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POLARISED ABSORPTION SPECTROSCOPY OF CHLOROPHYLL–LIPID MEMBRANES

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SUMMARY

A technique has been developed for measuring visible absorption spectra of chlorophyll in lipid membranes. An expression is derived which enables the directions of the transition moments of the different absorption bands to be determined from polarisation data. It is found that the transition moments of the principal blue and red absorption bands of chlorophyll *a* make angles of 26° and 36.5° respectively with the plane of the membrane. On the assumption that these two transitions lie in the plane of the porphyrin ring and are mutually perpendicular, it may be deduced that the plane of the porphyrin ring is tilted at approx. 48° to the membrane surface. For chlorophyll *b* the transition moments of the blue and red bands are found to make angles of 29.5° and 36.5° with the plane of the bilayer, giving an angle of tilt of the porphyrin ring of approx. 51°.

These results are compared with measurements of dichroism *in vivo*.

INTRODUCTION

The initial stages of photosynthesis are primarily physical processes involving energy and electron transfer[1]. Because of the complexity of the photosynthetic system, it is extremely difficult to obtain a detailed understanding of these processes. At present it is necessary to rely heavily on photophysical concepts obtained from the study of simpler systems. Thus the properties of chlorophyll in solution[2], in the solid state[3] and in monolayers[4] have been extensively studied.

There can be little doubt that the lamellar structure of the photosynthetic apparatus plays an important role in its function. Studies of chlorophyll in solution or crystalline form are unlikely to give much information directly relevant to the role of the membrane in photosynthesis. The monolayer appears more promising since it reproduces the essentially two dimensional nature of the membrane. However, the presence of air, rather than an aqueous phase on one side of the monolayer constitutes an obvious limitation.

In recent years techniques have been developed for the formation and study of isolated bimolecular lipid membranes separating two aqueous phases[5]. The incorporation of chlorophyll into these bilayers provides an attractive model system for

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studying some of the processes which are thought to be important in photosynthesis. In order to fully utilise the advantages of this model system, it is clearly important to characterise the system in as much detail as possible. With this aim we have developed a method for measuring the absorption spectrum of chlorophyll in the lipid membrane. This technique gives information both about the composition of the membrane and the orientation of the chlorophyll molecule in the membrane. A preliminary account of this work has appeared elsewhere[6].

METHODS

Materials

Chlorophylls *a* and *b* were extracted from pea leaves and purified by thin-layer chromatography according to Hager and Bertenrath[7]. Briefly, chlorophyll was extracted with acetone, and the extract was transferred to 60/80 light petroleum. The acetone was separated from the light petroleum fraction and the excess was removed by washing the mixture with saturated NaCl solution. After water was removed from the light petroleum fraction, the latter was dried with anhydrous Na_2SO_4 and evaporated to dryness. The residue was taken up in ether and applied to preparative thin layer chromatography plates. These were made from 48 g of Kieselguhr G, 12 g of Kieselgel G, 72 mg of $Ca(OH)_2$ and 12 g of $CaCO_3$ with 125 ml of 5 mM ascorbate buffer at pH 7.1. The plates were developed in a solution containing 60/80 light petroleum, 2-propanol and water in volume ratio of 100:5:0.25. The appropriate band (chlorophyll *a* or *b*) was eluted off with ether, and a further purification carried out on Kieselgel G plates developed in hexane–acetone mixture (80:30, v/v). The spectroscopic characteristics of the final products in ether solution were in good agreement with the published data for chlorophylls *a* and *b*.

Lecithin was extracted from fresh egg yolks and purified on an alumina column according to the method of Singleton *et al.*[8]. The lipid was stored in chloroform solution under nitrogen atmosphere in sealed ampoules at −20 °C until used.

The membrane forming solution was made up by dissolving weighed quantities of lecithin and chlorophyll in *n*-decane. Puriss grade *n*-decane obtained from Koch-Light Ltd was used without further purification. Normally the concentration of the solution was 1 % with respect to the dry weight of lecithin. The aqueous phase was 0.1 M KCl in 50 mM Tris buffer solution at pH 7.

Absorption spectra determination

In order to obtain absorption spectra of chlorophyll in the bilayer, we have designed a cell which will accommodate a series of bilayers and fits into the sample compartment of a Cary 14 spectrophotometer[6]. The bilayers are formed across circular apertures in teflon plates which slide into the cell. The plates are tilted at 45° to ease visual observation. As will be seen later, this is also a convenient angle for polarisation studies.

After aligning the cell in the spectrophotometer, membranes were formed using the brush technique. With the spectrophotometer slits fully opened, the light reflected by the membranes was sufficient to visually observe the transition from a thick film to the bilayer state. These observations were made at wavelengths which were not absorbed by chlorophyll in order to avoid any possible photochemical changes. When

the membranes had all thinned to the bilayer state, absorption spectra were recorded. A baseline was obtained by rescanning the spectrum after the membranes had been broken. Polarised spectra were obtained using polaroid polarisers as supplied by Cary.

In the normal arrangement for making absorbance measurements, circular apertures of 3 or 4 mm diameter were used, with a single 2 mm mask inserted at the focus of the light beam in the center of the cell. With 3-mm diameter apertures, this mask effectively confined the light beam to the bilayer region of the inner membranes but there was some overlapping of the surrounding Gibbs ring of the outer membranes. In order to investigate the effect of the Gibbs ring, some measurements were made with 4-mm diameter apertures at alternate positions, between which 2-mm masks were inserted. With this arrangement it was possible to ensure that no light passed through the Gibbs ring of any membrane. The effects of the ring could then be evaluated by comparing the results with those obtained with one single mask in the center of the cell. From these measurements it was deduced that light which passed through the Gibbs ring was scattered or defocussed and did not enter the measuring system. This scattering effect caused the baseline to shift uniformly to a lower absorbance level throughout the spectrum. The shift was greater for outer membranes. When this shift was subtracted from the measured absorbance, identical results were obtained for both methods of measurements. Because the arrangement with a single mask at the center of the cell gave a better signal to noise ratio and permitted more bilayers to be examined, this method was adopted as a standard procedure.

RESULTS

Initial experiments indicated an optimum chlorophyll–lecithin ratio in the membrane forming solution of about 1 : 1. Higher concentrations of chlorophyll were apt to produce less stable films, while lower concentrations gave smaller values of absorbance which made accurate measurements more difficult. Thus the majority of the data reported in this section were obtained with this ratio of chlorophyll–lecithin in the membrane forming solution.

The upper line in Fig. 1 is an example of an absorption spectrum as recorded. The spectrum is a superposition of two scans and demonstrates very well the repro-

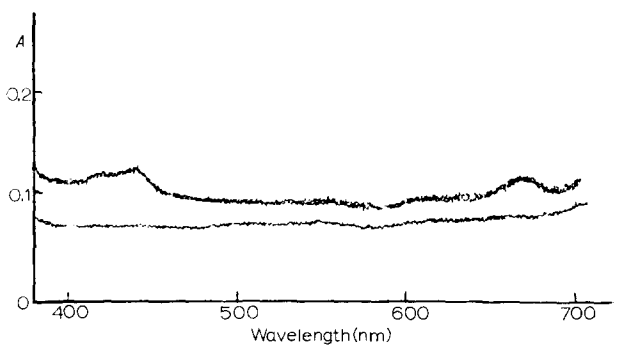

Fig. 1. Absorption spectrum as recorded. Upper trace (spectrum of six bilayers) is superposition of two scans. Lower trace is baseline recorded after breaking the bilayers. Membrane forming solution lecithin–chlorophyll *a* 1 : 1 in *n*-decane. Horizontal polarisation.

ducibility and stability of the system. The lower line shows the baseline recorded after breaking the bilayers. The fact that the baseline does not coincide with the absorption spectrum in regions of zero absorbance was due to the scattering effect mentioned above under Methods. Normally the absorption spectrum was replotted after subtracting the absorbance of the baseline and setting the absorbance at 500 nm to zero. For simplicity, only these replotted spectra are shown in subsequent figures. The noise level of the original recordings gives an uncertainty of ± 0.003 absorbance unit throughout the spectrum.

Polarised absorption spectra for chlorophylls *a* and *b* are shown in Fig. 2. In the case of horizontal polarisation the electric vector of the incident light is parallel to the plane of the bilayer, while for vertical polarisation the electric vector makes an angle of 45° with the normal to the bilayer. As reported previously[6], the ratio of

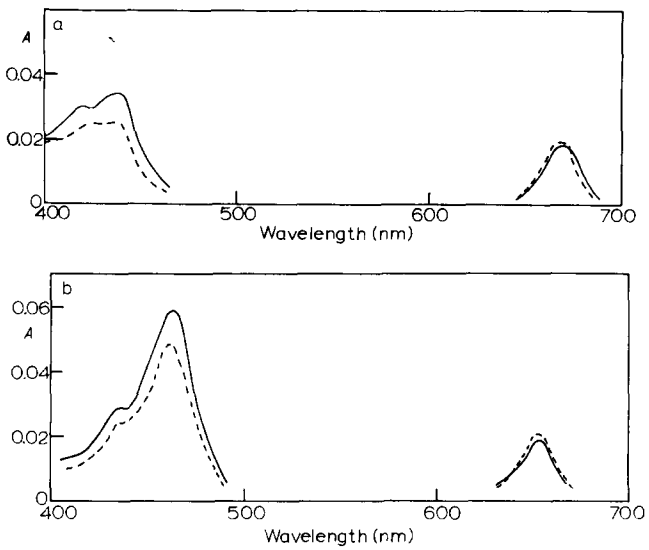

Fig. 2. (a) Polarised absorption spectra of chlorophyll *a* in lipid bilayer. Six bilayers, membrane forming solution lecithin–chlorophyll *a* 1:1. ———, horizontal polarisation; – – – –, vertical polarisation. (b) Polarised absorption spectra of chlorophyll *b* in lipid bilayer. Five bilayers, membrane forming solution lecithin–chlorophyll *b* 1:1. ———, horizontal polarisation; – – – –, vertical polarisation.

the blue to red peak heights show a small but consistent difference between the two directions of polarisation. The spectroscopic data are summarised in Table I. In some cases these values are slightly different from those reported in our preliminary communication[6]. These revisions have been made in the light of more accurate data following refinements in the experimental techniques.

In most spectra, the blue satellite band was only poorly resolved from the principal blue peak. Nevertheless, with chlorophyll *a*, there was a marked tendency for the blue satellite peak to be relatively higher than the principal blue peak when the incident light was vertically polarised. This was confirmed further by one set of bilayers which had incorporated more chlorophyll *a* than usual and in which the two peaks were particularly well resolved (Fig. 3).

TABLE I

SPECTROSCOPIC DATA

	Wavelengths of absorption maxima (nm)			*Intensity ratio Blue peak/Red peak*	
	Blue satellite	*Blue peak*	*Red peak*	*Horizontal polarisation*	*Vertical polarisation*
In ether					
Chlorophyll *a*	410	429.5	661.5		
Chlorophyll *b*	429	453	644		
In bilayer					
Chlorophyll *a*	419	439	672	1.8	1.3
Chlorophyll *b*	439	466	653	3.1	2.4

In some experiments bilayers were broken sequentially and absorption spectra were recorded for each stage. This enabled a plot to be made of absorbance against number of bilayers. As shown in Fig. 4 such plots are linear, as would be expected. This provides further confirmation that no errors are introduced by the Gibbs ring surrounding each bilayer. As explained under Methods any effect due to the ring would not occur uniformly, but would be much greater for the outer layers.

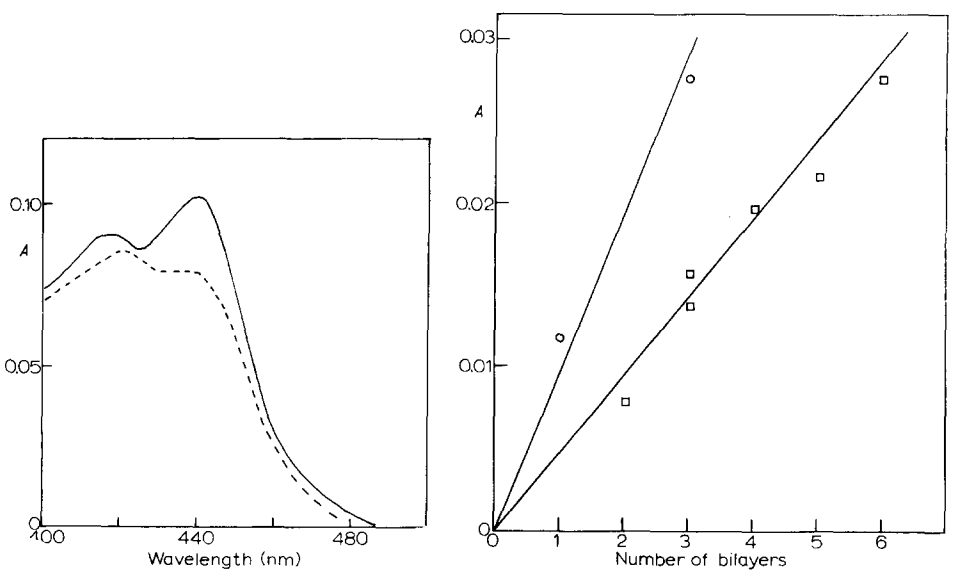

Fig. 3. Polarised absorption spectra of Soret band of chlorophyll *a* in lipid bilayer. Six bilayers, membrane forming solution lecithin–chlorophyll *a* 1:1. ———, horizontal polarisation; – – – –, vertical polarisation.

Fig. 4. Plot of measured absorbance against number of bilayers. □—□, chlorophyll *a*–lecithin 1.3:1; ○—○, chlorophyll *a*–lecithin 2:1. Unpolarised light.

DISCUSSION

Analysis of polarisation data

As shown in the appendix, dichroism of shape of a single lipid bilayer is negligibly small and hence the observed dichroism is an intrinsic effect arising from orientation of the chlorophyll molecules. In order to determine this orientation, we first derive an expression for the dichroic ratio. In Fig. 5, the x axis represents the direction of the incident light beam. The electric vector lies along the z axis for vertical polarisation and along the y axis for horizontal polarisation. *ABCO* is the plane of the bilayer which makes an angle α with the xy plane. For a given absorption band, the transition dipole moment is represented by the vector *OM*. *ON* is the projection of *OM* on the plane of the bilayer and Φ, the angle between *OM* and *ON*, defines the inclination of the transition moment to the bilayer. *ON* makes an angle $(\pi/2 - \theta)$ with the y axis.

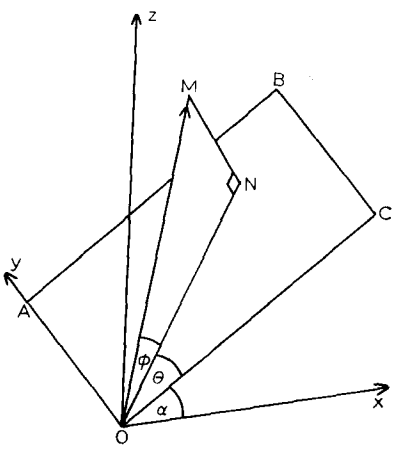

Fig. 5. Geometry of the chlorophyll–lipid bilayer system (see text for details).

From the geometrical configuration, the components of *OM* (M_y, M_z) along the y and z axes are given by

$$M_y = OM \sin\theta \cos\Phi \tag{1}$$

$$M_z = OM (\sin\alpha \cos\theta \cos\Phi + \cos\alpha \sin\Phi) \tag{2}$$

The dichroic ratio D, defined as the ratio of the absorbance for vertically polarised light to that for horizontally polarised light is given by

$$D = \frac{M_z^2}{M_y^2} \tag{3}$$

The lipid bilayer is a liquid crystalline structure with no long range order in the plane of the bilayer. Therefore, for a given angle Φ, the transition moment *OM* may point in any direction as long as it lies on the conical surface making an angle of $(\pi/2 - \Phi)$ with the normal to the membrane. Thus it is necessary to average M_z and M_y over all θ to obtain

$$D = \frac{\frac{1}{2\pi}\int_0^{2\pi} M_z^2 \, d\theta}{\frac{1}{2\pi}\int_0^{2\pi} M_y^2 \, d\theta} \tag{4}$$

$$= \sin^2 \alpha + 2 \tan^2 \Phi \cos^2 \alpha \tag{5}$$

Eqn 5 enables the direction of the transition moment to be calculated from the polarisation data. Thus, from the spectra in Fig 2, $D = 0.74$ at 439 nm and 1.05 at 672 nm for chlorophyll *a*. With $\alpha = 45°$ these values of D give $\Phi = 26°$ for the blue oscillator and $\Phi = 36.5°$ for the red oscillator. For chlorophyll *b* the corresponding values of D are 0.82 at 466 nm and 1.05 at 653 nm giving $\Phi = 29.5°$ and $36.5°$ for the blue and red oscillators respectively.

Angle of tilt of porphyrin ring

Determination of the directions of the transition moments of the principal chlorophyll absorption bands with respect to the plane of the bilayer does not immediately reveal the orientation of the porphyrin ring. However, the orientation may be determined if certain theoretical considerations are taken into account. The spectroscopy of molecules derived from the basic porphine structure has been treated theoretically by several authors[9-12] and summarised by Goedheer[2].

For present purpose the following spectroscopic properties of porphyrins are of importance. The principal electronic transitions observed in the absorption spectra are π–π^* transitions lying in the plane of the conjugated ring system. If the π electronic system is symmetrical, as in porphine, transitions lying along different directions in the plane of the ring are degenerate ("Round Field" spectrum). If the symmetry is of a lower order, as in tetrahydroporphine, transitions lying along the mutually perpendicular long and short axes of the ring become separated ("Long Field" spectrum).

The basic ring structure of chlorophylls is similar to that of dihydroporphine which has only one short 2-fold axis of symmetry. According to Rabinowitch[13], the spectrum of dihydroporphine is expected to resemble that of tetrahydroporphine more than that of porphine, because the circular symmetry of the conjugated system is destroyed by the hydrogenation of a single pyrrole ring. Therefore, the absorption band of dihydroporphine derivatives such as the chlorophylls, will be split into two components, one polarised in the direction of the symmetry axis of the conjugated band system and the other perpendicular to it. From his analysis, the long-wave (red) band of chlorophyll is due to a vibration parallel to the long axis, and the blue band of chlorophyll *a* (at 429 nm in ether) is polarised parallel to the short axis of the molecule. Goedheer, on the basis of fluorescence polarisation measurements[2,14] gave the same assignment to the direction of the chlorophyll *a* transitions and also proposed that the Soret band was composed of at least two mutually perpendicular components. The present absorption data are also consistent with these previous assignments.

We thus assume that the two transition moments responsible for the red and the principal blue bands are perpendicular to each other. With this assumption it

may be shown that the angle of tilt of the porphyrin ring is given by the expression

$$\sin^2 \gamma = \sin^2 \Phi_1 + \sin^2 \Phi_2 \quad (6)$$

where Φ_1 and Φ_2 are the values of Φ for the red and blue bands and γ is the angle between the plane of the porphyrin ring and the plane of the bilayer. From the values of Φ_1 and Φ_2 calculated in the previous section we obtain $\gamma = 48°$ for chlorophyll *a* and $\gamma = 51°$ for chlorophyll *b*. It should be mentioned that the angle of tilt is not unduly sensitive to the assumed angle between the red and blue transition moments. If it is supposed, for example, that this angle may be perturbed by up to 10° from its theoretical value of 90°, the uncertainty in the angle of tilt is still only $\pm 5°$.

The above analysis assumes a fixed value of γ. The alternative possibility that the porphyrin rings lie at varying angles cannot be ruled out on the basis of the present experiments. In this case the values deduced from the above analysis should be regarded as average values.

It was previously shown[6] that the number of chlorophyll molecules per cm² in the bilayer (y) may be determined from the expression $A = \varepsilon y N/M$ where A is the absorbance, ε the extinction coefficient, M the molecular weight and N Avogadro's number. Calculation of y from the above equation over a series of experiments indicate that y is normally between $2 \cdot 10^{13}$ and $4 \cdot 10^{13}$ when the membrane forming solution containes lecithin–chlorophyll 1:1. These values agree well with the independent measurements of Steinemann *et al.*[15]. From the concentration we calculate that the available area per molecule of chlorophyll is between 250 and 500 Å². Since the area of the porphyrin ring of chlorophyll is about 200 Å² (see ref. 16), a very high proportion of the available surface area would be covered if the porphyrin rings were lying flat on the membrane surface. This would only be conceivable if the rings were wholly outside the lipid polar groups. Elsewhere we have shown that the total membrane thickness as measured by optical reflectance is inconsistent with this possibility[17]. These considerations therefore provide confirming evidence that the rings must be tilted at an angle to the bilayer.

Chlorophyll in vivo

The spectral characteristics of chlorophyll *in vivo* have been reviewed by Butler[18]. In the chloroplast the two principal forms of chlorophyll *a* have absorption maxima at 673 and 683 nm while the chlorophyll *b* maximum is at 653 nm. Since the mechanisms of the red shifts are uncertain, it would be unwise to place much emphasis on the fact that the absorption maxima of chlorophyll *a* 673 and chlorophyll *b* in the chloroplast occupy similar positions to the absorption maxima of chlorophylls *a* and *b* in the lipid bilayer. However, chlorophyll *a* 673 is also fluorescent and exhibits little or no dichroism. These properties are also exhibited by chlorophyll *a* in a lipid membrane. (Fluorescence of the chlorophyll *a* lipid membrane system has been reported by Alamati and Läuger[19].) There is also little dichroism in the region of the chlorophyll *b* absorption maximum in the chloroplast. Thus the properties of chlorophyll *a* 673 and chlorophyll *b* *in vivo* are consistent with the view that they are in a lipid environment.

The low intrinsic dichroism observed in the photosynthetic system is generally interpreted as indicating a lack of overall orientation of the pigment molecules[2,19]. However, Eqn 5 demonstrates that $D = 1$ for all values of α when $\tan^2 \Phi = 1/2$,

i.e. $\Phi = 35.3°$. Thus a low dichroic ratio may also result from a partially ordered system in which the transition moment makes a constant angle of around 35° with the plane of the membrane but assumes a random orientation with respect to rotation about the normal to the membrane. This conclusion is in agreement with the previous studies of Kreutz[20].

ACKNOWLEDGMENTS

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APPENDIX

Dichroism of shape of a single lipid bilayer

We consider a system of parallel layers of equal cross-sectional area in which layers of refractive index n_1 and thickness d_1 alternate with layers of refractive index n_2 and thickness d_2. For this system Wiener[21] derived the following expressions for the refractive indices parallel and perpendicular to the layers ($n_\parallel$ and $n_\perp$).

$$n_\parallel{}^2 = \frac{d_1 n_1{}^2 + d_2 n_2{}^2}{d_1 + d_2} \tag{7}$$

$$\frac{1}{n_\perp{}^2} = \left(\frac{1}{d_1 + d_2}\right)\left(\frac{d_1}{n_1{}^2} + \frac{d_2}{n_2{}^2}\right) \tag{8}$$

For an absorbing layer the refractive index n must be replaced by the complex expression $(n - ik)$. In our case, we consider Layer 1 to be the chlorophyll–lipid membrane and Layer 2, the aqueous solution between successive membranes. Therefore Eqns 7 and 8 become

$$(n_\parallel - ik_\parallel{}^2) = \frac{d_1(n_1 - ik_1)^2 + d_2 n_2{}^2}{d_1 + d_2} \tag{9}$$

$$\frac{1}{(n_\perp - ik_\perp)^2} = \left[\frac{1}{d_1 + d_2}\right]\left[\frac{d_1}{(n_1 - ik_1)^2} + \frac{d_2}{n_2{}^2}\right] \tag{10}$$

Equating real and imaginary parts gives

$$n_\parallel{}^2 - k_\parallel{}^2 = \frac{d_1(n_1{}^2 - k_1{}^2) + d_2 n_2{}^2}{d_1 + d_2} \tag{11}$$

$$n_\parallel k_\parallel = \frac{d_1 n_1 k_1}{d_1 + d_2} \tag{12}$$

$$\frac{n_\perp{}^2 - k_\perp{}^2}{(n_\perp{}^2 + k_\perp{}^2)^2} = \left[\frac{1}{d_1 + d_2}\right]\left[\frac{d_1(n_1{}^2 - k_1{}^2)}{(n_1{}^2 + k_1{}^2)^2} + \frac{d_2}{n_2{}^2}\right] \tag{13}$$

$$\frac{n_\perp k_\perp}{(n_\perp{}^2 + k_\perp{}^2)^2} = \frac{d_1 n_1 k_1}{(d_1 + d_2)(n_1{}^2 + k_1{}^2)^2} \tag{14}$$

Exact solution of these equations is complex but an estimate of the dichroism to be expected from a single lipid membrane may be made with the aid of some approximations. The absorbance A at wavelength λ is related to the imaginary part of the refractive index by the expression $A = 4\pi k_1 d_1/2.3\lambda$. Inserting the measured maximum absorbance into expression and taking $d_1 = 60$ Å, we calculate that $k_1 < 0.15$ for the lipid–chlorophyll membrane. Since the real part of the refractive index is in the order of 1.4, we deduce that $k_1^2 \ll n_1^2$. It also follows that $k_\parallel^2 \ll n_\parallel^2$ and $k_\perp^2 \ll n_\perp^2$. Neglect of the k terms in Eqns 11 and 13 leads to expression identical with those of Eqns 7 and 8 while Eqn 14 becomes

$$\frac{k_\perp}{n_\perp^3} = \frac{d_1 k_1}{(d_1 + d_2) n_1^3} \tag{15}$$

Combining Eqns 7, 8, 12 and 15 gives

$$k_\parallel - k_\perp = \frac{d_1 n_1 k_1}{(d_1 + d_2)^{\frac{1}{2}}(d_1 n_1^2 + d_2 n_2^2)^{\frac{1}{2}}} - \frac{d_1 n_2^3 k_1 (d_1 + d_2)^{\frac{1}{2}}}{(d_1 n_2^2 + d_2 n_1^2)^{\frac{3}{2}}} \tag{16}$$

In our system $d_2 \gg d_1$. Thus we may further simplify Eqn 16 to obtain

$$k_\parallel - k_\perp = \frac{d_1 n_1 k_1}{d_2 n_2}\left[1 - \frac{n_2^4}{n_1^4}\right] \tag{17}$$

Taking $n_1 = 1.45$ and $n_2 = 1.33$ and arbitrarily taking $d_1/d_2 = 0.015$ (d_2 is not infinitely large since Wiener's equations only apply over a dimension comparable with the wavelength of light) we obtain

$$k_\parallel - k_\perp = 0.004\, k_1 \tag{18}$$

Thus the dichroism of shape is less than 1 % of the total absorption. Such an effect is too small to be detectable in the present experiments.

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