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ANISOTROPY OF PHOTOSYNTHETIC MEMBRANES AND THE DEGREE OF FLUORESCENCE POLARIZATION

J. F. BECKER, J. BRETON*, N. E. GEACINTOV and F. TRENTACOSTI

Radiation and Solid state Laboratory and Chemistry Department, New York University, New York, N.Y. 10003 (U.S.A.)

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SUMMARY

The degree of fluorescence polarization, P, of unoriented and magnetically oriented spinach chloroplasts as a function of excitation (400–680 nm) and emission wavelengths (675–750 nm) is reported. For unoriented chloroplasts P can be divided into two contributions, P_{IN} and P_{AN}. The latter arises from the optical anisotropy of the membranes which is due to the orientation with respect to the membrane plane of pigment molecules in vivo. The intrinsic polarization P_{IN}, which reflects the energy transfer between different pigment molecules and their degree of mutual orientation, can be measured unambiguously only if (1) oriented membranes are used and the fluorescence is viewed along a direction normal to the membrane planes, and (2) the excitation is confined to the Q_y ($\approx$ 660–680 nm) absorption band of chlorophyll in vivo. With 670–680 nm excitation, values of P using unoriented chloroplasts can be as high as $+14\,\%$, mostly reflecting the orientational anisotropy of the pigments. Using oriented chloroplasts, P_{IN} is shown to be $+5\pm1\,\%$. The excitation wavelength dependence studies of P_{IN} indicate that the carotenoid and chlorophyll Q_y transition moments tend to be partially oriented with respect to each other on a local level (within a given photosynthetic unit or its immediate neighbors).

INTRODUCTION

The study of the degree of polarization of fluorescence, P, of chlorophyll *a* in vivo has been pursued by a number of investigators in the hope of shedding some light on the mechanisms of energy transfer in photosynthetic membranes.The low values of P ($\approx$ 1–6 %) reported for *Chlorella* [1–5] or spinach chloroplasts [6–8] have usually been attributed to the efficient energy transfer between pigment molecules with a low degree of mutual order. There are two major difficulties in interpreting these earlier measurements. (1) Most of the measurements did not include

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea.

* Permanent address: Service de Biophysique, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, BP 2, 91190 Gif-Sur-Yvette, France

excitation wavelengths corresponding to the Q_y red absorption band of chlorophyll in vivo ($\approx$ 670–680 nm); an exception is the more recent paper by Whitmarsh and Levine [9]. Since the fluorescence also has Q_y polarization, excitation into higher singlet bands of chlorophyll (which have a different polarization), and excitation of accessory pigments, gives rise to lower values of P. (2) The pigments in vivo are oriented; the Q_y transition moments of a large fraction of chlorophyll *a* molecules tend to be oriented close to, or within the planes of the membranes [10–13]. The fluorescence of chlorophyll *a* in photosynthetic membranes is thus highly anisotropic [11].

In a preliminary report [14] we have shown that this orientational anisotropy contributes significantly to the magnitude of P, when suspensions of unoriented chloroplasts or whole *Chlorella* cells are used. This orientational effect is highly pronounced, and it is difficult if not impossible to draw quantitative conclusions regarding energy transfer in photosynthetic units which are based on values of P obtained with suspensions of unoriented chloroplasts or whole cells. Fortunately, the observation that spinach chloroplasts, whole *Chlorella* cells, etc. can be oriented in magnetic fields of about 10 kG [11], provides a technique for studying the degree of fluorescence polarization using oriented photosynthetic membranes under physiological conditions. The orientational contribution to P can be minimized by viewing the fluorescence along the normal to the planes of the membranes. Meaningful conclusions concerning the depolarization of fluorescence by energy transfer and the mutual orientation of pigments among which energy transfer takes place, can thus be drawn.

In this work, the anisotropic properties of photosynthetic membranes and their effect on measured values of P as a function of emission and absorption wavelengths are discussed in detail. Using magnetically oriented spinach chloroplasts, values of P reflecting only energy transfer and the mutual orientation of pigments, among which transfer takes place using Q_y excitation, are reported for the first time.

EXPERIMENTAL

Chloroplasts prepared from spinach leaves (obtained from a local market) by methods described previously [14] were suspended in a solution consisting of 0.4 M sucrose/0.004 M NaCl/0.01 M sodium ascorbate/0.02 M Tris buffer (pH 7.9) and 5 % Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), added to prevent settling of the chloroplasts.

The degree of fluorescence polarization P is defined by the equation:

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

where the angle between the excitation and fluorescence viewing directions is 90°, $I_{\parallel}$ is the fluorescence component polarized parallel to that of the excitation (denoted as "vertical" polarization), while $I_{\perp}$ is the perpendicular component. P was measured using an apparatus which was designed and especially built to fit between the poles of an electromagnet with a 5 cm pole-gap capable of yielding a steady magnetic field of 13 kG. This apparatus has also been described elsewhere [14]. It permits the

detection of fluorescence propagating in a direction along the magnetic field, and thus parallel to the normals of the oriented membrane planes [14].

The fluorescence was excited by means of a 750 W tungsten lamp. The excitation wavelengths were selected either by means of a monochromator or narrow bandpass filters, the nature of which will be indicated whenever appropriate. The fluorescence was also viewed either with a monochromator or through narrow wavelength bandpass filters. Sheet polarizers (type HN-22 Polaroid Corporation, Cambridge, Mass.) were used for both excitation and viewing. Light was transmitted into and out of the magnet bore by means of 1/4 inch fiber optics or quartz light pipes. The photomultiplier (RCA 7164R) and the electronic equipment were thus kept sufficiently far away from the magnet to avoid artefacts due to stray magnetic fields.

The excitation was mechanically chopped at 676 Hz and a lock-in amplifier was used as a phase sensitive detector. The output of this amplifier was displayed on an X-Y chart recorder.

The stray light levels were checked by removing the viewing filters from the fluorescence detecting optical train and placing them in the excitation light beam. Stray light levels were less than 1 % of the fluorescence intensity and were considered to be negligible (except as noted).

The residual polarization of light within the apparatus was tested using a concentrated methanolic extract of spinach chloroplasts. The fluorescence of such concentrated chlorophyll solutions are known to be depolarized. Horizontally polarized exciting light was used with a polarization which was perpendicular to both $I_{\parallel}$ and $I_{\perp}$ in Eqn. 1, thus, ideally P should be zero. The residual polarization of the apparatus was less than 1 % (typically +0.8 %): the values of P reported here for spinach chloroplasts have been corrected for this small residual polarization. We note in passing, that unoriented suspensions of chloroplasts and whole algal cells cannot be used for determining the residual polarization of the apparatus using horizontally polarized exciting light. We have shown previously that due to the optical anisotropy of photosynthetic membranes partially polarized light with $I_{\parallel} \neq I_{\perp}$ is produced even when horizontally polarized exciting light is used [14].

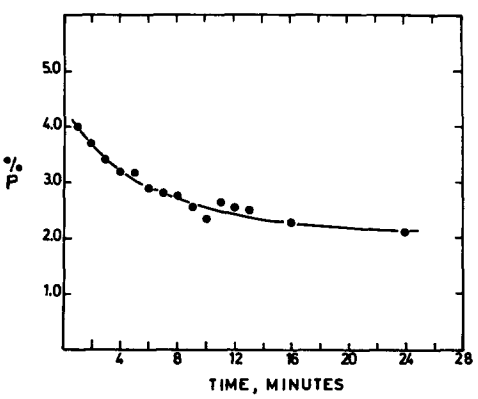

Fig. 1. Degree of polarization of fluorescence of a suspension of unoriented spinach chloroplasts as a function of time. Excitation wavelengths (350–580 nm) isolated with a broad band-pass Corning CS 4-72 filter. Fluorescence viewing wavelength $\lambda > 700$ nm (a cut-off filter was used).

We have observed that it is in general more difficult to obtain reproducible values of P when suspensions of unoriented chloroplasts were used, than when the chloroplasts were oriented magnetically. One reason for this behaviour is that the values of P change as a function of time after the sample has been poured into the sample cuvette or stirred vigorously. An example of this behaviour is shown in Fig. 1. The values of P decrease with time and approach a limiting value after about 30 min. Upon vigorous stirring, the initial polarization value is restored. We tentatively attribute this effect to a hydrodynamic and/or gravitational orientation of the chloroplasts.

Idealized membranes

We first consider a hypothetical membrane which can be represented by a rectangular slab. We consider the case in which the absorbing oscillators are completely at random but the emitting oscillators are lying in the membrane plane (at random within this plane). We then consider the emission of such slabs oriented within the three different orthogonal planes of a cartesian coordinate system as shown in Fig. 2. We further assume that energy absorbed by any single oscillator is efficiently redistributed by energy transfer to any other oscillator with equal probability. It is evident therefore, that for the orientation XZ shown in Fig. 2(A), $I_{\parallel} = I_{\perp}$ and the value of P will be zero. The values of P for orientations Figs. 2(B) and (C) will be -1 and $+1$ respectively.

If we now consider a random orientation of such rectangular membranes, we may assume as a first approximation that one-third of the membranes have a maximal projection in each of the three ortogonal planes as shown in Fig. 2. The orientations shown in this figure can thus serve as a useful model for a random suspension of these rectangular slabs in which each of the orientations XZ, XY and YZ are equally probable.

It is evident from Fig. 2, that for a random suspension, the overall value of P

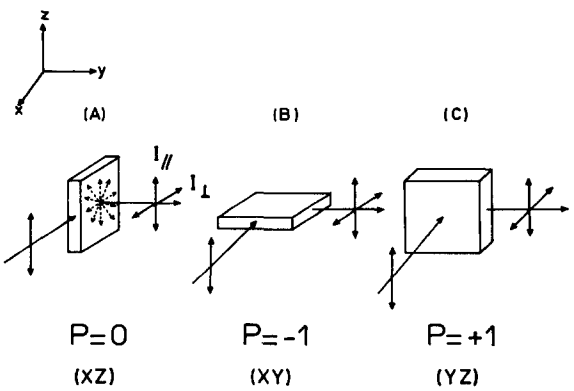

Fig. 2. Emission properties of hypothetical membranes with a random planar arrangement of emitting oscillators as shown in (A) and a three-dimensionally random orientation of absorbing oscillators (not shown in the figure). $I_{\parallel}$ and $I_{\perp}$ are the fluorescence components polarized parallel and perpendicular with respect to the polarization of the excitation. P is the degree of fluorescence polarization defined by Eqn. 1.

will be equal to zero, only if equal amounts of energy are absorbed by each of the three orientations.

Geometric photoselection

The amount of energy absorbed by each of the three orientations when an unpolarized exciting light beam is utilized is not the same [14]. In orientation YZ (Fig. 2(C)) the rectangular slab faces the light beam and thus presents a larger cross-section for absorption of light than both orientations XZ and XY in Fig. 2. We propose to call this effect geometric photoselection. Since slab YZ contributes more to the fluorescence intensity than the other slabs, the overall value of P for a suspension of randomly oriented slabs will be finite with $P > 0$.

The geometric photoselection effect is illustrated for magnetically oriented

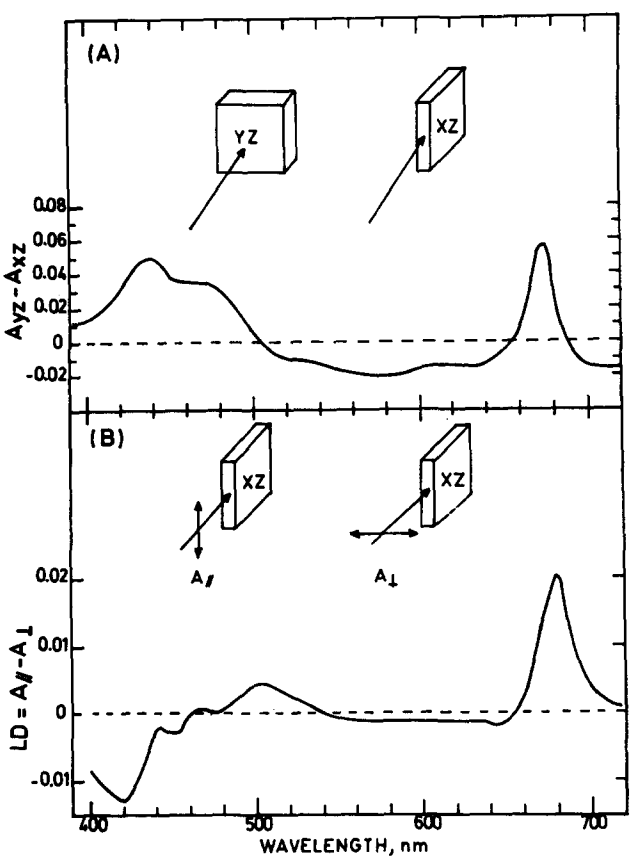

Fig. 3. Anisotropic absorption properties of spinach chloroplast membranes. (A) The geometric photoselection effect; the differences in absorbance $A_{YZ}-A_{XZ}$ for the same suspension of chloroplasts is plotted for two different directions of the light beam using unpolarized light. The graph was derived from data given in Van Nostrand's dissertation [24]. The $A_{YZ}-A_{XZ}$ spectrum is similar to the absorbance of an unoriented suspension of chloroplasts, but with a shifted baseline (due to light scattering effects). (B) Linear dichroism (LD) of membranes; $A_{\parallel}$ and $A_{\perp}$ are the absorbances for light polarized parallel and perpendicular with respect to the planes of the membranes.

spinach chloroplast membranes in Fig. 3(A). It is shown that when the membranes are oriented with their planes perpendicular to the incident light beam, more light is absorbed than when they are oriented with their planes parallel to this direction. The incident light beam does not need to be polarized.

Photoselection by polarized light

Different amounts of light are also absorbed by membranes when their edges are turned towards the direction of the light beam. This situation is depicted in Fig. 3(B). The differences in absorption for the two polarizations are depicted in terms of the linear dichroism LD, where

$$\mathrm{LD} = A_{\parallel} - A_{\perp} \tag{2}$$

where $A_{\parallel}$ and $A_{\perp}$ are the light absorbances for light polarized parallel and perpendicular to the membrane planes respectively. Since the linear dichroism is strongly wavelength dependent, differences in the amount of light absorbed by the two orientations will also depend on the wavelength of the exciting light. Using red light, more light will be absorbed with a polarization parallel to the membrane planes since the Q_y transition moment vectors tend to be oriented in this plane. The opposite tends to be observed in the blue (Soret) region of the spectrum, since the planes of the porphyrin rings tend to be tilted out of the membrane planes and the transitions within the Soret band are degenerate and polarized within the plane.

Fluorescence anisotropy and the emission wavelength dependence of P

The preferred orientation of the Q_y oscillators close to or within the plane of the membranes gives rise to the relatively large linear dichroism in the region of 680 nm (Fig. 3(B)). The fluorescence, which also has a Q_y origin and peaks at $\approx$ 685 nm, is strongly polarized parallel to the membrane planes. If we denote the intensity of the fluorescence polarized parallel to the membrane plane by $F_{\parallel}$ and the perpendicular component by $F_{\perp}$, we find that the ratio $F_{\parallel}/F_{\perp}$ is wavelength dependent. This wavelength dependence is due to the heterogeneity of chlorophyll in vivo and reflects the orientation of the Q_y transition moment vectors of the different chlorophyll *a* forms with respect to the membrane planes. The larger the ratio $F_{\parallel}/F_{\perp}$ the greater the degree of orientation of Q_y close to the membrane planes. The data shown in Fig. 4(B) indicates that the shorter wavelength forms of chlorophyll *a* in vivo are less well oriented than the longer wavelength forms. These results are consistent with an analysis of the linear dichroism data [12, 13].

We now consider the dependence on emission wavelength of the degree of fluorescence polarization P. This value determined with a suspension of randomly oriented chloroplasts will be denoted by $P(O)$. We distinguish contributions to $P(O)$ from two sources: (1) the contribution from the orientational anisotropy which is due to the orientation of chlorophyll in vivo, termed P_{AN}. (2) The intrinsic contribution P_{IN}. This is the component which reflects the energy transfer among different chlorophyll molecules and the relative orientation of these molecules.

We can then write

$$P(O) = P_{\mathrm{IN}} + P_{\mathrm{AN}} \tag{3}$$

The contribution of P_{AN} to $P(O)$ will be larger the greater the orientation of

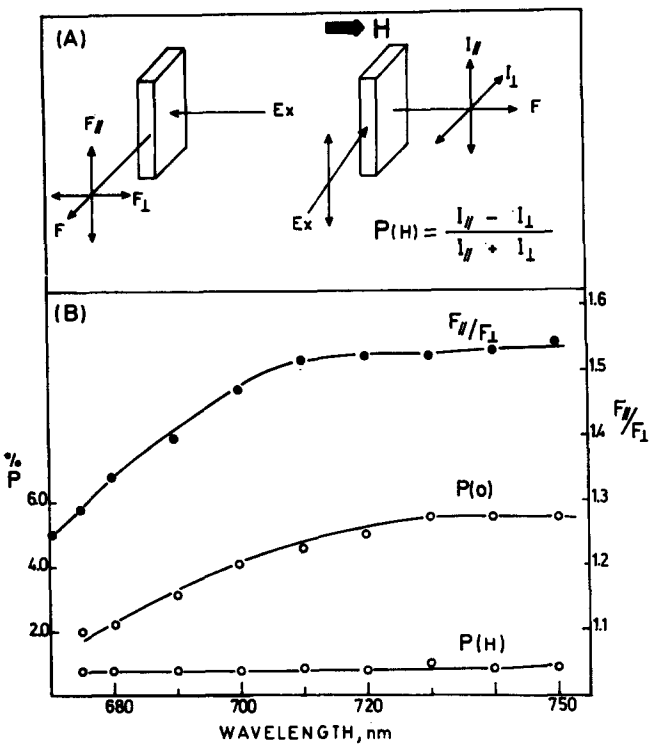

Fig. 4. Fluorescence anisotropy $F_{\parallel}/F_{\perp}$ of spinach chloroplast membranes and degree of fluorescence polarization of randomly oriented ($P(O)$) and magnetically oriented ($P(H)$) chloroplasts as a function of emission wavelength. Broad band-pass (350–580 nm) excitation was used in these experiments. The experimental configurations for $F_{\parallel}/F_{\perp}$ and $P(H)$ are shown in (A).

the Q_y transition moment vectors. The strong resemblance between the $F_{\parallel}/F_{\perp}$ and $P(O)$ curves shown in Fig. 4(B) support this conclusion; in this particular experiment, blue light was used for excitation so that P_{IN} is relatively small (the absorbing and emitting oscillators are not the same). $P(O)$ therefore represents primarily P_{AN}, rather than P_{IN}. Thus $P(O) \approx P_{AN}$ in this case, and the effects of chlorophyll orientation can be observed for randomly oriented spinach chloroplasts due to the effects of geometric photoselection described above. In general the greater the value of $F_{\parallel}/F_{\perp}$ at a given wavelength, the greater will be the value of P_{AN} at that wavelength.

The orientational anisotropy effects can be eliminated if the fluorescence is viewed along the normal to the membrane planes which is the symmetry axis of the photosynthetic membranes. If the fluorescence is viewed along a direction parallel to the membrane planes, $P \rightarrow P_{AN}$ [11], where in this case P_{AN} is defined by

$$P_{AN} \approx \frac{F_{\parallel}-F_{\perp}}{F_{\parallel}+F_{\perp}} = \frac{F_{\parallel}/F_{\perp}-1}{F_{\parallel}/F_{\perp}+1} \tag{4}$$

It is therefore evident that oriented membranes must be used in order to obtain information about P_{IN} and that the fluorescence viewing direction is crucial. The recommended experimental configuration is shown on the right hand side of Fig. 4(A).

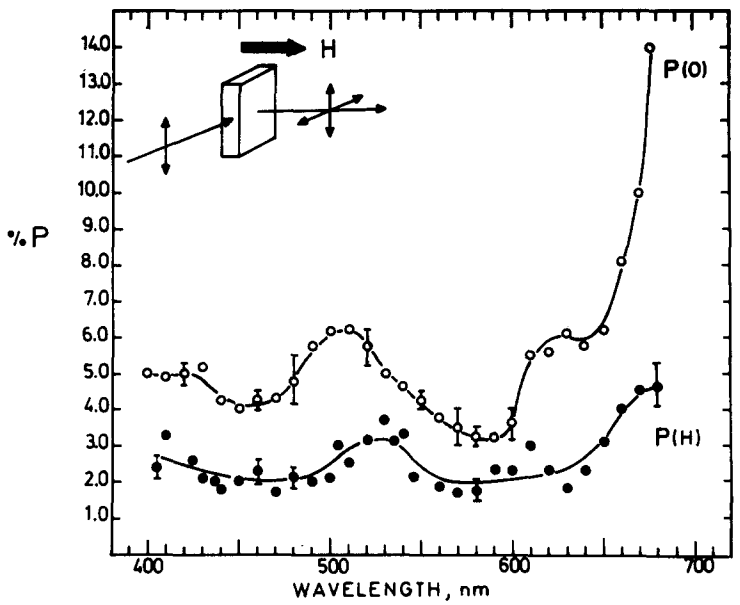

Fig. 5. Degree of fluorescence polarization as a function of excitation wavelength of unoriented ($P(O)$) and oriented ($P(H)$) spinach chloroplasts. The recommended experimental configuration for measuring P is also shown. A sharp cut-off (700 nm) filter was used for viewing the fluorescence for excitation above 600 nm, and a Corning CS 2-64 filter was used for excitation below 600 nm.

When the membranes are oriented in a magnetic field, their planes are perpendicular to the field [12, 15] and the fluorescence must be viewed along a direction parallel to the magnetic field. The degree of fluorescence polarization measured under these conditions is denoted by $P(H)$ where

$$P(H) \approx P_{IN} \tag{5}$$

It is evident that for all emission wavelengths $P(H) < P(O)$ which is demonstrated in Fig. 4(B). The orientational anisotropy is removed and the emission wavelength dependence of $P(H)$ is flat when blue excitation light is utilized; typical values of $P(H)$ range from 0.5 to 2 %. There are similar variations in $P(O)$ which can vary strongly from sample to sample.

The dependence of P on the excitation wavelength

The excitation wavelength dependence of both $P(O)$ and $P(H)$ are shown in Fig. 5. The sample utilized in the determination of $P(O)$ gave particularly high values, which were as large as +14 % upon excitation within the Q_y band; more typical values are about 8–12 % for excitation within the Q_y band. The wavelength dependence of $P(O)$ is similar to the one published by Goedheer [6]; this author, however, did not extend the excitation wavelengths beyond 640 nm.

The effect of orientation of absorbing oscillators within the planes on P

There is a uniform decrease in the degree of fluorescence polarization when the magnetic field is switched on and the fluorescence is viewed along the magnetic field as shown in the insert of Fig. 5. In both the $P(O)$ and $P(H)$ curves there is a bump in

the region of 500–530 nm. A similar maximum is observed in the linear dichroism spectrum in Fig. 3(B) which is attributed to the preferred orientation of the long axes of the carotenoid molecules within the membrane planes [12, 13].

As the excitation wavelength approaches the Q_y red absorption band of chlorophyll there is a significant increase in both the $P(O)$ and $P(H)$ values. This increase is attributed to the fact that the absorption and emission upon excitation within the Q_y band originate from the same group of oscillators. Excitation at shorter wavelengths gives rise to an intrinsic depolarization as the energy cascades from the higher lying singlet states to the lowest lying Q_y singlet state of chlorophyll. Excitation within the Q_y band therefore should always give the highest values of P. Meaningful interpretations about energy transfer in vivo based on measurements of the degree of fluorescence polarization can therefore be made only if the excitation wavelength corresponds to the Q_y band of chlorophyll.

We return to a discussion of the shape of the $P(O)$ curve in Fig. 5. At all wavelengths $P(O) > P(H)$ and the orientational anisotropy manifests itself both in emission (Fig. 4) and the absorption. We assume that a real suspension of unoriented chloroplasts can be represented crudely by the three projections XZ, XY, and YZ shown in Fig. 2. We know from linear dichroism spectra that the Q_y and carotenoid oscillators tend to lie in the planes of the membranes. We now consider the contributions of each of the configurations in Fig. 2 to the fluorescence components $I_{\parallel}$ and $I_{\perp}$. In the carotenoid and Q_y region of the absorption spectrum, membrane XZ will absorb more light than membrane XY (see Fig. 3(B)). Membrane XZ will thus emit more fluorescence than XY. If P_{IN} is sufficiently larger than zero for excitation within the $\lambda = 500$–530 nm or $\lambda > 660$ nm regions, $I_{\parallel}$ will be larger than $I_{\perp}$ if the effects of membranes XZ and XY are summed. The contribution of membrane YZ to $I_{\parallel}$ will also be larger than to $I_{\perp}$ because of its geometric orientation and the orientation of the absorbing and emitting oscillators within the plane.

Effect of random orientation of absorbing oscillators on P

Absorbing oscillators which are completely randomly oriented (three dimensional randomness) can also contribute to the anisotropy since in such a case both membranes XZ and XY will contribute equal amounts of fluorescence since they absorb equal amounts of energy; however, since the emitting oscillators lie in the planes of the membranes, $I_{\parallel} = I_{\perp}$ for XZ and $I_{\perp} > I_{\parallel}$ for XY; thus in such a case, the net effect of membranes XZ and XY will be to produce a negative value of $P(O)$ when the absorbing oscillators are randomly oriented. Membrane YZ on the other hand, because of the geometric photoselection effect, will absorb more light than the other two configurations and will yield $I_{\parallel} > I_{\perp}$ which tends to give a positive value of $P(O)$. The overall effect of the three membranes in such a case is to give a value of $P(O)$ close to zero. Moreover, small positive values, or small negative values of $P(O)$ may be observed which depend on the relative effects of membrane XY as compared to membrane YZ. We observe positive values of $P(O)$ at all wavelengths of excitation and conclude that the anisotropy contributed by membranes YZ is greater than that contributed by membranes XY in the regions where the linear dichroism is close to zero.

Relative pigment orientation and its effect on P

When the magnetic field is switched on and the fluorescence is viewed as

indicated in Fig. 5, $P(H)$ should be zero if $P_{IN} = 0$; small residual effects in $P(H)$ may still be observed due to the anisotropic emission properties of the membranes as discussed above, and due to imperfect orientation of the membranes in the magnetic field. The background values of $P(H)$ of 0.5–2 % shown in Figs. 4 and 5 are attributed to these effects.

The higher values of $P(H)$ in the carotenoid and Q_y absorption bands of chlorophyll definitively indicates that P_{IN} is not zero. This means that depolarization by energy transfer within the photosynthetic units is not complete. These finite values of $P(H)$ can be interpreted in two ways:

(1) a larger number of energy transfer steps between pigment molecules which possess a high degree of mutual orientation.

(2) a very small number of transfer steps among pigment molecules possessing a low degree of mutual orientation. In this case the polarization would be due mainly to the return of the energy to the molecule which was originally excited, which is an event of low probability [16].

It should be noted that the orientation of oscillators close to or within the plane, does not imply that the transition moment vectors possess a high degree of orientation within the plane of the membrane with respect to each other. Thus in the presence of extensive energy transfer and a low degree of mutual orientation $P(H)$ could still be zero, even if all of the oscillators lie perfectly in the plane of the membranes.

Possibility (2), a very small number of energy transfer steps, appears to be unlikely in view of the high trapping efficiency of the excitation by the reaction centers which are associated with 200–300 antenna molecules in photosynthetic units [17]. Furthermore, energy transfer between photosynthetic units probably occurs as well [18]. In view of these considerations, it appears most reasonable to interpret the degree of fluorescence polarization in terms of possibility (1).

It is concluded that the carotenoid and Q_y transition moment vectors are not only oriented closely to the membrane planes, but also are oriented with respect to each other within the membrane plane. This relative partial orientation probably exists at a local level within a given photosynthetic unit or its immediate neighboring units. Because of the short ($<$ ns) lifetime of the fluorescence [17], it cannot range too far from the site of absorption of the light quantum and the degree of polarization does not provide information about any possible longer range order.

In principle, the intrinsic degree of fluorescence polarization P_{IN} depends both on the number of transfer steps during the lifetime of the excitation and on the degree of mutual orientation of oscillators within the membrane planes. The transfer rate in vivo is not known [17] and thus only qualitative conclusions regarding the mutual orientation of pigments can be made at this time. Furthermore, conclusions regarding the mechanisms of energy transfer within photosynthetic units [5, 9, 17] which are based on the degree of fluorescence polarization data above appear to be too speculative and will not be discussed further.

Using oriented membranes, two types of information regarding the orientation of pigments can be obtained.

(1) The overall orientation of transition moments with respect to the membrane planes can be obtained by studying the fluorescence anisotropy $F_{\parallel}/F_{\perp}$ (Fig. 4), linear dichroism (Fig. 3), or anisotropy in the 90° light scattering [19] employing

suspensions of magnetically oriented membranes. In the measurement of the fluorescence anisotropy, the small effects of P_{IN} which are superimposed on the values of $F_{\parallel}/F_{\perp}$ depending on the mode of excitation [11], can be minimized by using blue (Soret region) broad band exciting light. Since, there is little absorption anisotropy in this case, the emission anisotropy will reflect only the orientation of the emitting Q_y oscillators.

(2) The local order and mutual orientation of pigments with respect to each other within a given photosynthetic unit and its immediate neighbors can be estimated from measurements of $P(H) \approx P_{IN}$ using magnetically oriented samples. This method is basically a photoselection technique applied to oriented membranes.

Excitation and viewing within the Q_y band and effects of DCMU

The red band of chlorophyll in vivo is actually a superposition of a number of different Q_y absorption bands of the different spectroscopic forms [20] of chlorophyll *a*. Similarly, the fluorescence spectrum can be considered as the superposition of the emission of several forms of chlorophyll [21]. Excitation at different wavelengths and viewing the fluorescence at different wavelengths thus may reveal how energy is channeled among the different spectroscopic forms of chlorophyll. Furthermore, there are at least two suitable methods for increasing the lifetime of the excitation and thus observing its effect on the degree of fluorescence polarization. An increased number of transfer steps is expected under the conditions when the reaction centers are closed by using either high light intensities, or the photosystem II inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea). Under these conditions the fluorescence lifetime increases [22, 23] and P_{IN} may decrease if the energy transfer occurs among a group of poorly oriented oscillators and a larger number of these molecules is sampled by the excitation during its lifetime. On the other hand, if the excitation ranges over a set of molecules with a high degree of mutual order, there will be little effect of the increased lifetime on P_{IN}.

Using conventional broad-band light sources for excitation, and combinations of interference and cut off optical filters for isolating the excitation and viewing

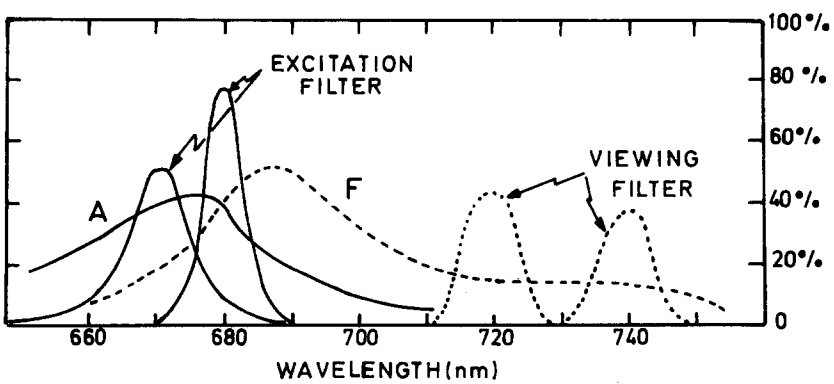

Fig. 6. Transmittance of filter combinations used for the experiments described in Table I. Interference and sharp cut-off filters were used to minimize the stray light levels. The absorbance (*A*) of spinach chloroplasts and fluorescence spectrum (*F*) are shown for reference.

TABLE I

TYPICAL MEASUREMENTS OF THE DEGREE OF POLARIZATION OF FLUORESCENCE OF MAGNETICALLY ORIENTED SPINACH CHLOROPLASTS USING THE FILTER COMBINATIONS SHOWN IN FIG. 6

Each value is the average of about six determinations; the variations from the mean were calculated from the sample variance and reflect a 90 % confidence limit.

Excitation wavelength 670 nm		
Viewing wavelength	720 nm	740 nm
Without DCMU	2.2±0.8 %	5.7±0.6 %
With DCMU (10^{-5} M)	0.8±0.2 %	3.0±0.6 %
Excitation wavelength 680 nm		
Viewing wavelength	720 nm	740 nm
Without DCMU	5.4±1.1 %	4.8±0.3 %
With DCMU (10^{-5} M)	5.2±0.7 %	5.3±0.9 %

wavelengths, the choice of combinations is limited. Using such combinations of filters, whose transmittances are shown in Fig. 6, we were able to excite either at 670 or 680 nm and view the fluorescence at either 720 or 740 nm. The stray-light levels were less than 2 % in all cases except in the E680-F720 (excitation at 680 and viewing at 720 nm) case, where the stray-light level was $\approx$ 5 %; this signal was subtracted from the measured values of $I_{\parallel}$ and $I_{\perp}$ before $P(H)$ was calculated. Following Mar and Govindjee [5], as well as Whitmarsh and Levine [9], the effects of excitation lifetime on P were determined by adding 10^{-5} M DCMU to the suspensions of chloroplasts; this was found to be more convenient than varying the light intensity. In these experiments the exciting light intensity was kept low to prevent the saturation of the reaction centers. Upon adding the DCMU, the fluorescence intensity at 720 and 740 nm increased by factors of 1.3–1.6, depending on the sample.

Typical results obtained upon excitation at 670 and 680 nm and viewing at 720 and 740 are shown in Table I. Variations by as much as ± 1 percentage point from the values given in Table I for a particular sample of chloroplasts were encountered with other samples. However, a DCMU effect is consistently observed upon excitation at 670 nm, while no effect was observed with excitation at 680 nm. This effect may be due to the lower degree of mutual orientation of the shorter wavelength spectroscopic forms of chlorophyll in vivo. Linear dichroism and fluorescence polarization experiments show that the spectroscopic forms of chlorophyll which absorb predominantly at 680 nm are more highly oriented than those which predominate at 670 nm. The effects of DCMU upon excitation at 670 nm indicate that the increased number of transfer steps occur over a set of pigments which have a low degree of mutual orientation, while the opposite appears to be true with 680 nm excitation.

CONCLUSIONS

The optical properties of photosynthetic membranes show that they are highly anisotropic. The degree of polarization of fluorescence must therefore be determined using oriented membranes or whole cells. The fluorescence must be viewed along a normal to the planes of the membranes. Meaningful information about energy transfer and the mutual orientation of pigments among which energy transfer occurs can be obtained only by exciting within the Q_y band of chlorophyll in vivo. Preliminary results obtained here with excitation within the red Q_y band of chlorophyll in spinach chloroplasts and the associated effects of DCMU, indicate that important information concerning the energy transfer pathways in photosynthetic membranes may be obtained using narrow bandwidth (e.g. a dye laser) excitation. Such studies should prove particularly fruitful at lower temperatures where the absorption bands of the different spectroscopic forms of chlorophyll are better resolved than at room temperature.

We finally note that, while the results shown in this paper were obtained with spinach chloroplasts, analogous effects were also observed with suspensions of whole cells of *Chlorella pyrenoidosa.*

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REFERENCES

1 Arnold, W. and Meek, E. S. (1956) Arch. Biochem. Biophys. 60, 82–90
2 Goedheer, J. C. (1957) Thesis, the State University, Utrecht
3 Teale, F. W. J. (1960) Biochim. Biophys. Acta 47, 69–75
4 Lavorel, J. (1964) Biochim. Biophys. Acta 80, 20–36
5 Mar, T. and Govindjee (1971) Proc. of the 2nd Int. Congress on Photosynthesis Research, Stresa, Vol. 1, pp. 271–281, Dr. W. Junk N.V. Publishers, The Hague.
6 Goedheer, J. C. (1966) in Biochemistry of Chloroplasts (Goodwin, T. W. ed.), pp. 75–82, Academic Press, New York
7 Cederstrand, C. and Govindjee (1966) Biochim. Biophys. Acta 120, 177–180
8 Goedheer, J. C. and Gulyaev, B. A. (1971) Proc. of the 1st European Biophysics Congress, Vienna, (Broda, E., Locker, A. and Springer-Lederer, eds.), pp. 43–47, Verlag Wiener. Mediz. Akad., Vienna
9 Whitmarsh, J. and Levine, R. P. (1974) Biochim. Biophys. Acta 368, 199–213
10 Breton, J. and Roux, E. (1971) Biochem. Biophys. Res. Commun. 45, 557–563
11 Geacintov, N. E., Van Nostrand, F., Becker, J. F. and Tinkel, J. B. (1972) Biochim. Biophys. Acta 267, 65–79
12 Breton, J., Michel-Villaz, M. and Paillotin, G. (1973) Biochim. Biophys. Acta 314, 42–56
13 Geacintov, N. E., Van Nostrand, F. and Becker, J. F. (1974) Biochim. Biophys. Acta 347, 443–463
14 Breton, J., Becker, J. F. and Geacintov, N. E. (1973) Biochem. Biophys. Res. Commun. 54, 1403–1409
15 Becker, J. F., Geacintov, N. E., Van Nostrand, F. and Van Metter, R. (1973) Biochem. Biophys. Res. Commun. 51, 597–602
16 Hemenger, R. P. and Pearlstein, R. M. (1971) Abstracts of the Conference on Primary Photochemistry of Photosynthesis, Argonne, Illinois, p. 14
17 Knox, R. S. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 183–221, Academic Press, New York

18 Paillotin, G. (1976) J. Theor. Biol. 58, 237–252
19 Swenberg, C. E. and Geacintov, N. E. (1976) in Excited States of Biological Molecules (Birks, J. B., ed.), pp. 288–300, John Wiley and Sons, Inc. New York
20 French, C. H. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2893–2897
21 Litvin F. F. and Sineshchekov, V. A. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 620–661, Academic Press, New York
22 Müller, A., Lumry, R. and Walker, M. S. (1969) Photochem. Photobiol. 9, 113–126
23 Briantais, J. M., Merkelo, H. and Govindjee (1972) Photosynthetica 6, 133–141
24 Van Nostrand, F. (1972), Thesis, New York University, New York