simeq \tfrac{1}{2}(a_{\parallel} - a_{\perp}) \sin 2\theta$$

to obtain α in degrees:

$$\alpha = -\frac{180}{4\pi \log e}(A_{\parallel} - A_{\perp}) \sin 2\theta \tag{1}$$

A laboratory built sample holder that fits in the sample compartment of the spectropolarimeter has been used. This device allows an accurate measurement of the angle θ ($\pm 1°$) and, more generally, a precise determination of the position of the quartz plate in the space.

The relation between the sign of α (and then of $A_{\parallel} - A_{\perp}$) and the sign of θ has been checked by inserting in the light beam a sheet of polaroid that acts as a dichroic sample.

Samples were usually orientated so that the condition $\theta = -45°$ giving a maximum for α in Eqn 1 is fulfilled. In these conditions $\alpha = 33\,\Delta A$.

The ORD signal, being independent of θ, is easy to eliminate. Furthermore, in the case of oriented chloroplasts, the ORD contribution is usually much smaller than the linear dichroism signal.

Linear dichroism measurements by this technique have several advantages over the other methods:

(1) Use of an unmodified, commercially available apparatus.

(2) High sensitivity: because it is a zero method and because only orientated species contribute to the observed signal. Values as low as $2 \cdot 10^{-5}$ absorbance unit can be detected. A great sensitivity for this technique has been reported[22] in a study of the orientation of chlorophyll aggregates in monolayers.

(3) Only one measurement is required to obtain the spectrum. There is no need to modify polarizer or sample position as it is when using a spectrophotometer. This completely eliminates the problem of residual polarization found with gratings, optical components and detectors.

(4) Possible use in the ultraviolet range, as shown by this work which is, to our knowledge, the first reported study of the linear dichroism of a complex membrane as far as 185–180 nm. This method might be applied to other biological materials where orientated chromophores are known or expected.

RESULTS

Orientation by spreading

In the range of 800–300 nm the linear dichroism spectrum had the same characteristics whether lamellae or intact chloroplasts were used. For ultraviolet measurements the best resolution was achieved when using lamellae free of stroma and soluble proteins (as prepared by EDTA treatment). The linear dichroism spectrum for such material is shown together with the absorption spectrum (Fig. 2).

We checked the dependence of α upon $\sin 2\theta$ (Eqn 1) and found an agreement within 2%. More than 300 preparations, with absorbance varying from 0.02 to 1, were analyzed and the shape of the linear dichroism spectra was found to be remark-

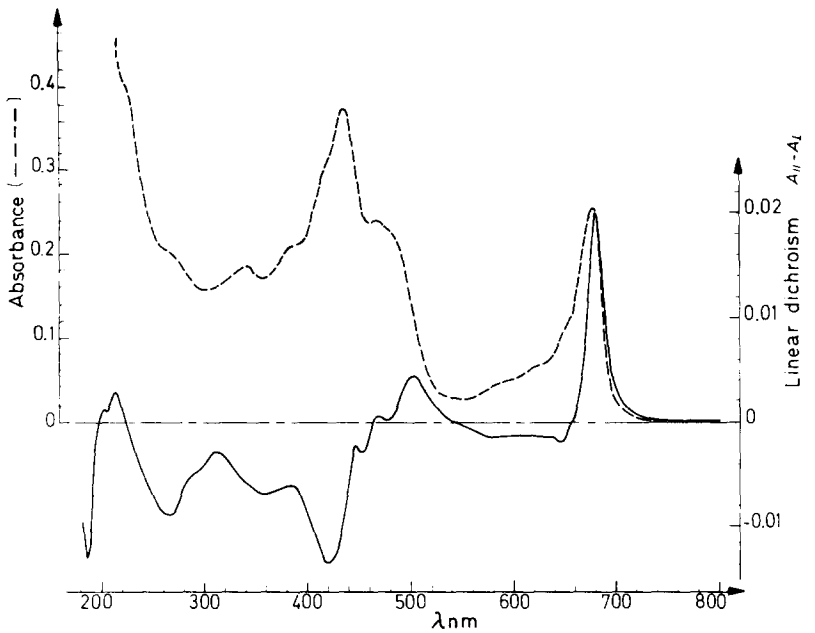

Fig. 2. Linear dichroism and absorption spectra of orientated spinach chloroplast lamellae isolated from EDTA-washed grana. Orientation by the spreading technique (polyvinyl alcohol added). ———, linear dichroism spectrum recorded with a spectropolarimeter (ORD corrected). Bandwidth: 2 nm. — — —, absorption spectrum.

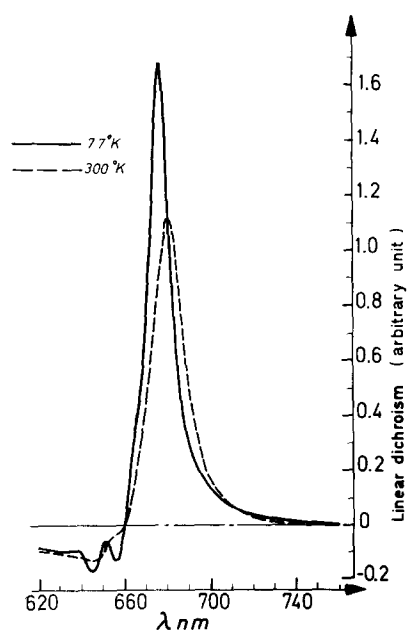

Fig. 3. Linear dichroism spectra of intact spinach chloroplasts as recorded with a spectropolarimeter at 300 °K and 77 °K. Orientation by spreading. Condition $\alpha = 33$ ΔA fulfilled. ORD corrected. Bandwidth: 2 nm. Absorbance at red maximum: 0.30 at 300 °K, 0.39 at 77 °K.

ably constant (this justifies the condition $a_{\parallel} - a_{\perp} \ll 1$ of Eqn 1). The ratio $\Delta A/A$ varied according to the degree of orientation that was achieved in the spreading operation, and was always less for lamellae than for intact chloroplasts.

The linear dichroism spectra, taken at 77 °K and 300 °K, for the red band of chloroplasts orientated by spreading are reported (Fig. 3). The chlorophyll *b* peak at 650 nm and a shoulder near 670 nm were resolved at 77 °K.

Orientation by air-drying

For air-dried preparations we needed to tilt the plate with respect to the beam, by an angle *i*, in order to record a linear dichroism signal. The rotation of the polarization plane induced by the quartz plate needed to be cancelled by putting into the light beam a properly tilted quartz plate without deposited material. Then the linear dichroism spectrum was very similar to the one obtained with spreading-orientated samples. Only minor differences in the wavelengths of zero-line crossings were observed. On those preparations we checked the dependence of α against the values of $\sin^2 i/\cos i$ (see Discussion). The agreement was within 5% ($0 < i < 50°$).

Orientation by magnetic field

For chloroplasts suspended in glycerol–water (2:1, v/v) and orientated in the magnetic field, the linear dichroism spectra were identical to the ones obtained by the spreading technique.

Using cells with different path lengths, and maintaining the absorbance at a constant value, we checked that the relative intensities of the different bands were not affected by varying the chloroplast concentration by an order of magnitude. Using solutions of 0.35 M NaCl in glycerol, the variations of the linear dichroism signals as a function of the refractive index of the suspension medium were followed. For low refractive indices a selective scattering distortion[19] was observed; it was reduced as *n* increased. The magnitude of this effect was prominent in the long wavelength side of the red band (Table I). There were also some modifications in the positions of maxima and minima and of the zero-line crossings. However, these distortions did not change the overall structure of the linear dichroism spectra.

In our spectropolarimeter, the detector is located about 35 cm from the sample. Such a situation is unfavourable for scattering material and we can expect a distortion by selective scattering. A better situation is found with the Perkin–Elmer spectro-

TABLE I

DEPENDENCE OF THE SELECTIVE POLARIZED SCATTERING CONTRIBUTION AT 710 nm ON THE REFRACTIVE INDEX *n* OF THE SUSPENSION MEDIUM

$$R^* = \frac{\text{optical rotation at 710 nm}}{\text{optical rotation at red maximum}}$$

Spinach chloroplasts orientated by a 10.5-kG magnetic field. Suspension medium: solution of 0.35 M NaCl in glycerol. Refractive indices are measured with Abbe refractometer.

n:	1.35	1.37	1.39	1.41	1.43	1.45	1.47
R^*:	0.252	0.204	0.170	0.131	0.102	0.064	0.051

photometer where the orientated sample can be positioned in contact with the detector. The 750–400-nm linear dichroism spectra obtained with this apparatus, for chloroplasts suspended in glycerol and orientated by a magnetic field, showed the characteristics depicted in the corresponding region of Fig. 2. However, the sensitivity of this measurement, two orders of magnitude less than the sensitivity of the optical rotation technique, did not allow a more detailed comparison.

Numerical data

The preparations giving the higher ratio $\Delta A/A$ have been selected. The numerical values for $\Delta A/A$ at 681 nm for the different techniques of orientation are collected in Table II.

TABLE II

VALUES OF $\Delta A/A$ AT 681 nm FOR SPINACH CHLOROPLAST MATERIAL ORIENTATED BY THE THREE DIFFERENT TECHNIQUES

Orientation by	*Spreading**	*Air-drying***	*Magnetic field****
$\Delta A/A$ at 681 nm	0.115	0.127 ($i=30°$)	0.425 ($\boldsymbol{H}=10.5$ kG)

* For spreading-oriented chloroplasts ΔA and A measurements are performed perpendicularly to the sample.

** For air-dried chloroplast lamellae A is recorded perpendicularly to the sample and ΔA with the sample tilted by an angle $i=30°$ with respect to the light beam.

*** A is measured in non soriented suspension and ΔA at the steady state of orientation ($\boldsymbol{H}=10.5$ kG).

DISCUSSION

Orientation of the photosynthetic membranes

The three methods of orientation of the photosynthetic material described here involve distinct mechanisms for the orientation process:

(1) Spreading involves a velocity gradient and some drying. This drying is not necessary for orientation as we have obtained identical spectra with intact chloroplasts suspended in the isolation medium (1% methyl cellulose added) by flow in a capillary tube (0.31 mm internal diameter; average velocity 40 $cm \cdot s^{-1}$).

(2) For air-drying, gravity and drying are involved. The conclusion that air-drying of a photosynthetic membrane does not modify the orientation of pigments has been given by Morita *et al.*[17].

(3) When using a magnetic field, the orientation occurs by a cooperative effect *via* an anisotropy in the magnetic susceptibility of some membrane constituents[20,23]. There are neither physical nor experimental evidence of a possible reorientation of individual pigments (to a detectable level).

The fact that these three methods led to identical spectra favours the assumption that the spectra represent the true linear dichroism signals of photosynthetic membranes.

When isolated lamellae are oriented by air-drying it is clear from X-ray data

that the planes of the membranes are parallel to the quartz plate. From the positive sign of the linear dichroism in the red it is then possible to determine the orientation of the membranes obtained by the other orientation techniques. It has been found that for the spreading more membranes are orientated parallel to the spreading axes than perpendicular to it. When using a magnetic field it has been detected that more membranes are orientated perpendicular to the field than parallel to it, thus confirming the results of Geacintov *et al.*[20].

Contribution of the artifacts in linear dichroism measurements

Apart from the flattening effect described by Duysens[24], that occurs in absorbance measurements of suspensions and whose contribution could be usually neglected in this study by virtue of the similarity of the linear dichroism spectra of chloroplasts and isolated lamellae, we analyzed and/or detected three different artifacts inherent in linear dichroism measurements on oriented membranes:

(1) The first one was textural dichroism. If anisotropic objects containing unorientated pigments are orientated, then a linear dichroism signal with the shape of the absorption spectrum will occur. From the work of various authors[25,26] it is possible to show that textural dichroism will be:

$$A_{\parallel} - A_{\perp} = 8f(1-f)(n-n')(L_{\parallel} - L_{\perp})A$$

where f is the fraction of the total volume of the suspension occupied by the objects; n, refractive index of the external medium; n', refractive index of the object; $L_{\parallel} - L_{\perp}$, factor depending upon the anisotropy of shape of the object, $L_{\parallel} - L_{\perp}$ can vary from 1 to -1; A, absorbance of the suspension.

Three experimental tests led to the conclusion that this artifact is negligible in our experiments:

(a) The shape of the linear dichroism spectrum was very different from that of the absorption spectrum.

(b) The shape of the linear dichroism signals was the same for intact chloroplasts and for isolated lamellae, although $L_{\parallel} - L_{\perp}$ should be different.

(c) By using cells of 1, 2, 3 and 10 mm, and maintaining the absorbance at a constant level, we checked that variations of f by an order of magnitude did not change the relative intensities of the different bands.

(2) The second possible artifact is an effect of selective scattering. Geacintov *et al.*[19] showed that linear dichroism measurements in the red could be affected by polarized selective scattering of the pigments. We indeed detected here an effect of the refractive index of the external medium on the shape of the red band that confirmed those results. As this selective scattering has the shape of anomalous dispersion of the refractive index in the absorption bands of the pigments[27] it might explain some of the features of our linear dichroism spectra, namely the negative sign observed for the 420-nm band. However, in the experiments with the Perkin–Elmer apparatus light was collected under a large solid angle and selective scattering is thus minimized[28]. In this case we clearly observed a negative sign for $\lambda < 440$ nm; so we conclude that scattering effects are not prevailing in our linear dichroism measurements.

(3) The third effect, which we propose to call polarized selective reflection,

occurs every time that a linear dichroism measurement concerns a sample whose plane is not perpendicular to the light beam. When a glass plate is tilted with respect to a linearly polarized light beam, light polarized in the incident plane is not reflected to the same degree as light polarized perpendicularly to the incident plane. This effect depends on the refractive index of the glass. If unorientated pigments are deposited on such a plate, the same process occurs, but as n' varies in absorption bands of the pigments, the effect becomes dependent on the anomalous dispersion of the pigments. This will give rise to a linear dichroism signal with a dispersive shape. Such a signal must appear in linear dichroism measurements on chloroplasts, since the orientation of the pigments can only be detected on lamellae whose planes are not perpendicular to the light beam. This effect has been detected on photosynthetic pigments randomly deposited on a glass plate and also in some types of monomolecular layers[29]. This artifact is difficult to evaluate from the present results as it is directly linked to the measurement itself, and since it depends on the thickness of the pigmented layer. However, if chloroplasts are incubated with rhodamine B (ref. 6) and then orientated (by the spreading technique), a positive peak at 560 nm is observed. The difference linear dichroism spectrum between rhodamine-treated and non-treated chloroplasts has the shape of the absorption band of rhodamine (Breton, J., unpublished). This indicates that dispersive contributions are not predominant in our linear dichroism spectra.

In brief, though our linear dichroism spectra must be distorted by dispersive terms, which need to be further analyzed, the contribution of the orientation of pigments is predominant; all the following discussion will neglect the contribution of the artifacts described above.

Analysis of polarization data

As chloroplasts orientated by the different techniques result in identical linear dichroism spectra, we conclude that no evidence is obtained of a preferred direction in the lamellar plane. The only orientation detected is that of the transition moments with respect to the normal to the lamellae plane.

Let us consider a set of transition moments, corresponding to only one absorption band which is perfectly polarized, isotropically distributed around the nor-

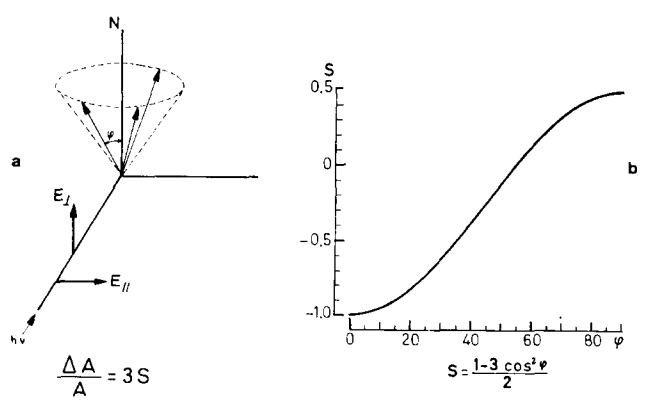

Fig. 4. Dependence of the linear dichroism intensity on the orientation of the transition dipole moments.

mal N to the plane of the chloroplast lamellae, and which make an angle φ with N (Fig. 4a).

It is known that the absorbance is proportional to the average of the square of the scalar product between the electric vector of incident light and the transition moment. After averaging the square of the sine of the radial angle characterizing one transition moment, we obtain:

$$A_{\perp} = 3A\cos^2\varphi \qquad A_{\parallel} = \tfrac{3}{2}A\sin^2\varphi$$

with $A=(A_{\parallel}+2A_{\perp})/3$ representing the absorption in solution.

Therefore, the linear dichroism signal has the shape of the absorption band for the transition under consideration, but its intensity and its sign will be φ dependent according to:

$$\Delta A = A_{\parallel} - A_{\perp} = 3A\frac{1-3\cos^2\varphi}{2}$$

$$\frac{\Delta A}{A} = 3S \tag{2}$$

$S=(1-3\cos^2\varphi)/2$ is called the orientation parameter. Its variations with φ are depicted in Fig. 4b. Its average is 0 for randomly distributed φ angles. With linear dichroism measurements, we obtain essentially the average value of S. For a particular value of this average, different distributions of φ angles are possible. In order to simplify we shall assume all the φ identical as is done for simple model systems[29–31].

The absorbance of unpolarized light propagated along N is not equal to the absorbance in solution; it is also φ dependent:

$$A_N = A_{\parallel} = \tfrac{3}{2}A\sin^2\varphi \qquad A_N = A(1+S) \tag{3}$$

Let us consider the situation for chloroplasts orientated by the different techniques:

(1) Magnetic field induced orientation: The absorbance is taken in unorientated suspension, and the dichroism with the light beam perpendicular to the magnetic field. If we neglect the contribution of the flattening effect[20,24], which will reduce the observed linear dichroism signal, Eqn 2 is valid: looking at Eqn 2 and with the result $\Delta A/A=0.425$ (Table II), we obtain $S=+0.14$ at 681 nm.

(2) Preparations oriented by air-drying: The measurement of absorption is done perpendicularly to the quartz plate, so Eqn 3 is valid. The linear dichroism spectra are recorded by tilting the plate by an angle i with respect to the beam, so introducing $\sin^2 i/\cos i$ as a coefficient in Eqn 2 (ref. 17). We checked the dependence of α upon i and found it was correct (Results). So, for $i=30°$:

$$\frac{\Delta A}{A_N} = \frac{1}{2\sqrt{3}} \times \frac{3S}{1+S} \tag{4}$$

Eqn 4 and the result $\Delta A/A=0.127$ give $S=+0.17$ at 681 nm.

(3) Preparation orientated by spreading: The measurements of linear dichroism and of absorption are done perpendicularly to the sample. The plane of the mem-

branes are parallel to the spreading axis and if we assume an isotropic distribution of the planes of the membranes around this axis a 0.5 factor has to be introduced in Eqn 2 (average value of the square of a director sine). The measured absorption A_M will be φ dependent; by averaging we obtain:

$$A_M = A\left(1 + \frac{S}{4}\right)$$

and

$$\frac{\Delta A}{A_M} = \frac{6S}{4+S} \tag{5}$$

Looking at Eqn 5 and with the result 0.115 for $\Delta A/A$ we obtain $S = +0.08$ at 681 nm.

As seen from these different S values, the orientations obtained by the spreading technique are rather weak ($S=0.08$) and those obtained by air-drying are the best ($S=0.17$). Magnetic field induces high orientation if we take into account the fact that the 10.5-kG field of our electromagnet does not saturate the orientation of spinach chloroplasts. From the polarized fluorescence data of Geacintov *et al.*[20] we can estimate the orientation at 10.5 kG at about 66% of the maximum. That would lead to an S value of 0.21.

In the following discussion we shall give only approximate values of φ for different reasons:

(a) The exact extent of orientation of the photosynthetic material is not presently known. The membranes may be to some degree folded and the pigments on the edges of the lamellae are not orientated with respect to the normal at the main lamellar plane. Moreover, in intact chloroplasts the distribution of the lamellae around the major axis is not known precisely. All these factors would always led to experimental values of S that are smaller than the real value. This appears particularly clear for the measurements in the magnetic field where S has been calculated assuming that all the lamellae are perpendicular to the field.

(b) Eqn 2 is only valid for fully polarized transitions. This is not always the case here, namely in the Soret region where x and y transitions overlap.

(c) The absorption bands of the chloroplast pigments often overlap; this will further complicate the interpretation of the spectra.

Orientation of pigments in vivo

(1) Chlorophyll *a*: In the spectral range of 730–680 nm, where the well polarized[32] Q_y transition moment of chlorophyll *a* is involved, we found $0.15 < S < 0.2$ ($61° < \varphi < 64°$). This is interpreted as the y directions of the tetrapyrolic rings of Ca-680 and longer wavelength forms of chlorophyll *a* lying close to the lamellar plane.

In the region of 680–660 nm the linear dichroism signal decreases sharper than the absorption signal. S becomes very low though it remains positive. This means a low orientation of Q_y transition moments of Ca-670 or an orientation with φ slightly greater than 55°. The small shoulder in this region (Fig. 3) could be attributed to this chlorophyll species.

In the spectral range of 450–370 nm the Soret band of chlorophyll *a* is mainly

involved. Fluorescence polarization measurements[32] and linear dichroism experiments on chlorophyll molecules orientated in stretched films[33] have shown that some of the transitions in this region are x polarized. The negative sign of the linear dichroism observed here can be interpreted as the x directions pointing somewhat out of the lamellae plane. However, as orientation of the y directions of the different forms of chlorophyll *a* are different, we have no indication concerning their respective x directions. If the x directions of Ca-670 are unorientated as we suggested for their y directions, it becomes possible to evaluate the tilting of the x directions of Ca-680 and longer wavelength forms upon the membrane plane. Assuming that only half the absorbance in this region accounts for Ca-680 and longer wavelength forms, we found that at 425 nm $S \simeq -0.18$ ($\varphi \simeq 48°$). If the x directions of Ca-670 are also oriented around the normal, then this value reflects only an overall orientation of x transition moments.

The 385-nm shoulder in the absorption spectrum of chloroplasts, that has been attributed to a y polarized transition of chlorophyll *a*[33], appears in our linear dichroism spectra with the same positive sign as for the red band.

The negative sign observed in the region 650–550 nm might come from the Q_x transitions of chlorophyll *a*. However, a negative contribution can also be expected there from dispersive terms (see Discussion on artifacts).

(2) Carotenoids: In the spectral range of 530–500 nm carotenoid molecules are the main absorbing species and the linear dichroism is positive. The optical transition being polarized along the polyenic chain of the molecule, the long axes of carotenoid molecules lie close to the lamellar plane.

(3) Chlorophyll *b*: The small positive band at 650 nm at 77 °K (Fig. 3) is attributed to Q_y transitions of chlorophyll *b* molecules showing a low degree of order, but with φ greater than 55°.

In the region of 500–450 nm chlorophyll *a*, chlorophyll *b* and carotenoids absorptions overlap. However, the second maximum of carotenoids absorption does not appear (Fig. 2) with the high dichroism expected. This is explained if the dichroism of chlorophyll *b* in this region is negative. The φ value for the x directions of chlorophyll *b* would then be smaller than 55°.

Orientation of structural proteins

In the spectral range of 290–260 nm there is a shoulder in the absorption spectrum and a large negative linear dichroism signal. Chlorophyll *a* does not seem to be responsible of this absorption as there is a dip at these wavelengths in the absorption spectrum in ether[34]. Searching for an orientation of plastoquinones, we found no modification of the linear dichroism signal in this region following an heptane extraction of oriented lamellae (though the 500-nm signal is slightly reduced). On the other hand, magnetic circular dichroism spectra on isolated spinach chloroplast lamellae show an S-shaped signal between 295 and 285 nm (Breton, J., unpublished) that can be assigned to tryptophan residues[35] of structural proteins. So the negative linear dichroism signal in this region (Fig. 2) can be attributed (at least partly because there are other aromatic amino acids) to an orientation of tryptophan residues of the structural proteins. As π–π^* transitions, occurring in the plane of the molecule, are involved, it can be inferred that the plane of these tryptophan residues tends to lie perpendicular to the lamellar plane.

Three bands in the linear dichroism spectra of oriented lamellae occur at 216, 204 and 186 nm. ORD signals below 240 nm for suspension of isolated lamellae have been attributed to α-helical regions of structural proteins[36]. In synthetic α-helical homopolypeptides three bands have been also detected and ascribed using linear dichroism measurements[37]:

222 nm: n–π^* transitions
206 nm: π–π^* transitions polarized parallel to the great axes of the α-helix
191 nm: π–π^* transitions polarized perpendicular to the great axes of the α-helix

In our spectra the 204-nm transition appears as parallel and the 186-nm as perpendicular to the lamellar plane. So it can be concluded that the long axes of the α-helices of structural proteins lie parallel to the plane of the thylakoid membrane.

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