FIELD CONCENTRATION AND TEMPERATURE DEPENDENCE OF FLUORESCENCE POLARIZATION OF MAGNETICALLY ORIENTED CHLOROPLASTS

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ABSTRACT Chloroplasts in higher magnetic fields align with their equatorial plane perpendicular to the field. Because of the nonrandom orientation of the chromophores in the membrane the fluorescence radiation will be partially polarized. The chloroplast concentration, magnetic field, and temperature dependence of the fluorescence polarization has been investigated. The results are compared with a simplified model calculation. It is shown that the concentration dependence can be related to the linear dichroism of the fluorescence radiation and self-absorption. Taking these effects into account results in the calculation of a higher fluorescence polarization (*FP*) ratio and higher inclination of chlorophyll dipoles to the membrane plane. Analyzing the magnetic field dependence of the *FP* ratio, we conclude that in a magnetic field not only will the chloroplasts be aligned, but the thylakoid stacks as well. A decrease in the *FP* ratio was observed around 20°C. It is suggested that this decrease reflects a phase transition in the photosynthetic membrane.

INTRODUCTION

Application of a magnetic or electric (dc or ac) field to a suspension of whole cells or membrane fragments changes their isotropic distribution (1–7). The alignment of chloroplasts and membranes in a magnetic field has been observed directly with the light microscope and by x-ray and neutron diffraction (8–11). The alignment mechanism has its origin in the interaction of the magnetic field with the diamagnetically anisotropic molecules inside the membrane and in the interaction of the electric field with permanent or induced electric dipoles or charge movements associated with the membrane. This interaction creates a torque on the membrane acting against the thermal randomization. It is believed that the moderate fields (~1 T [tesla], or 10 kG [gauss], and 100 V/cm) necessary for aligning cells and membranes do not change the molecular structure of the membrane. Chloroplasts align in magnetic field with their equatorial plane perpendicular to the field.

In the case of photosynthetic membranes the magnetic alignment leads to fluorescence polarization. If the fluorescence is viewed perpendicular to the field, the intensity of the fluorescence component ($F_\perp$) perpendicular to the field increases while the component parallel to the field ($F_\parallel$) decreases with increasing magnetic fields, reaching saturation values at higher fields (1, 2). The increase of the fluorescence polarization ratio (*FP*, defined as $FP = F_\perp/F_\parallel$) is due to the fact that the dipole moments of the fluorescing molecules (chlorophyll *a*) are closer to being parallel to the membrane plane. The theory of fluorescence polarization in magnetically oriented photosynthetic systems has been worked out by different groups but especially by Knox and Davidovich (12). Aligned chloroplasts show linear dichroism (13, 14): the absorption of light polarized perpendicular to the field is higher than for polarized light parallel to the field (if the incoming light is perpendicular to the field).

In this paper we report the results of a study of fluorescence polarization of spinach chloroplasts aligned in a magnetic field. We measured its dependence on the chloroplast concentration, on the magnetic field, and on the temperature in the range 0–1.6 T and 5–40°C, respectively. We compare the results with a simplified model calculation.

EXPERIMENTAL PROCEDURE

Chloroplasts were isolated from fresh spinach. After being homogenized at 5°C in 0.01 M KCL and 0.3 M saccharose Tris buffer (40 mM, pH 7.2) using a blender, the chloroplasts were filtered and centrifuged at 300 *g* for 3 min. The supernatant was then centrifuged at 2800 *g* for 10 min. Usually the measurements were carried out within a few hours after the preparation, but in a few cases they were continued on the next day. After longer times a gradual degradation in *FP* was observed.

Our concentrations are expressed in arbitrary units. Usually we started with 20 g of spinach leaves; this resulted in 10 ml of a concentrated chloroplast suspension. This concentration was taken as $c = 1$. The OD is ~0.9 for $c = 0.05$ and $\lambda = 685$ nm, though this varies with different preparations. For comparisons, measurements on the same preparation were taken.

The exciting light came from a tungsten filament lamp (150 W) through a monochromator: $\lambda_e = 430 \pm 10$ nm. Occasionally a polarizer in the exciting light was used. The fluorescence radiation perpendicular to the exciting light and the magnetic field was viewed by a photomultiplier (PM) (E. M. I. Electronics Limited, Wells, England, model 9558 B) through an analyzer (Zeiss polaroid, Carl Zeiss, Inc., Jena, GDR), a

630-nm cut-off red filter, and a Zeiss interference filter. Two methods were adopted. With the dc method the PM current was measured by a picoammeter (Model MV40, VEB Pracitronic, Inc., Dresden, GDR, with external zero suppression) with the use of a static analyzer. With the ac method the analyzer was rotated at ~10 Hz, and the ac component of the PM signal was measured by a Keithley 840 lock-in amplifier (Keithley Instruments, Inc., Cleveland, OH). The reference signal to the amplifier was generated by a double electrical switch contact placed on the rotating analyzer. The dc method measured the $F_\perp$ and $F_\parallel$ components separately; the ac method gave a signal proportional to $(F_\perp - F_\parallel)$.

The cuvette ($5 \times 5 \times 10$ mm^3) containing the chloroplast suspension was placed into a copper block, whose temperature was controlled by a water thermostat. The temperature of the suspension was measured by a copper-constantan thermocouple. The magnetic field (maximum 1.6 T) was generated by an electromagnet.

RESULTS AND DISCUSSION

A brief outline of the theoretical considerations and assumptions follows.

(*a*) At saturating magnetic fields all the membranes are oriented with their planes perpendicular to the field. (That is, we will take the shape factor to be 1. See reference 12.)

(*b*) The radiating dipole moment, d, of the fluorescing chlorophyll *a* molecule makes an angle ψ with the direction normal to the membrane plane (Fig. 1); it is randomly distributed about the x axis so that we have to average only for α with oriented chloroplasts. But with unoriented chloroplasts (zero magnetic field) the average has to be taken for both α and ψ angles. These averages will be denoted by $\langle \ldots \rangle_\alpha$ and $\langle \ldots \rangle_\psi$.

(*c*) There is a complete energy transfer before fluorescence takes place (no polarization memory). This means that every molecule is excited with equal probability.

If the fluorescence radiation is not absorbed in the suspension, the following relations hold for the fluorescence polarization (in saturating magnetic fields):

$$F_\perp/F_0 = (3/2)\sin^2\psi \qquad (1a)$$

$$F_\parallel/F_0 = 3\cos^2\psi \qquad (b)$$

$$FP = F_\perp/F_\parallel = (1/2)tg^2\psi. \qquad (c)$$

(Here F_0 is the isotropic fluorescence intensity, in zero magnetic field, along any analyzer.) We notice that the following relation,

$$2F_\perp + F_\parallel = \text{constant}, \qquad (2)$$

is always satisfied for oriented, partially oriented, and unoriented chloroplasts, independently of ψ. (See reference 2, Fig. 1.) This has a very simple physical interpretation: the electric field radiated by emitting dipoles has three perpendicular components. The resultant intensity is constant if the absorbed light intensity is constant. But we cannot observe the component polarized in the viewing direction. Because our system is cylindrically symmetric, the light intensity of the nonobservable component is equal to $F_\perp$.

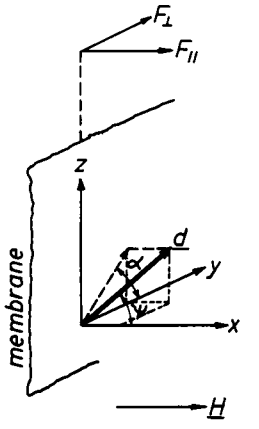

FIGURE 1 Orientation of the membrane and dipoles relative to the magnetic field. ψ, the angle between x and d; α, the angle between y and the projection of d to the (y,z) plane; y-excitation light and z-fluorescence viewing directions.

The Dependence of the Fluorescence Polarization on the Chloroplast Concentration

To get a reliable value for ψ, the rather strong (and different) dependence of $F_\perp$ or $F_\parallel$ on chloroplast or cell concentration should be taken into account. This problem was pointed out in reference 14 but was not fully analyzed. Because the self-absorption within a chloroplast or cell is rather high, a much higher FP ratio is obtained than extrapolation to zero concentration would yield.

The observed $F_\perp$ component usually is decreasing while $F_\parallel$ is increasing with concentration (see Fig. 2). As an oriented chloroplast medium is optically highly anisotropic, the concentration dependence of the two-compo-

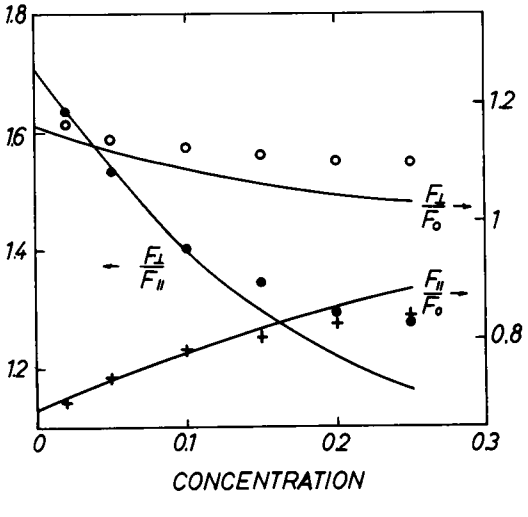

FIGURE 2 Concentration dependence of the fluorescence polarization ratio (●) and the parallel (+) and perpendicular (○) component of the fluorescence intensity. Full lines calculated from Eq. 7a–c. H = 1.6 T; λ_f = 700 nm.

nent system is essentially a linear dichroism problem for the fluorescence radiation.

To solve the problem we take two dipoles (molecules), d_1 and d_2, along the z (viewing) direction: d_1 is in an excited state, d_2 in the ground state. It is supposed that d_2 has a finite transition probability at the fluorescence wavelength of d_1. The absorption by d_2 of the d_1 radiation will be proportional to $(E \cdot d_2)^2$ and for the y ($\perp$) component (Fig. 1):

$$a'(E_y d_{2y})^2 \sim a' \sin^2 \psi_1 \cos^2 \alpha_1 \sin^2 \psi_2 \cos^2 \alpha_2.$$

Here a' is the absorption for two molecules. For oriented dipoles this has to be averaged over α_1, α_2:

$$a' \langle (E_y d_{2y})^2 \rangle_{\alpha_1,\alpha_2} \sim \tfrac{1}{4} \sin^2 \psi_1 \sin^2 \psi_2.$$

So the fluorescence radiation of d_1 will be decreased by the absorption of d_2:

$$F_\perp \sim \tfrac{1}{2} \sin^2 \psi_1 [1 - (\tfrac{1}{2}) \sin^2 \psi_2]. \tag{3}$$

We can take here $\psi_1 = \psi_2$. To get the fluorescence radiation for the whole chloroplast suspension, Eq. 3 has to be integrated first for all d_2 dipoles above d_1 and then for all d_1. The result is

$$F_\perp \sim 1 - e^{-1/2 a \sin^2 \psi \cdot c} \tag{4}$$

where a includes the absorption coefficient and several geometrical factors, and c is the chloroplast concentration.

Similar calculations yield for $F_\parallel$ and for the unoriented case (F_0):

$$F_\parallel \sim 1 - e^{-a \cos^2 \psi \cdot c} \tag{5}$$

$$F_0 \sim 1 - e^{-1/3 a \cdot c}. \tag{6}$$

From Eqs. 4–6 we get:

$$\frac{F_\perp}{F_0} = \frac{1 - e^{-1/2 a \sin^2 \psi \cdot c}}{1 - e^{-1/3 a \cdot c}} \tag{7a}$$

$$\frac{F_\parallel}{F_0} = \frac{1 - e^{-a \cos^2 \psi \cdot c}}{1 - e^{-1/3 a \cdot c}} \tag{b}$$

$$\frac{F_\perp}{F_\parallel} = \frac{1 - e^{-1/2 a \sin^2 \psi \cdot c}}{1 - e^{-a \cos^2 \psi \cdot c}}. \tag{c}$$

Eq. 7a–c reduce to Eq. 1a–c for $c \to 0$. Also $FP = 1$ at $\psi = 54.74°$; the "magic" angle remains valid for finite concentration too.

Eq. 7a–c have been compared with our experimental results. The procedure was the following. A fit was made to Eq. 7c at low concentration (Fig. 2). This yielded the parameters a and ψ. Then, with these parameters, Eq. 7a and b were calculated (Fig. 2). Although these equations reproduce the general trend of the experimental results, there are some systematic deviations at higher concentrations and for $F_\perp/F_0$ and $F_\parallel/F_0$.

These later deviations can be accounted for by a slight difference in the excitation between the oriented and unoriented case, which is not taken into account in the theoretical derivation.

To find some reasons for the deviations at higher concentration (Fig. 2) we considered several possible sources. First, there is the self-absorption of the fluorescence radiation within the same chloroplast, which can be quite high (15). At least approximately, the self-absorption can be taken into account with a constant, ϵ, in the exponent of Eq. 7c:

$$\frac{F_\perp}{F_\parallel} = \frac{1 - e^{-1/2 a \sin^2 \psi \cdot (\epsilon + c)}}{1 - e^{-a \cos^2 \psi \cdot (\epsilon + c)}}. \tag{8}$$

With $\epsilon \neq 0$, we get a finite absorption even at zero chloroplast concentration. We checked the effect of ϵ on the fit to Eq. 8. It improves the fit, but some deviations remain at higher concentration. As a second source for these deviations we considered light scattering. It is proportional to the concentration, but we have to assume that for the $F_\parallel$ component the scattering is higher than for $F_\perp$. This gives a linear correction to Eq. 8:

$$\frac{F_\perp}{F_\parallel} = \frac{1 - e^{-1/2 a \sin^2 \psi \cdot (\epsilon + c)}}{1 - e^{-a \cos^2 \psi \cdot (\epsilon + c)}} (1 + nc) \tag{9}$$

where n is a new parameter, accounting for the light scattering. It turns out that Eq. 9 gives quite a good fit to the experimentally observed FP ratios. This is shown for different fluorescence wavelengths in Fig. 3. The fitting parameters of Eq. 9 are included in Table I.

The main consequence of introducing the self-absorption (parameter) is a much higher FP ratio, extrapolated to

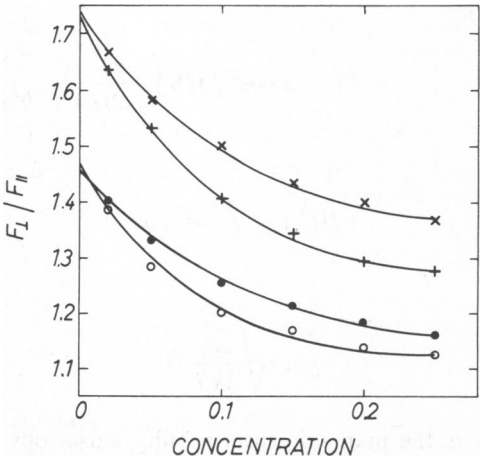

FIGURE 3 Concentration dependence of the fluorescence polarization ratio for different fluorescence wavelengths. ●, 650 nm; ○, 675 nm; +, 700 nm; ×, 725 nm. Full lines from Eq. 9 with parameters given in Table I.

TABLE I
PARAMETERS OF EQ. 9 AT DIFFERENT FLUORESCENCE WAVELENGTHS

λ_f	ψ	$F_\perp/F_\parallel$	a	ϵ	n
nm					
650	63°	1.93	7.3	0.1	0.3
675	64.5°	2.2	9.7	0.09	0.35
700	67°	2.78	6.7	0.1	0.6
725	67°	2.78	5.1	0.13	0.7

zero concentration and zero ϵ. This means that the chlorophyll dipoles are more inclined to the membrane plane than would be deduced from the simple *FP* extrapolation to zero concentration. The increasing ψ with increasing wavelength (Table I) again underlines its importance for an effective energy transfer to the reaction centers.

The self-absorption probably explains the rather wide range of reported *FP* values. We found, e.g., that springtime spinach (purchased on the market) had a higher *FP* for the extrapolated to zero chloroplast concentration ($FP \sim 1.7$) than the summer spinach ($FP \sim 1.4$). This can be accounted for by a higher chlorophyll content of the chloroplast for the summer spinach resulting in a higher value for ϵ.

Magnetic Field Dependence of the Fluorescence Polarization

Assuming that the effect of the magnetic field will rotate the chloroplast as a whole, Knox and Davidovich (12) derived formulas for the magnetic field dependence of the fluorescence intensity $F_\perp$ and $F_\parallel$. With a slightly different notation and for $b = 1$ (shape factor) one has

$$\frac{F_\perp}{2F_\perp + F_\parallel} = (1 + \cos^2\psi)/4 - (1 - 3\cos^2\psi)/8\left(\frac{e^{h^2}}{hD(h)} - \frac{1}{h^2}\right) \quad (10)$$

where

$$D(h) = \int_0^h e^{x^2}\,dx$$

and

$$h = \sqrt{\frac{\Delta\chi}{2kT}}H,$$

and $\Delta\chi$ is the magnetic susceptibility anisotropy for the whole chloroplast. (It can be shown generally that Eq. 2 will be valid for all values of H, assuming the absorption does not change. So $2F_\perp + F_\parallel$ is a convenient normalization.)

We wanted to fit our measured values to Eq. 10. But a linear increase in $F_\perp$ observed at higher fields (Fig. 4) gives a deviation from Eq. 10. (These equations describe a saturation of $F_\perp$ and $F_\parallel$ at higher fields. An increase of $F_\perp$ for spinach chloroplasts at higher fields was observed by Geacintov et al., too [2].) A possible cause of the deviation is that the assumption of the rigidity of the whole chloroplast in magnetic fields is not valid. In a magnetic field there is a torque on the thylakoid stacks. This torque will rotate the chloroplast as a whole. But inside the chloroplast the stacks can rotate individually against a linear, elastic force. This linear force may arise from the membranes connecting the grana stacks. The rotation of the grana stacks will increase $F_\perp$ and $F_\perp/F_\parallel$ linearly.

To compare $F_\perp$ with Eq. 10 we subtracted a linear portion, extrapolated from higher fields, from the observed $F_\perp$ value. The corrected values were fitted to Eq. 10. As can be seen from Fig. 4, the agreement with Eq. 10 is quite good, not only in shape (as reported earlier) but over the whole magnetic field interval. The location of the inflection point at quite a low field (~0.3 T) is surprising.

Polydispersity of the chloroplasts can be ruled out as a reason for the deviation of $F_\perp$ from Eq. 10 at higher fields. A high polydispersity would lead to a deviation from Eq. 10 at lower fields as well, which is not observed within experimental error (Fig. 4). So we conclude that the elastic rotation of the grana stacks inside the chloroplast is a more realistic assumption.

From the fitting we get for the magnetic anisotropy, $\Delta\chi = 3.4 \times 10^{-20}$ cm^3. Assuming a value of $\Delta\chi \sim 3.4 \times 10^{-28}$ cm^3 for a chlorophyll *a* molecule (12), it follows that at least $\sim 10^8$ molecules of chlorophyll *a* per chloroplast should be present to account for the orientation. It seems that this number is too high and we should assume that other molecules with high magnetic anisotropy also contribute to $\Delta\chi$. This assumption is supported by the observation that for summer-time spinach chloroplasts, where the chlorophyll *a* content is higher (see the previous paragraph), the inflection point in the magnetic field dependence occurs approximately at the same field.

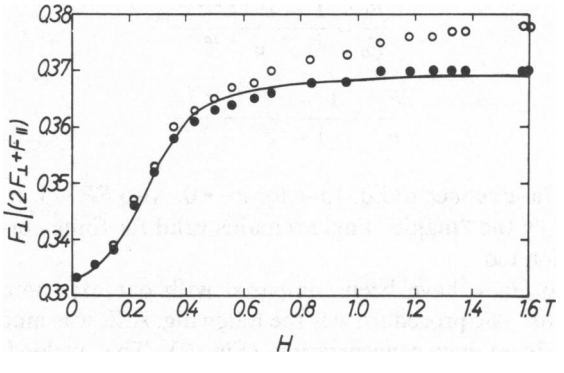

FIGURE 4 Magnetic field dependence of the perpendicular component of the fluorescence intensity. ○, measured values; ●, corrected measured values; full line from Eq. 10 with $\psi = 59.3°$, $\sqrt{\Delta\chi/(2kT)} = 6.5\ \mathrm{T}^{-1}$ and $\lambda_f = 725$ nm.

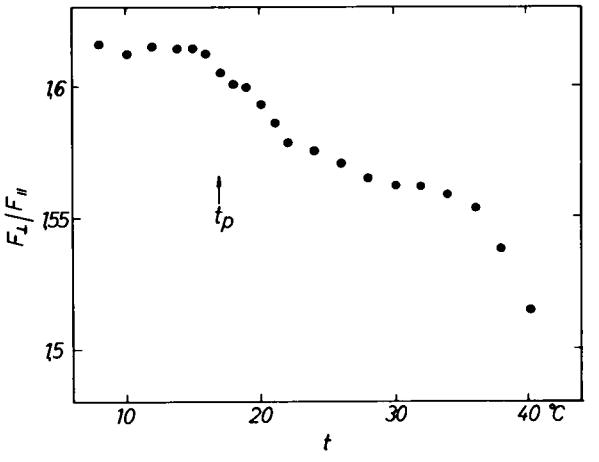

FIGURE 5 The temperature dependence of the fluorescence polarization ratio. H = 1,6 T; λ_f = 725 nm.

Temperature Dependence of the Fluorescence Polarization Ratio

The temperature dependence of the *FP* was measured between 5 and 40°C in constant magnetic field (1.6 T) (Fig. 5). The *FP* remains constant up to 17–22°C (depending on the sample and its fluorescence wavelength). Above this temperature (t_p) there is a decrease in *FP*. This behavior is approximately reversible for temperatures up to ~30°C. Above this temperature the decrease is irreversible.

For a possible explanation of the change in *FP* around t_p we suggest that it reflects a phase transition in the membrane. Such a phase transition around 20°C has been reported (16). If the membrane above t_p becomes less rigid, then the rotational diffusion of the chloroplast complexes will be faster, resulting in a decrease of the *FP* (the averaged value of ψ decreases). The wavelength dependence of t_p may be connected with different environments of the different chloroplast groups.

The above explanation is very tentative; the problem needs further investigation.

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