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Fluorescence anisotropy of chlorophyll *a* and chlorophyll *b* in castor oil

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A detailed study of the fluorescence anisotropy of chlorophyll *a* and chlorophyll *b* in castor oil is presented. Scanning across the wavelength of emission the value of the fluorescence anisotropy shows a considerable variation. This indicates that differently polarized emission moments are present. When excitation spectra of the fluorescence anisotropy are recorded at two distinct emission wavelengths, two separate curves are found that cross each other several times. The difference between the two spectra yields information about the extent to which absorption transitions overlap. It is found that over the whole spectrum of visible light, absorption transition bands overlap, so that the value of the fluorescence anisotropy cannot be interpreted in terms of a single angle between absorption and emission moment. It rather yields an average direction of the overlapping transitions. Castor oil at room temperature is not sufficiently viscous to quench the rotational depolarization of the chlorophyll fluorescence. A limiting value of the fluorescence anisotropy is obtained using time-resolved measurements.

Introduction

Chlorophylls are among the most important biological dye molecules. They are responsible for the absorption of sunlight photons during the primary steps of photosynthesis, the subsequent transfer of excited-state energy, and finally the initiation of charge separation in the reaction center proteins. The relative orientation of the chlorophyll molecules plays an important role in the energy-transfer process. The efficiency of the energy transfer is dependent on the mutual directions of emission and absorption moment in the donor and acceptor molecule, respectively. Thus, the energy transfer path between initial absorption and final charge separation will be markedly influenced by the orientation of the various pigments in the photosynthetic system. It is therefore important to characterize these orientations, both within the protein complex and in the photosynthetic membrane in order to obtain a better insight into the energy-transfer process. The first step in this study, however, must be the determination of the directions of the transition moments in the molecular frame of the chlorophylls themselves.

Although much attention has been paid to chlorophyll *a* and *b*, the most common pigments in algae and green plants, knowledge of the directions of their transition moments is still not complete. This is in the first place due to the low symmetry of the molecules, but secondly because the transition bands overlap across the whole absorption wavelength region. Thus at every wavelength of light with which the molecules are illuminated, more than one transition band is excited.

One of the easiest and most commonly used techniques to study the anisotropic optical properties of dye molecules is fluorescence anisotropy (FA), or fluorescence polarization. A dilute solution of the dye in a viscous solvent is excited with vertically polarized light, and the fluorescence polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the exciting light vector are detected at right angles. The FA is then defined as:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (1)$$

This parameter is a more convenient one than the fluorescence polarization p, defined as:

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (2)$$

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Abbreviation: FA, fluorescence anisotropy.

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since the denominator in Eqn. 1 denotes the total fluorescence. It then follows that the FA of different subpopulations simply add up to the measured value of r [1]. It is well known that the FA is a direct measure for the angle ε between absorption and emission moment in the dye molecule [2,3], provided that only well-separated transitions are involved:

$$r = 0.2(3\cos^2\varepsilon - 1) \tag{3}$$

In the case of mixed transitions (of which chlorophyll and indole [4] are good examples) we have the weighted sum of the anisotropies belonging to the different 'overlapping' transitions:

$$r = \sum_{ij} f_{ij} r_{ij} = 0.2\left(3\sum_{ij} f_{ij}\cos^2\varepsilon_{ij} - 1\right)$$

$$\sum_{ij} f_{ij} = 1 \tag{4}$$

where ε_{ij} is the angle between the absorption moment, i, and the emission moment, j, in a specific molecule, and f_{ij} is the probability that the transition moments i and j are involved. In the simplest case we have one emission moment and two perpendicular absorption moments:

$$r = 0.2\{3[f_1\cos^2\varepsilon_1 + (1-f_1)\sin^2\varepsilon_1] - 1\} \tag{5}$$

We have assumed here that only dipole transitions are involved, that is, a dipole moment is associated with each transition. In our present investigation we are not concerned with the origin of these transition moments, but simply study their vector properties in the macrocyle of the dye molecule. It is important to note here that the considerations of overlapping bands, Eqns. 4 and 5, are based on the general property of fluorescent molecules that the emission spectrum is independent of the excitation wavelength [1,2,33,34]. This is the result of fast internal conversion from the higher excited states to the ground vibrational level of the lowest excited electronic state from which the fluorescence emission occurs [1,2,33,34].

In complex molecules, like chlorophyll, it is very difficult to separate the different transitions that are involved when the molecule is excited at a given wavelength and the emission is recorded at some other wavelength. What we measure is some average value, containing information about the directions of various transition moments that contribute to the experiment, as given by Eqn. 4. If we assume however, that the chlorophylls in our viscous solvent have the same anisotropic absorption and emission properties as in vivo, we can use our findings in studies of the polarized absorption and emission of complete photosynthetic systems.

The FA of chlorophylls has been studied by a large number of workers [5–18]. These studies, in combination with theoretical calculations, helped to assign the absorption bands to orthogonal transitions, lying roughly either along the y or the x direction [19] in the chlorophyll macrocycle [10,15–18,20–23].

In order to find the definite transition moment, directions in the xy-plane one has to incorporate chlorophyll in some macroscopically ordered medium [23–25]. In that case, however, the average orientation of the chlorophylls is unknown, and we have to determine additional order parameters: two when measuring absorption, five when measuring fluorescence [26]. The convenient situation of perfect orientational order is obtained when chlorophyll is incorporated in a host crystal. Boxer and co-workers [27,28] succeeded in incorporating zinc pyrochlorophyllide a into a crystal of human hemoglobin. Using time-resolved and steady-state fluorescence anisotropy they extracted an angle of $95° \pm 2°$ with respect to the x-axis for the $Q_y(0\text{–}0)$ transition of ZnPChl a. Interestingly, a value of 0.366 $\pm$ 0.002 was found for the limiting anisotropy of this molecule, much higher than ever found in anisotropy studies of chlorophyll a in viscous solvents or frozen glasses.

Here we present a detailed study of the fluorescence anisotropy of chlorophyll a and chlorophyll b in castor oil. The emission spectra of the fluorescence anisotropy and also excitation spectra have been recorded at distinct emission wavelengths. We will discuss how the results should be analyzed in terms of average directions of the overlapping transition moments. In addition, we discuss the influence of the spectral resolution of the emission wavelength and the influence of the viscosity of the solvent on the limiting values of the fluorescence anisotropy.

Materials and Methods

Chlorophyll a and chlorophyll b were extracted from fresh spinach according to Terpstra and Lambers [29], and purified using thin-layer chromatography. No attempt was made to disentangle these chlorophylls from their stereoisomers, but it was found that after purification of chlorophyll a only 4% of the pigment was chlorophyll a' following the method of Watanabe [30]. Castor oil was obtained from OPG, Utrecht, The Netherlands. The concentrations of chlorophyll a and b in castor oil were $5 \cdot 10^{-6}$ M.

The experimental set-up consisted of a 1600 W stabilized Xenon lamp, a 0.25 m Jarrell-Ash monochromator with 0.5 mm and 2 mm slits for the excitation and emission anisotropy measurements, respectivly (dispersion 3.3 nm/mm) and a Glan-Thompson prism for polarizing the light vertically. Fluorescence was detected in the 90° geometry with a cooled RCA 31034 pho-

tomultiplier tube through a polaroid sheet (either horizontally or vertically) and either an Oriel 0.125 m monochromator (6.4 nm bandwidth) or an interference filter (660 ± 7, 680 ± 8, 737 ± 13 and 750 ± 12 nm). Only a small spot (2 mm ϕ) of the cuvette was illuminated in order to restrict stray light. In some cases corrections were made for stray light by repeating the measurements with a cuvette only containing castor oil. The true FA can then easily be found due to the additive property of fluorescence anisotropies [1] by subtracting the stray light anisotropy r_s of the blank sample from the measured anisotropy r_m, weighted with the respective intensities I_s and I_m:

$$r = \frac{I_m r_m - I_s r_s}{I_m - I_s} \tag{6}$$

Lenses were used to focus the light on the entrance slit of the monochromators and to produce a parallel light beam prior to its passage through the GT prism and the polaroid sheet. Data were collected using a PAR Lock-in amplifier, triggered by an optical chopper in the excitation beam, and an Apple II-microcomputer with ADC-card.

The polarization bias of the set-up was measured by exciting a dilute acetone solution of chlorophyll *a* with horizontally polarized light and detecting the horizontally and vertically polarized fluorescence. As in this case both polarized intensities should be equal, their measured ratio is a measure for the polarization bias of the set-up [1]. The polarization bias was found to be wavelength-dependent, and was therefore determined over the whole chlorophyll emission spectrum. The temperature of the sample was regulated with a Colora thermostat-bath and kept at 4°C for most measurements.

Time-resolved measurements were carried out in Daresbury (U.K.), using synchrotron radiation as a tunable light source with high repetition frequency [31]. The optical part of the set-up was as described above. Detection was now performed with a cooled Philips XP2233B PM tube, and data were collected using the single-photon counting technique with an Ortec TAC and Techno MCA and stored on a DEC minicomputer.

Decay curves of the parallel and perpendicular components of the fluorescence were measured. From Eqn. 1 and $I_{tot} = I_{\parallel} + 2I_{\perp}$ these can be written as:

$$I_{\parallel}(t) = 1/3 I_{tot}(t)[1 + 2r(t)]$$

$$I_{\perp}(t) = 1/3 I_{tot}(t)[1 - r(t)] \tag{7}$$

Both the fluorescence lifetime $I_{tot}(t)$ and the fluorescence anisotropy $r(t)$ were assumed to have a two-exponential decay, and the measured decay curves $I_{\parallel}(t)$ and $I_{\perp}(t)$ were deconvoluted simultaneously [32] using a Marquardt non-linear least-squares procedure.

Results

Wavelength dependence of the fluorescence anisotropy

The absorption and emission spectra of chlorophylls *a* and *b* are shown in Fig. 1. Fig. 2 shows the FA at varying emission wavelengths when both chlorophylls are excited at their respective $Q_y(0-0)$ band maximum. A distinct decrease of the FA with increasing wavelength can be seen, though the FA tends to increase again at high wavelengths. The excitation FA spectra at two different emission wavelengths chosen from Fig. 2, 680 and 750 nm for chlorophyll *a* and 660 and 737 nm for chlorophyll *b*, are shown in Figs. 3 and 4, respectively. Differences can be seen across the whole spectral range, but especially at the $Q_y(0-0)$ bands and around 380 nm. Note also the band at 495 nm in chlorophyll *b*, and the increase of the FA at the red edge of the absorption when the emission is detected at 737 nm.

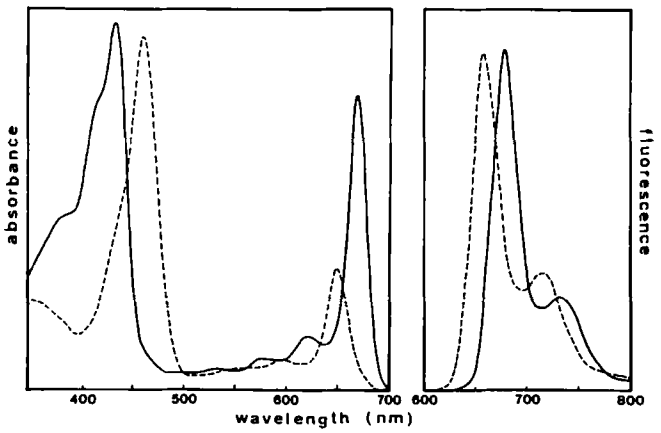

Fig. 1. Absorption (left) and fluorescence (right) spectra of chlorophyll *a* (———) and chlorophyll *b* (– – –) in castor oil.

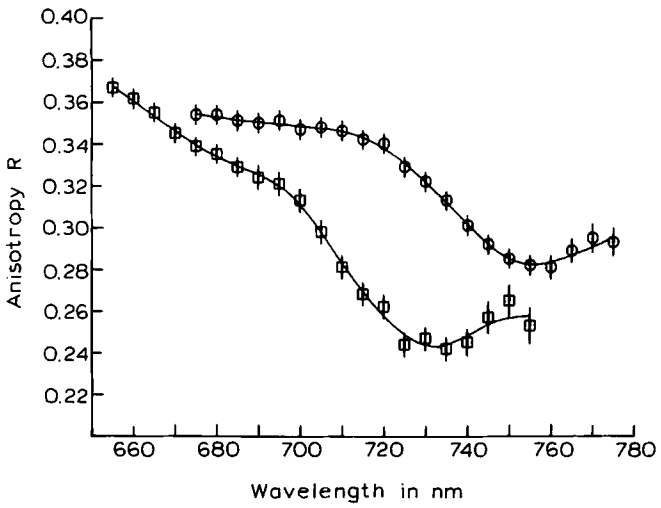

Fig. 2. Emission wavelength dependence of the fluorescence anisotropy of chlorophyll *a* (○) and chlorophyll *b* (□) in castor oil at 4°C. Wavelength of excitation is 668 nm for chlorophyll *a* and 649 nm for chlorophyll *b*.

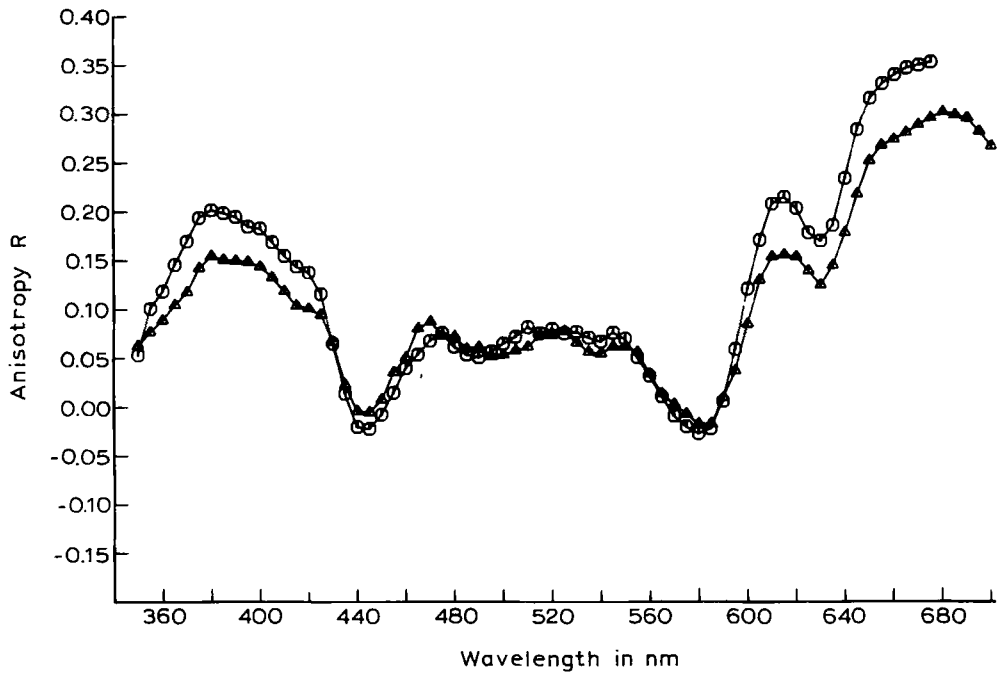

Fig. 3. Excitation wavelength dependence of the fluorescence anisotropy of chlorophyll *a* in castor oil at 4° C. Wavelengths of emission are 680 nm (○) and 750 nm (△).

A complicating factor in our measurements was the fluorescence emission of the castor oil itself, with an excitation maximum at 387 nm and an emission maximum at 431 nm. In the excitation spectrum of the chlorophylls, however, no 387 nm peak showed up, and the spectrum of the FA was in excellent agreement with the one recorded in paraffin oil. We conclude therefore that the fluorescence of the castor oil had a negligible effect on our measurements.

Temperature dependence of the fluorescence anisotropy

Fig. 5 shows the temperature dependence of the FA detected at 660 nm and 680 nm for chlorophyll *a* and *b*, respectively, when the excitation is at the $Q_y(0-0)$

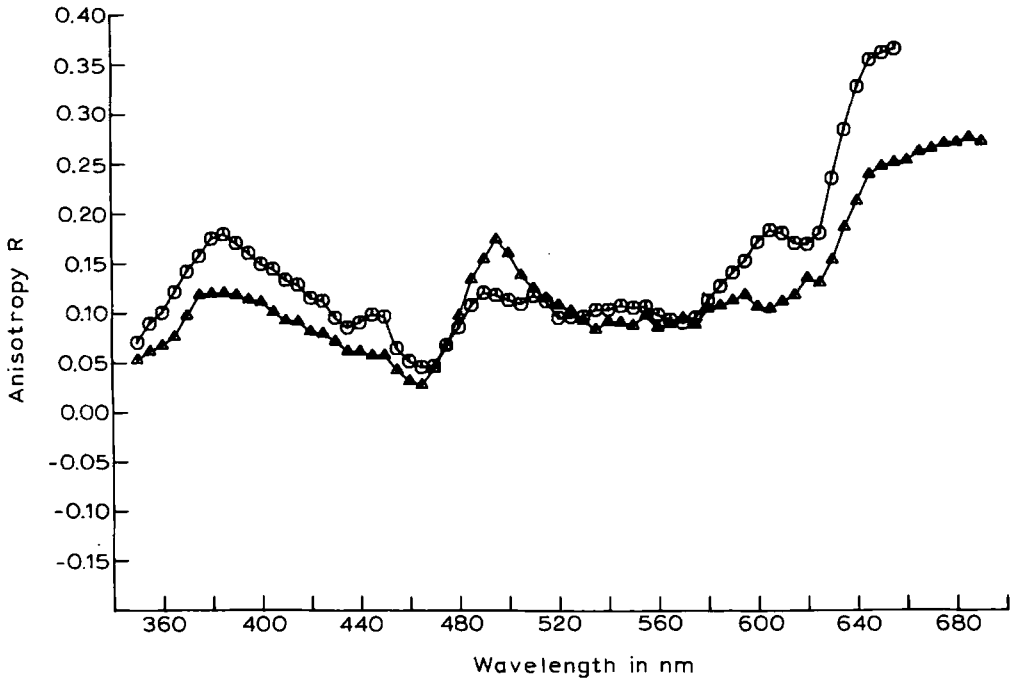

Fig. 4. Excitation wavelength dependence of the fluorescence anisotropy of chlorophyll *b* in castor oil at 4° C. Wavelengths of emission are 660 nm (△) and 737 nm (○).

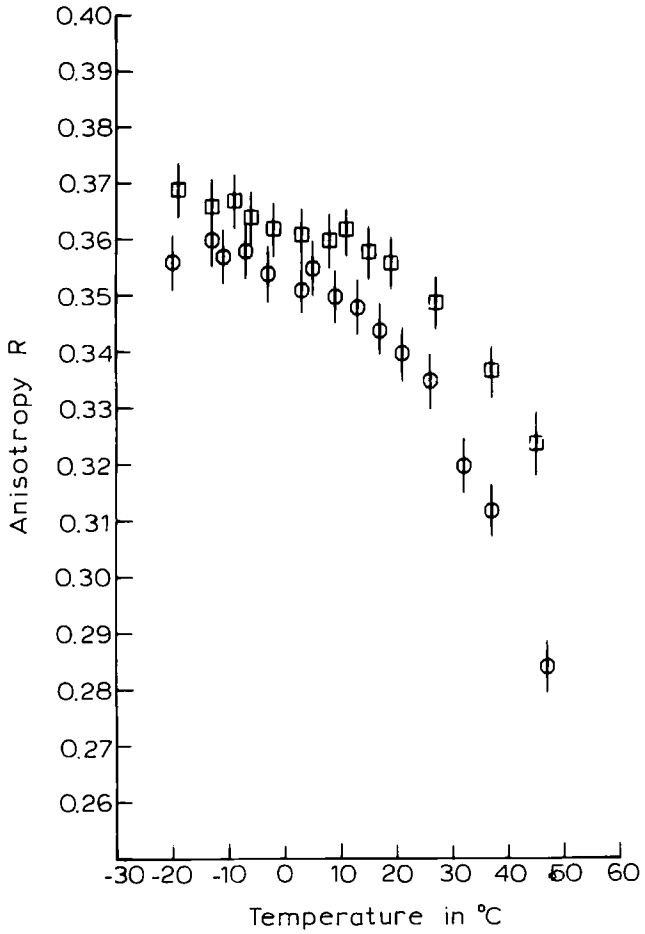

Fig. 5. Temperature dependence of the fluorescence anisotropy of chlorophyll *a* (○) and chlorophyll *b* (□) in castor oil. Excitation and emission wavelengths are 668 and 680 nm for chlorophyll *a* and 649 and 660 nm for chlorophyll *b*.

band maximum. We see that depolarization due to the reorientational motions of the molecules is quenched only at temperatures lower than −20 °C and is clearly present at 4 °C. We have therefore carried out time-resolved FA measurements and calculated r_0 (FA at time $t = 0$) from the two exponential fit of $r(t)$. Fig. 6 shows the anisotropy decay of chlorophyll *a* at room temperature, obtained by manipulating the recorded spectra of $I_{\parallel}(t)$ and $I_{\perp}(t)$ according to Eqn. 1. Comparison of the experimental decay with a theoretical fit is done by convoluting $r(t)I(t)$ and $I(t)$ with the measured lamp profile and taking their quotient. Here $r(t)$ and $I(t)$ are two-exponential fits found from the deconvolution procedure using Eqn. 7 [32]. We found for the FA at time zero: $r_0 = 0.365 \pm 0.004$ for chlorophyll *a* and $r_0 = 0.373 \pm 0.004$ for chlorophyll *b*.

Discussion

Emission wavelength dependence of the fluorescence anisotropy

The variation of the FA with the excitation wavelength is a well known spectroscopic property. Less well known is the variation of the FA with the emission wavelength. Such a dependence was briefly reported for chlorophyll *a* [15,16,27]. Kaplanova and Vacek [13] reproted the emission FA of chlorophyll *a* in frozen solution, but on excitation with a broad band in the 400–500 nm region. They found negative values around the 680 nm emission, and a positive maximum around 720 nm, a similar curve to that shown in our Fig. 2 but turned upside down. The form of the spectrum was explained in terms of three (slightly different polarized) emission bands. The variation of the emission FA is much larger for chlorophyll *b* than for chlorophyll *a*, and also reaches a plateau value for chlorophyll *a*, while it still increases for chlorophyll *b* at the high energy side of the fluorescence spectrum. This could indicate that an additional emission band exists at this side close to the excited absorption band, or that hot bands are involved [33]. The interesting conclusion from Fig. 2 is that the wavelength at which the emission is detected determines the recorded excitation FA spectrum. Thus the value of the limiting anisotropy, $r_0 = 0.330$ determined by Gouterman and Stryer [9], which is frequently cited in the literature, is in fact not reliable, as it was measured using a broad spectral detection.

Overlapping bands

Figs. 3 and 4 show the excitation FA spectra recorded at two different emission wavelengths. Indeed we see that the wavelength of detection influences the form of the spectra. It is interesting to note that the curves exhibit several crossover points. This indicates that at the corresponding wavelengths more than one transition is excited and that we have overlapping bands. We shall now assume that electronic excitation is followed by fast internal conversion processes to the ground vibrational level of the lowest excited state from which fluorescence emission occurs [1,2,33,34]. These processes are much faster than the fluroescence lifetime of the molecule. This assumption is justified by the observation that the emission spectrum is independent of the wavelength of excitation.

Consider the planar configuration of two orthogonal absorption moments $\bar{\mu}_1$ and $\bar{\mu}_2$ and two emission moments $\bar{\nu}_1$ and $\bar{\nu}_2$. The emission moments make a small angle δ with each other and lie in between $\bar{\mu}_1$ and $\bar{\mu}_2$. Let the angle between $\bar{\mu}_1$ and $\bar{\nu}_1$ be ε_1 and between $\bar{\mu}_2$ and $\bar{\nu}_2$ be ε_2, as shown in Fig. 7. Suppose that at wavelength A only $\bar{\mu}_1$ is excited, while at wavelength B only $\bar{\mu}_2$ is excited, and that both are excited together with weighting factors f_1 and $f_2 = 1 - f_1$ at an intermediate wavelength. When the fluorescence arises from $\bar{\nu}_1$ the FA is $r_{11} = 0.2(3\cos^2\varepsilon_1 - 1)$ at A and $r_{21} = 0.2(3\cos^2(\varepsilon_2 + \delta) - 1)$ at B. When the fluorescence originates from $\bar{\nu}_2$ we have $r_{12} = 0.2(3\cos^2(\varepsilon_1 + \delta) - 1)$ and $r_{22} = 0.2(3\cos^2\varepsilon_2) - 1)$ at A and B, respectively. Fig. 8 shows the FA spectra detected at the two emission wave-

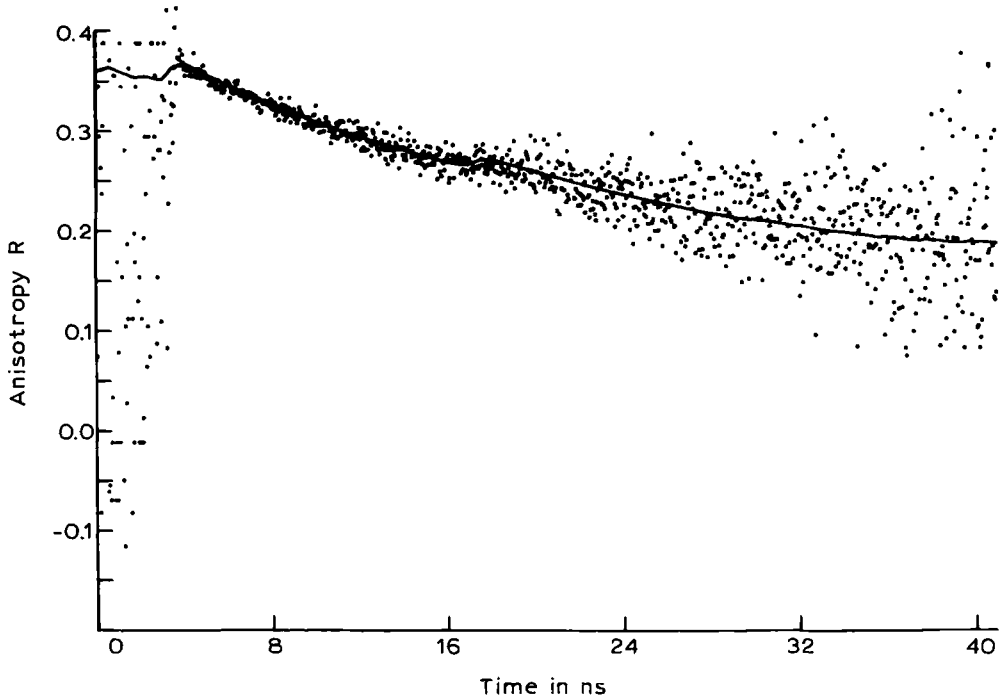

Fig. 6. Fluorescence anisotropy decay of chlorophyll *a* in castor oil at room temperature. Excitation is at 668 nm, emission at 680 nm. The experimental decay of $r(t)$ obtained from the convoluted curves of $I_{\parallel}$ and $I_{\perp}$ according to Eqn. 1 is shown, together with the theoretical curve found by convoluting $r(t)I(t)$ and $I(t)$ and taking their quotient. Here $r(t)$ and $I(t)$ are two exponential fits found from the deconvolution procedure using Eqn. 7. Average lifetime of chlorophyll *a* was 5.8 ns.

lengths on excitation at wavelengths intermediate between A and B. At the crossover point we have:

$$f_1 r_{11} + f_2 r_{21} = f_1 r_{12} + f_2 r_{22} \tag{8}$$

It follows that for $\varepsilon_1 + \varepsilon_2 + \delta = \pi/2$ Eqn. 8 yields:

$$f_1 \cos^2\varepsilon_1 + f_2 \sin^2\varepsilon_1 = f_1 \sin^2\varepsilon_2 + f_2 \cos^2\varepsilon_2 \tag{9}$$

It can be immediately seen that the only non-trivial solution of Eqn. 9 is $f_1 = f_2 = 1/2$. In this case the measured anisotropy at the crossing of the curves is $r = 0.1$, independent of any of the angles ε_1, ε_2 and δ.

Figs. 3 and 4 show that the FA values at the crossing points deviate from 0.1, indicating that either the absorption moments are not orthogonal, or that more than two absorption transitions overlap. Our results show

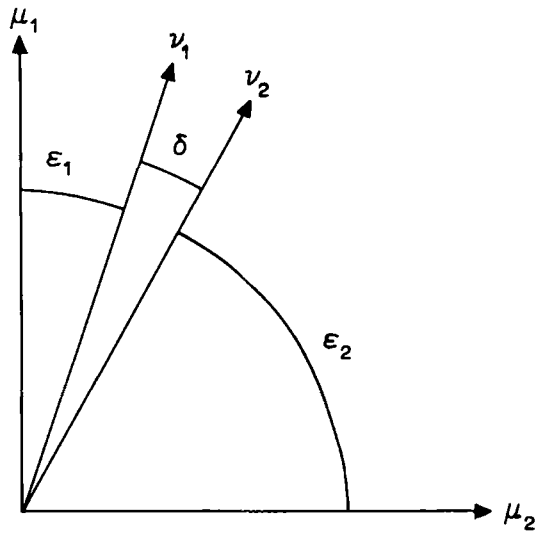

Fig. 7. Transition moment directions in an imaginary molecule. $\bar{\mu}_1$ and $\bar{\mu}_2$ are two perpendicular absorption moments, while $\bar{\nu}_1$ and $\bar{\nu}_2$ are two emission moments.

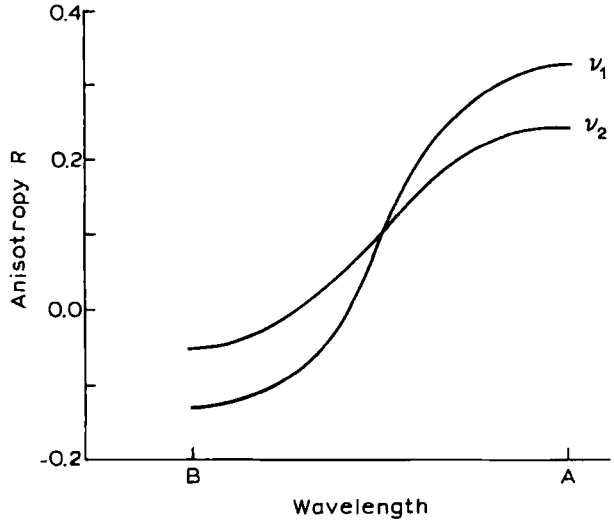

Fig. 8. Excitation wavelength dependence of the fluorescence anisotropy of the imaginary molecule in fig. 7, detected at the two emission wavelengths of the moments $\bar{\nu}_1$ and $\bar{\nu}_2$. At wavelength A only $\bar{\mu}_1$ is excited, while at wavelength B only $\bar{\mu}_2$ is excited. In between A and B both $\bar{\mu}_1$ and $\bar{\mu}_2$ are excited.

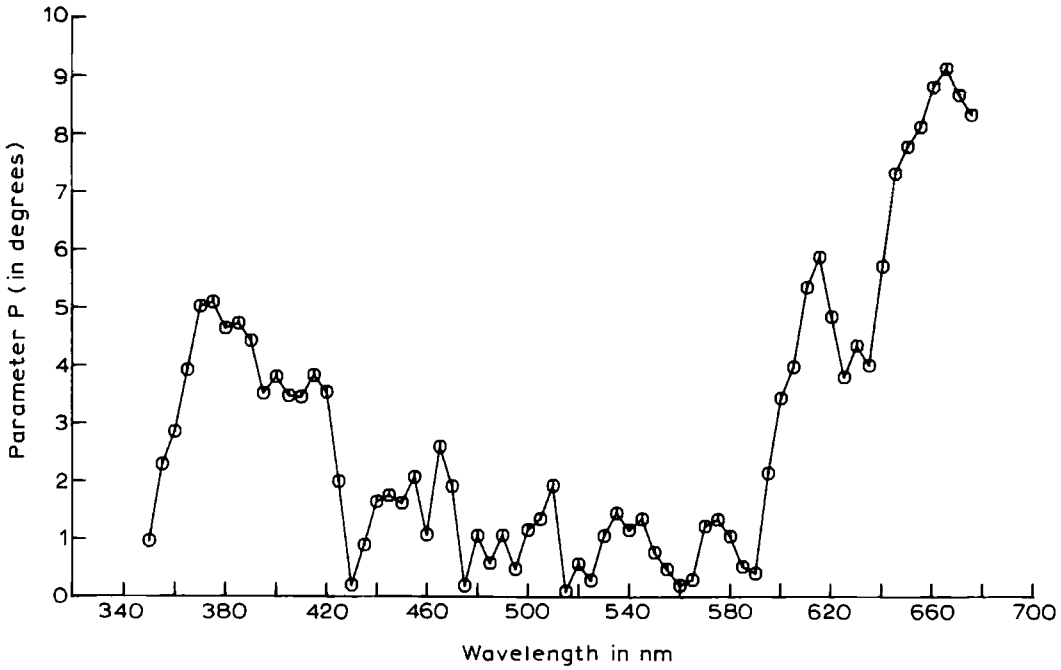

Fig. 9. Pureness P (in degrees) as defined by Eqn. 10 of chlorophyll a in castor oil. In the case of pure transitions P reaches the same maximal value at each transition wavelength.

furthermore that the differenc in the r-value, Δr, at the two emission wavelengths varies considerably across the FA spectrum. This parameter Δr can be intuitively correlated with the 'pureness' of the absorption transition. Thus Δr is large at the Q_y bands that are often assumed to be (almost) pure, whereas it is small around the Q_x bands (580 nm and 560 nm for chlorophyll a and b, respectively) which arise from overlapping transitions. A good measure of overlap appears to be the difference, P, of the two effective angles calculated from the two anisotropies at every excitation wavelength. From Eqn. 3 we now have:

$$P = \left| \arccos\sqrt{\frac{5r_1+1}{3}} - \arccos\sqrt{\frac{5r_2+1}{3}} \right| \tag{10}$$

where r_1 and r_2 are the two anisotropies shown in Figs. 3 and 4. For chlorophyll a and b the spectrum of the 'pureness' P is given in Figs. 9 and 10, respectively. In

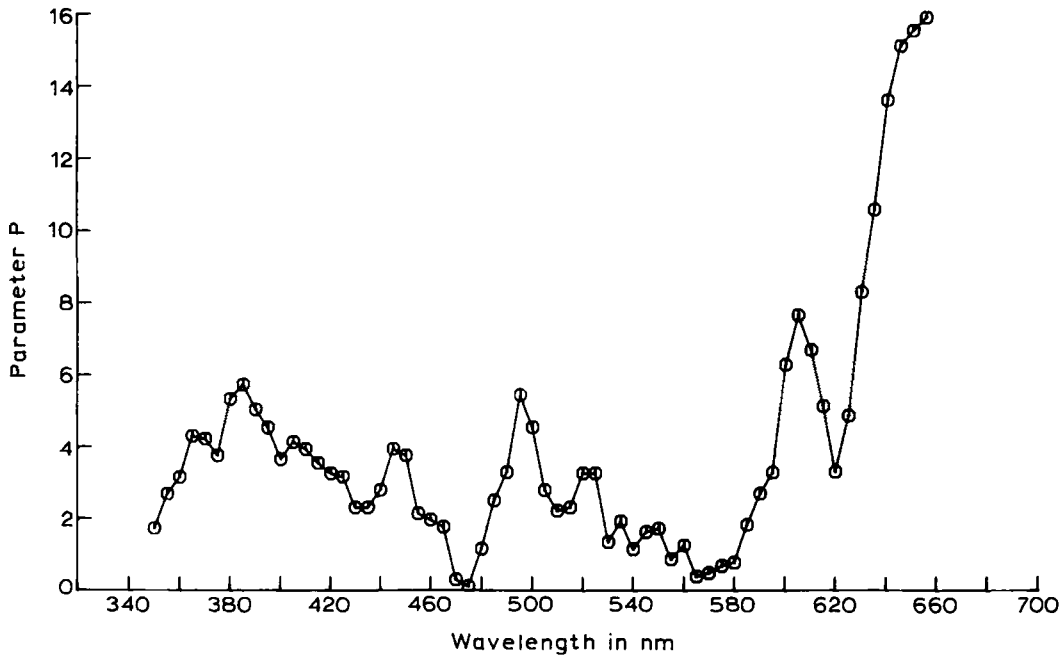

Fig. 10. Pureness P (in degrees) as defined by Eqn. 10 of chlorophyll b in castor oil.

the case of pure transitions we expect the curve to reach the same maximal value at each transition wavelength. Since this is not the case we conclude that the transitions overlap across the whole region of absorption. Nevertheless we note that the maximal values are found both for chlorophyll *a* and *b* around their Q_y-band. However, one cannot necessarily conclude that these transitions are pure, as a plateau is only found for the strongest chlorophyll *a* emission band.

Limiting anisotropy

Much attention has been paid in the literature to the fact that the limiting values of the FA 0.4 and -0.2 are seldom found experimentally [9,27,34,35]. It was suggested that internal rotations in the molecule cause a depolarization on the picosecond timescale so that even in highly viscous or frozen solutions the limiting values are not encountered [36]. A value of 0.4 was indeed observed with a picosecond time resolution [37,38]. It is clear from Fig. 5 that in castor oil at room temperature considerable rotational depolarization occurs, and that the limiting anisotropy can only be obtained from extrapolation to low temperatures or from time-resolved experiments. The limiting anisotropy is the value of the FA when depolarization arising from the overall rotational motions of the molecule has been quenched. At 4°C we found the value of r to be about 3% (for chlorophyll *a*, 2% for chlorophyll *b*) lower than that obtained from extrapolation to low temperatures. On assuming for the sake of simplicity a monoexponential lifetime $1/\tau \exp(-t/\tau)$ and anisotropy decay $r_0 \exp(-t/\phi)$ we find

$$r = \int_0^\infty r(t)F(t)\,dt = r_0 \frac{\phi}{\phi + \tau} \tag{11}$$

so that $\tau/\phi = 0.03$ for chlorophyll *a* and $\tau/\phi = 0.02$ for chlorophyll *b*. We may now obtain the limiting anisotropy from a simple multiplication of the FA values obtained at 4°C by the factors 1.03 and 1.02 for chlorophyll *a* and *b*, respectively. The limiting anisotropy found from time-resolved experiments coincides surprisingly well with the value found by Kuki and Boxer [27] for zinc pyrochlorophyllide *a* in crystals of hemoglobin. This supports their opinion that the value of 0.4 is not found for chlorophyll *a* because even in the Q_y-band an *x*-directed transition is partially excited. On the other hand, in a large and asymmetric molecule like chlorophyll one would expect internal rotations and torsions to take place and to cause depolarization of the emission light, so that even with a pure Q_y-band the maximum value of 0.4 is not reached.

Conclusions

The fluorescence anisotropy of chlorophyll *a* and *b* varies considerably on detection at different emission wavelengths. The emission is caused by differently polarized transitions, probably as a result of vibronic coupling in the molecule. Consequently, a well-defined emission wavelength must be used for recording the excitation fluorescence anisotropy spectra.

The measured fluorescence anisotropy cannot be interpreted in terms of a single angle between absorption and emission moments if two or more absorption bands are excited simulaneously. Rather it yields an average value of the cosine square of all the angles of the excited transitions.

Information about the pureness of the transition can be obtained on measuring the excitation spectrum of the fluorescence anisotropy at two distinct emission wavelengths.

Furthermore castor oil at room temperature is not sufficiently viscous to quench the rotational depolarization of the chlorophyll fluorescence. The limiting value of the anisotropy can be obtained either at temperatures far below zero, or from time-resolved measurements.

Acknowledgements

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