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Orientation of the Porphyrin Ring in Artificial Chlorophyll Membranes

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Summary. A sensitive photometric method is described by which the dichroism of lipid bilayer membranes in aqueous phase can be measured. The method is applied to black films with incorporated chlorophyll *a* and *b*. With chlorophyll *a* a relatively large dichroism is found in the Soret band and a much weaker dichroism in the red band. From the experimental data, the angles β_B and β_R between the "blue" and "red" transition moments and the membrane can be obtained. β_B and β_R are then used to calculate the angle γ of the porphyrin ring with respect to the membrane surface. For chlorophyll *a* and three different lipids, values of γ between 44 and 49° are found.

Lipid bilayer membranes with incorporated chlorophyll *a* have recently been used for the study of photochemical reactions of chlorophyll with aqueous redox systems (Tien & Verma, 1970; Trissl & Läuger, 1970; Van & Tien, 1970). For a detailed understanding of the reactions of excited chlorophyll in lipid membranes it is necessary to know the structure of these composite membranes. By light absorption measurements it has been shown that up to 3×10^{13} chlorophyll *a* molecules per cm^2 may be incorporated into the lipid bilayer (Cherry, Hsu & Chapman, 1971; Steinemann, Alamuti, Brodmann, Marschall & Läuger, 1971). At these concentrations the pigment molecules interact with each other and energy transfer occurs, as revealed by fluorescence quenching (Alamuti & Läuger, 1970). Experimental evidence has been presented that the porphyrin ring of the chlorophyll molecule is in contact with the aqueous phase adjacent to the membrane (Steinemann *et al.*, 1971). Though there is some indication for a preferred orientation of chlorophyll in a bilayer from polarization measurements (Cherry *et al.*, 1971) as yet it has not been possible to determine the angle between the porphyrin ring and the membrane surface. In this paper, we describe a

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sensitive method for the measurement of the dichroism of bilayer membranes with incorporated pigment molecules. Using this method it is possible to measure the angle between the transition moments of the blue and red absorption peaks of chlorophyll and the membrane surface. From the known angle between the two transition moments the orientation of the porphyrin can then be calculated. It is to be expected that this polarization technique may be useful in a number of other studies of membrane structure.

Materials and Methods

Principle of the Method

The main problem encountered in dichroism measurements of lipid bilayers is the very low light absorption of the membrane. To achieve a sufficient signal-to-noise ratio, the following method is used. The electrical vector of the plane-polarized light passing through the membrane is rotated at a constant frequency ν and the transmitted light is monitored with a photomultiplier. For an arbitrary angle α between the dichroitic membrane and the light beam, the output voltage of the photomultiplier consists of a d-c signal with a small superimposed a-c component of frequency 2ν. The amplitude of the a-c signal is a function of the optical anisotropy of the membrane and of the angle α. The a-c signal is fed into a lock-in amplifier (tuned to the frequency 2ν), together with a reference signal which has the same frequency and a fixed phase angle with respect to the main signal. In this way a phase-sensitive rectification of the photomultiplier signal is achieved which greatly reduces noise frequences other than 2ν, as well as 2ν noise which is out of phase with the main signal.

Design of the Photometer

The block diagram of the apparatus is shown in Fig. 1. The light source was an Osram 150 W halogen-tungsten lamp combined with a Hewlett-Packard Model 6966A d-c power supply (stabilization better than 10^{-4}). The lamp house was equipped with a water-cooled jacket. To obtain a high light intensity, interference filters (Balzers, band-

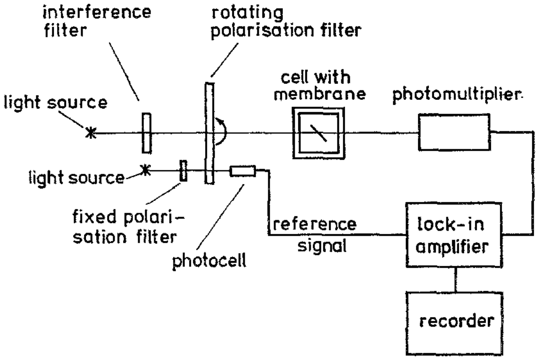


Fig. 1. Block diagram of the polarization photometer

width 5 to 10 nm) were used instead of a monochromator. After passing the interference filter, the light beam was polarized with a Polaroid filter. The filter was rotated at a frequency of 35 Hz with a cogwheel drive. The light beam entered through the hollow axis of the mount and passed through the central part of the filter. The membrane was formed on a teflon diaphragm submersed in an aqueous electrolyte solution. The diaphragm had an opening in the form of a horizontal slit (length 12 mm, height 5 mm). Only the central part of the black film was illuminated by the light beam which had a circular cross-section of 2 mm in diameter. By rotating the diaphragm, the angle between the normal to the membrane and the light beam could be varied between 0 and ±55°. To avoid mechanical disturbances of the membrane, a rotation rate of about 1 degree/sec had been used; the rotation was carried out by a small electric motor. The photomultiplier (EMI 9558B) was powered with a John Fluke Model 405B high-voltage power supply (stabilization better than 5×10^{-5}). To minimize the shot noise of the photocathode, the full intensity of the light source was used and, accordingly, the number of dynodes reduced to 5 (dynode-to-dynode voltage, 90 V); the last 6 dynodes were electrically connected with the anode. After passing a preamplifier stage, the photomultiplier signal was fed into a lock-in amplifier (Princeton Applied Research Model 220, combined with auxiliary amplifiers Models 210 and 211). The reference signal for the lock-in amplifier was taken from the rotating polarization filter, using an additional small light-source, a photocell and a fixed polarization filter (*see* Fig. 1).

Measuring Procedure and Calibration of the Instrument

To correct for the phase shift between the main signal and the reference signal, the variable phase angle of the lock-in amplifier had to be adjusted prior to the measurements. This was done in the following way. After the membrane was in the final black state, the angle α between the normal to the membrane and the light beam was repeatedly varied between $\alpha = 0$ and $\alpha = 45°$ and each time the output signal $S(\alpha)$ of the lock-in amplifier was observed. The phase angle was then adjusted so that the difference $|S(45) - S(0)|$ assumed a maximum. The phase angle for maximum value of $|S(45) - S(0)|$ was found to be a constant, irrespective of whether a bilayer membrane or a reflecting plate was brought into the light beam. All measurements were then performed with this phase angle.

For the calibration of the instrument, the cell with the membrane (Fig. 1) was replaced by a glass plate in air, which was tilted at an angle α with respect to the light beam. This gave a large optical anisotropy, so that the amplitude ΔV of the a-c component of the photomultiplier signal could be directly measured without the lock-in amplifier. The signal S at the output of the lock-in amplifier was found to be proportional to ΔV (*see below*):

$$S = k \Delta V. \tag{1}$$

Thus, the calibration factor k can be obtained by measuring S and ΔV in the presence of a large optical anisotropy. To check that Eq. (2) is valid down to very low values of ΔV, the signal S as well as the d-c component V_0 of the photomultiplier have been measured at different light intensities. It has been found that S was proportional to V_0 even at the lowest intensities. As both V_0 and ΔV are proportional to the light intensity, Eq. (2) is therefore verified in the whole range of ΔV.

The photomultiplier signal, corrected for the residual polarization of the instrument, is then given by (ω = angular velocity, t = time):

$$V^*(\omega t) = V_0 + \Delta V^* \cos 2\omega t \tag{2}$$

where

$$\Delta V^* = \frac{1}{k}[S(\alpha) - S(0)]. \tag{3}$$

As shown below [Eq. (11)] the experimental quantity used to evaluate the orientation of the pigment molecule is $\Delta V^*/V_0$. Therefore, in each experiment the d-c component V_0 of the photomultiplier signal has been recorded in addition to $[S(\alpha) - S(0)]$.

For the measurement of the unpolarized absorption of the membrane (Steinemann & Läuger, 1971), the membrane was oriented normal to the light beam. The photomultiplier signal was monitored with a differential voltmeter (John Fluke Model 873A). As the light reflection of the membrane is negligible under these circumstances, the absorption is given by the quantity $\Delta V'/V'$, where $\Delta V'$ is the change in the photomultiplier signal when the membrane is destroyed and V' is the mean photomultiplier signal.

Formation of the Membranes

Bilayer membranes were formed at 20 °C from a solution of 0.3% (w/v) phospholipid plus various amounts of chlorophyll *a* or *b* in *n*-decane as described previously (Steinemann *et al.*, 1971). The aqueous phase contained 0.1 M KCl. Dioleoyllecithin was synthesized after the method of Baer and Buchnea (1959); phosphatidylserine from Koch-Light was purified on a silicic acid column; phosphatidylethanolamine was obtained from Sigma, and chlorophylls *a* and *b* from Fluka. The purity of the lipids was checked by thin-layer chromatography.

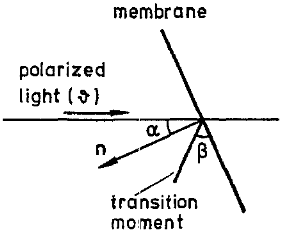
Theory

The intensity of the light which is transmitted through the membrane is influenced both by absorption and reflection. As the reflection coefficient for polarized light depends on the mutual orientation of the electrical vector and the membrane, the output signal of the instrument contains a reflection component. At first, the separation of reflection and absorption seems to be principally difficult because of the change of refractive index in the vicinity of an absorption band (anomalous dispersion). A closer inspection, based on the Drude theory of refractive index (van der Plaats, 1915; Wolf & Herzfeld, 1928), however, shows that the contribution of anomalous dispersion is negligibly small even at the highest pigment concentrations in the membrane. Accordingly, the measured signal has been corrected for reflection by subtracting the small signal obtained from a lecithin membrane without chlorophyll. In the following sections, therefore, we treat the dichroism and the light reflection of the membrane separately.

Dichroism of the Membrane

To describe the dichroism of the membrane, we assume that the normal *n* to the membrane forms an angle α with the light beam (Fig. 2). The angle

Fig. 2. Definition of the angles α and β. n is the normal to the membrane. The electrical vector of the light makes an angle ϑ with the plane of incidence (the plane formed by n and the light beam)



between the linear transition moment of the pigment molecule and the membrane is denoted by β. If N is the number of pigment molecules per unit area of the membrane, $N/\cos\alpha$ pigment molecules lie within the light beam of unity cross-section. We further introduce the absorption cross-section σ of a pigment molecule, which is a function of α and β as well as of the angle ϑ between the electrical vector of the light beam and the plane of incidence. In all practical cases, the inequality $|\sigma N/\cos\alpha| \ll 1$ is fulfilled. Then the following relation holds between the incident light intensity I_0 and the transmitted light intensity I:

$$I = I_0 \left[1 - \frac{N\sigma(\alpha, \beta, \vartheta)}{\cos\alpha}\right]. \tag{4}$$

The absorption cross-section σ is highest if the angle ψ between the electrical vector of the light and the transition moment is zero. This maximum value of σ is denoted by σ_p. For an individual pigment molecule the absorption cross-section at other angles is then given by

$$\sigma' = \sigma_p \cos^2\psi. \tag{5}$$

To calculate the mean absorption cross-section σ of a pigment molecule in the membrane, one has to account for the fact that the orientation of the transition moment is random with respect to a rotation about the normal to the membrane. Thus,

$$\sigma = \sigma_p \overline{\cos^2\psi} \tag{6}$$

where the bar denotes the average over the azimuthal angle. For a linear transition moment, $\overline{\cos^2\psi}$ is given by (*see* Appendix):

$$\overline{\cos^2\psi} = A + B\cos 2\vartheta \tag{7}$$

$$A \equiv \tfrac{1}{2}\sin^2\alpha \sin^2\beta + \tfrac{1}{4}\cos^2\beta(1+\cos^2\alpha) \tag{8}$$

$$B \equiv \tfrac{1}{4}\sin^2\alpha(3\sin^2\beta - 1). \tag{9}$$

Introducing the transmitted light intensities for parallel and perpendicular orientation of the electrical vector with respect to the plane of incidence:

$$I_{\parallel}=I(\vartheta=0), \qquad I_{\perp}=I(\vartheta=\pi/2)$$

we may express the dichroism of the membrane by the quantity

$$\delta=\frac{I_{\parallel}-I_{\perp}}{I_{\parallel}+I_{\perp}}\approx\frac{I_{\parallel}-I_{\perp}}{2I_0}. \tag{10}$$

As the photomultiplier signal is proportional to I, Eq. (2) may be used to express δ in terms of experimental variables; with $|\Delta V^*| \ll V_0$ and $\vartheta=\omega t$ one obtains:

$$\delta\approx\frac{V^*(0)-V^*(\pi/2)}{2V_0}=\frac{\Delta V^*}{V_0}. \tag{11}$$

Introduction of Eqs. (4), (6) and (7) into Eq. (10) gives

$$\delta=-\frac{N\sigma_p}{\cos\alpha}B. \tag{12}$$

The product $N\sigma_p$ may be determined by measuring the unpolarized absorption of the membrane at normal incidence ($\alpha=0$). In this experiment, the transmitted light is given by

$$I=I_0(1-\sigma_m N). \tag{13}$$

σ_m is the mean absorption cross-section of a pigment molecule in the membrane at $\alpha=0$. Thus, $\sigma_m N$ is directly obtained from the absorption measurement:

$$\sigma_m N=\frac{I_0-I}{I_0}=\frac{\Delta V'}{V'} \tag{14}$$

(*see* Materials and Methods). From Eqs. (6)–(8), σ_m is calculated to be

$$\sigma_m=\sigma(\alpha=0)=\tfrac{1}{2}\sigma_p\cos^2\beta. \tag{15}$$

The final result is obtained by combining Eqs. (9), (12) and (15):

$$\delta=\sigma_m N\frac{\sin^2\alpha}{\cos\alpha}\left(\frac{3}{2}-\frac{1}{\cos^2\beta}\right). \tag{16}$$

It is seen from this equation that the dichroism vanishes for $\cos^2\beta=2/3$, or $\beta\simeq 35°$, and becomes negative for $\beta>35°$. Eq. (16) will be used later to calculate β from the experimental values of $\sigma_m N$ and $\delta(\alpha)$.

It is obvious that the measurement of a single angle β is not sufficient to specify the orientation of the porphyrin plane. Polarized absorption and fluorescence measurements, however, have shown that chlorophyll possesses two transition moments which are mutually perpendicular and which lie in the plane of the porphyrin ring. From the angles β_1 and β_2 which the two transition moments form with the membrane, the angle γ between the porphyrin plane and the membrane may be obtained using the trigonometric formula

$$\sin^2\gamma = \frac{1}{\sin^2\rho}(\sin^2\beta_1 + \sin^2\beta_2 - 2\sin\beta_1 \sin\beta_2 \cos\rho) \tag{17}$$

where ρ is the angle between the transition moments.

Light Reflection of the Membrane

To a first approximation, the bilayer membrane may be considered as thin homogeneous and isotropic film of thickness d and refractive index n embedded into an infinite medium of refractive index n_0. For a non-absorbing film, the incident and transmitted light intensities I_0 and I are related by the reflection coefficient R (*see*, for instance, Heavens, 1965):

$$I = I_0(1-R), \tag{18}$$

$$R = \frac{4r^2 \sin^2\eta}{1 - 2r^2 \cos 2\eta + r^4}, \tag{19}$$

$$\eta = \frac{2\pi n d}{\lambda} \cos\alpha', \tag{20}$$

$$n \cdot \sin\alpha' = n_0 \sin\alpha \tag{21}$$

where λ is the wavelength of the light. The Fresnel coefficients r for polarized light with the electrical vector parallel ($r_{\parallel}$) and perpendicular ($r_{\perp}$) to the plane of incidence are given by

$$r_{\parallel} = \frac{n_0 \cos\alpha' - n\cos\alpha}{n_0 \cos\alpha' + n\cos\alpha}, \tag{22}$$

$$r_{\perp} = \frac{n_0 \cos\alpha - n\cos\alpha'}{n_0 \cos\alpha + n\cos\alpha'}. \tag{23}$$

For a bilayer membrane both $r_{\parallel}$ and $r_{\perp}$ are small ($r_{\parallel}^2 \ll 1$, $r_{\perp}^2 \ll 1$), so that the approximation

$$R \approx 4r^2 \sin^2\eta \tag{24}$$

may be used. For the experimentally observed quantity

$$\delta_r \equiv \left(\frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}\right)_{\text{reflection}} \approx \frac{\Delta V^*}{V_0} \tag{25}$$

the following relation is obtained from Eqs. (18) and (24):

$$\delta_r = 2(r_{\perp}^2 - r_{\parallel}^2)\sin^2\eta. \tag{26}$$

Light Reflection of a Thick Plate

For a check of the performance of the instrument, a thick glass plate of refractive index n in a liquid of refractive index n_0 may be used as a reflection standard. In this case, I_0 and I are related by the Fresnel coefficient r in the following way (*see*, *e.g.*, König, 1928):

$$I = I_0 \frac{1-r^2}{1+r^2} \tag{27}$$

so that δ_r is given by

$$\delta_r = \frac{r_\perp^2 - r_\parallel^2}{1 - r_\parallel^2 r_\perp^2} \approx r_\perp^2 - r_\parallel^2. \tag{28}$$

Results

Thick Glass Plate

Prior to the experiments with bilayer membranes, the instrument had been tested using a glass plate immersed into different liquids of known refractive index n_0. The observed values of δ_r are shown in Fig. 3. The experiment with benzene as the immersion liquid has been used to deter-

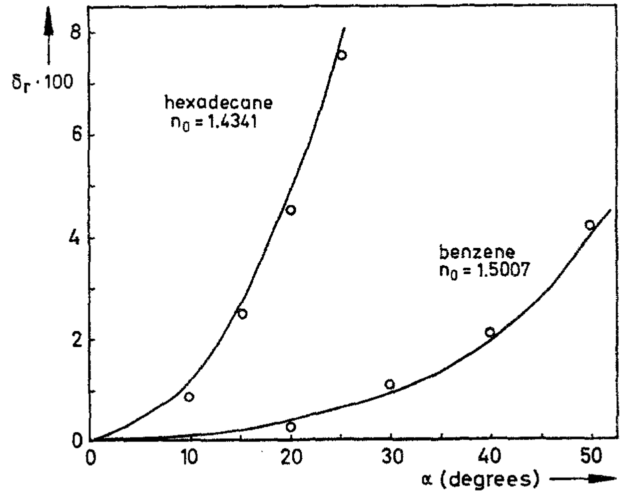


Fig. 3. Test of the instrument with a thick glass plate (refractive index n) immersed into a liquid of refractive index n_0. The values of n_0 have been measured separately. δ_r is the relative difference in the transmitted light intensities for light polarized parallel and perpendicular to the plane of incidence [Eq. (25)]; α is the angle between the light beam and the normal to the plate. The lines are theoretical curves calculated from Eqs. (22), (23) and (28) using a n value of 1.5277 which gave the best fit in the experiment with benzene as an immersion liquid

mine the refractive index n of the glass plate; i.e., the theoretical curve $\delta_r(\alpha)$ has been drawn according to Eqs. (22), (23) and (28) using an n value which gave the best fit with the experimental points. With this value ($n = 1.5277$) the $\delta_r(\alpha)$ curve for hexadecane has been calculated. The agreement between calculated and observed δ_r values is satisfactory.

Lecithin Membrane without Chlorophyll

If a nonabsorbing membrane, such as a pure lecithin bilayer in the wavelength range of 400 to 700 nm, is brought into the light beam at $\alpha \neq 0$, the measured signal arises from the unequal reflection of light polarized parallel and perpendicular to the plane of incidence. In Fig. 4 the signal δ_r [defined by Eqs. (25) and (26)] from a lecithin membrane at $\lambda = 439$ nm is plotted as a function of the angle α (open circles). The continuous curve has been calculated from Eq. (26) using values for the refractive index of the aqueous KCl solution ($n_0 = 1.340$) and for the membrane thickness ($d = 60$ Å) which were taken from the literature (Cherry & Chapman, 1969). For the refractive index of the membrane a value of $n = 1.425$ has been chosen which gave the best fit with the experimental points. Previous measurements of the reflectivity of black lecithin membranes gave values

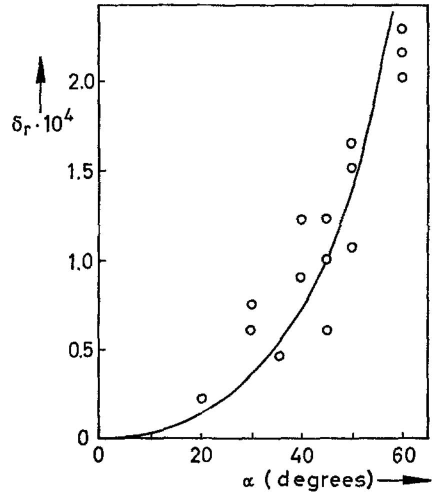


Fig. 4. Light reflection of a lecithin membrane without chlorophyll ($\lambda = 439$ nm). δ [defined by Eq. (26)] is plotted as a function of the angle α between the light beam and the normal to the membrane. The experimental values (open circles) have been obtained from four different membranes. The curve has been calculated from Eqs. (20)–(23) and Eq. (26) using the values $n_0 = 1.340$, $n = 1.425$, $d = 60$ Å

of n between 1.66 (Huang & Thompson, 1965) and 1.37 (Cherry & Chapman, 1967). Later, Cherry and Chapman (1969) demonstrated that a better theoretical description of the reflectance is obtained if the optical anisotropy of the film is taken into account; they calculated from their measurements values of 1.464 and 1.486 for the refractive index parallel and perpendicular to the film, respectively. The δ_r values given in Fig. 4 have been used to correct the dichroism measurements for the reflectance of the membrane.

Dichroism of Chlorophyll Membranes

In a first series of experiments, the dichroism δ of lecithin/chlorophyll *a* membranes has been studied at different orientations of the membrane with respect to the light beam. In Fig. 5 the observed values of δ are plotted as a function of $\sin^2 \alpha/\cos \alpha$ at $\lambda = 439$ nm for three different compositions. As predicted by the theory [Eq. (16)], δ is found to be proportional to $\sin^2 \alpha/\cos \alpha$.

In Fig. 6 the quantity $\delta/\sigma_m N$ is plotted as a function of the molar ratio X of chlorophyll to lecithin at a fixed angle $\alpha = 45°$ for the same wavelength. It is seen that $\delta/\sigma_m N$ is constant within the experimental limits up to at least $X = 0.7$. According to Eq. (16), this means that the orientation of the transition moment corresponding to $\lambda = 439$ nm is independent of the chlorophyll concentration in the membrane. The same is true for the transition moment at $\lambda = 671$ nm.

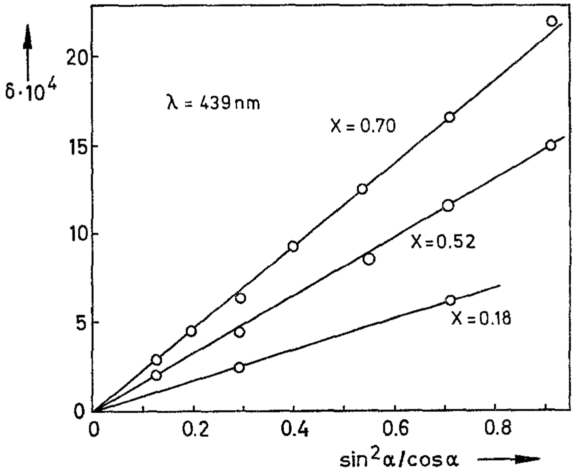


Fig. 5. Dichroism δ [defined by Eq. (10)] of a membrane made from a mixture of chlorophyll *a* and dioleoyllecithin ($\lambda = 439$ nm). The molar ratio [chl]/[lec] in the film-forming solution is denoted by X. The values of δ are corrected for reflection (*compare* Fig. 4)

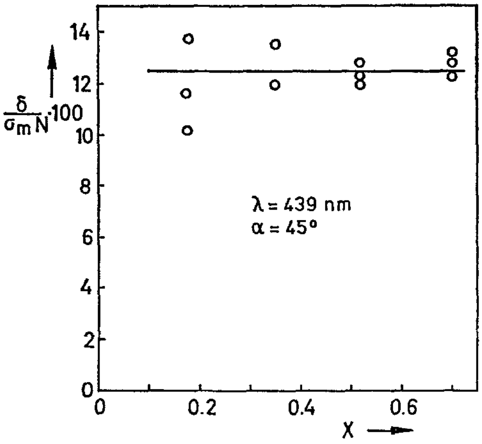


Fig. 6. Dichroism δ divided by the unpolarized absorption $\sigma_m N$ of the membrane at $\alpha = 45°$ and $\lambda = 439$ nm. X is the molar ratio of chlorophyll *a* to dioleoyl-lecithin in the film-forming solution. The values of δ are corrected for reflection. Each point corresponds to a different membrane

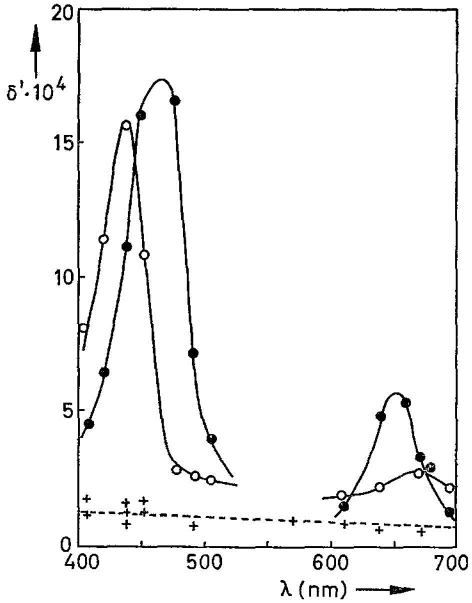


Fig. 7. Dichroism δ' (not corrected for reflection) at $\alpha = 45°$ as a function of wavelength λ for a dioleoyllecithin/chlorophyll membrane. ○ chlorophyll *a* ($X = 0.70$). • chlorophyll *b* ($X = 0.45$). + δ_r [defined by Eq. (25)] of a lecithin membrane without chlorophyll ($\alpha = 45°$)

The dichroism as a function of wavelength is represented in Fig. 7. The dichroism spectrum of chlorophyll *a* shows two peaks at 440 and 670 nm, (chlorophyll *b*: 460 and 650 nm) corresponding to the blue and red bands

Table 1. Summary of the results obtained with different membranes

Lipid	Chl.	X	$(\delta/\sigma_m N)_B$	$(\delta/\sigma_m N)_R$	β_B	β_R	γ
Dioleoyl-PC	*a*	0.18 – 0.70	0.12	0.02	27° ± 2°	34° ± 1°	46° ± 3°
PE	*a*	0.52	0.16	0.00	23° ± 2°	35° ± 1°	44° ± 3°
PS	*a*	0.52	0.09	−0.01	29° ± 3°	36° ± 2°	49° ± 5°
Dioleoyl-PC	*b*	0.4 – 1.2	0.10	0.11	29° ± 2°	28° ± 2°	(42° ± 4°)

The dichroism δ was measured at $\alpha = 45°$ (values corrected for reflection). $(\delta/\sigma_m N)_B$ refers to 439 nm for chlorophyll *a* and to 452 nm for chlorophyll *b*; $(\delta/\sigma_m N)_R$ to 671 nm for chlorophyll *a* and to 659 nm for chlorophyll *b*. PC = phosphatidylcholine (lecithin); PE = phosphatidylethanolamine; PS = phosphatidylserine. X = molar ratio chlorophyll/lipid in the film-forming solution. β_B and β_R are the angles between the membrane and the transition moments corresponding to the blue and red absorption bands, respectively. γ is the angle between the porphyrin ring and the membrane. The value of γ for chlorophyll *b* is set in parentheses because the mutual orientation of the transition moments is less certain in this case.

in the ordinary absorption spectrum. The dichroism of chlorophyll *a* at 670 nm is much smaller than at 440 nm; the ratio of the absorption cross-sections, σ_m (670)/σ_m (440), however, is equal to 0.8; i.e., of the same order as found for chlorophyll *a* in organic solvents. As discussed below, the observed wavelength dependence of the dichroism is a consequence of the fact that the blue and red absorption bands originate from different transition moments which make a right angle with each other.

The results obtained with chlorophyll *a* and *b* and different lipids are summarized in Table 1. It is seen that for chlorophyll *a* there is relatively little variation of $\delta/\sigma_m N$ from one lipid to the other for both the blue and the red absorption band.

From $(\delta/\sigma_m N)_B$ and $(\delta/\sigma_m N)_R$, the angles β_B and β_R between the "blue" and "red" transition moments and the membrane have been calculated using Eq. (16) and are given in Table 1. β_B and β_R may then be used to calculate the angle γ between the porphyrin ring and the membrane, if ρ is known [Eq. (17)]. Measurements of the dichroism of chlorophyll in liquid crystals indicate that the absorption occurs in the plane of the porphyrin ring for all bands in the visible part of the spectrum (Goedheer, 1955, 1966). Information about the mutual orientation of the transition moments in chlorophyll *a* mainly comes from fluorescence polarization measurements (Bär, Lang, Schnabel & Kuhn, 1961; Gouterman & Stryer, 1962; Surma & Frackowiak, 1970). These studies led to the conclusion (*compare* Goedheer, 1966) that the main blue absorption band of chlorophyll *a* at 430 to 440 nm is polarized perpendicular to the main emission oscillator in the red (which corresponds to the main red absorption band). In the case of chlorophyll *b*, the situation is less clear. The fluorescence polarization spectrum (Gouterman & Stryer, 1962) is similar to the spectrum of chlorophyll *a* but shows less marked maxima and minima. Goedheer (1966) points out that the most plausible interpretation of the chlorophyll *b* spectrum is the assumption of a similar system of transitions as present in chlorophyll *a*. We may therefore tentatively assume that also in the case of chlorophyll *b* the dichroism measured in the blue and red absorption bands originates from mutually perpendicular transition moments. $\rho = 90°$ has therefore been used throughout for the calculation of the angle γ (*see* Table 1).

In the previous paper (Steinemann *et al.*, 1971) the number N of chlorophyll molecules per cm^2 has been calculated from the optical absorption of the membrane using the extinction coefficient $\varepsilon_{432} = 1.3 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ of chlorophyll *a* in solution. The value of N can now be corrected for the orientation of the pigment molecules in the membrane. If σ_s is the absorption cross-section of randomly oriented chlorophyll, then we see from Eqs. (6), (15), and (A.12) that $\sigma_m = (3/2)\, \sigma_s \cos^2 \beta$. The value of N given in the previous paper has, therefore, to be divided by the factor $(3/2) \cos^2 \beta_B \simeq 1.2$ to get the true number of chlorophyll molecules per cm^2.

Discussion

The dichroism measurements presented above have shown that chlorophyll possesses a preferential orientation in a lipid bilayer membrane. For three different phospholipids, the angles γ between the porphyrin plane of chlorophyll *a* and the membrane were found to be in the range of 44 to 49°, the variation between the individual phospholipids lying within the limits of experimental error. A similar angle ($\gamma = 42° \pm 4°$) was calculated for chlorophyll *b*; this value, however, is somewhat doubtful because of the lack of precise information on the mutual orientation of the blue and red transition moments of chlorophyll *b*. It should be emphasized that in the evaluation of the angles β_B, β_R and γ the assumption has been made that all chlorophyll molecules in the bilayer contribute equally to the dichroism; i.e., that the angle between the transition moment and the membrane has a fixed value. It is obvious that this assumption is never strictly fulfilled, because, in general, a distribution about a mean value $\bar{\beta}$ has to be expected. It may be shown, however, that the calculated value of β is not very sensitive to a small uncertainty of the orientation. For instance, according to Eq. (A.17) of the Appendix, with an observed value of $(\delta/\sigma_m N)_B = 0.12$ at

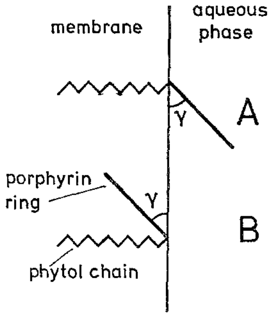


Fig. 8

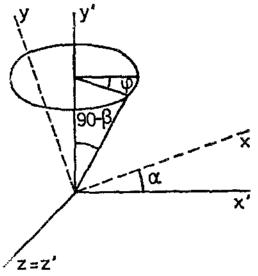


Fig. 9

Fig. 8. Two possible conformations of the chlorophyll molecule with respect to the membrane surface

Fig. 9. Cartesian coordinate system for the calculation of the angle ψ

$\alpha = 45°$ for chlorophyll *a*, the error $\bar{\beta}_B - \beta_B$ would be less than 1°, if a homogeneous distribution of width $2\Delta\beta = 10°$ is assumed.

If the angle γ between the porphyrin plane and the membrane surface is known, there still exists a choice between several possible conformations of the chlorophyll molecule. From the hydrophobic nature of phytol chain, however, it is very likely that the phytol chain is inserted into the hydrocarbon core of the membrane. Then two possible geometrical arrangements remain which are represented in Fig. 8. In conformation *A* the porphyrin ring is surrounded by water, whereas in conformation *B* the porphyrin ring is in a hydrophobic environment. It may be seen from the structural formula of chlorophyll *a* that adjacent to the phytol ester link there is a localized hydrophilic region on the porphyrin moiety, which is represented by the carbonyl and ester groups of the cyclohexane ring as well as by the phytol ester link itself; the larger part of the porphyrin ring, however, is hydrophobic. From the chemical structure of chlorophyll *a* the conformation *B* seems therefore more probable. Specifically, we propose that the porphyrin moiety is oriented toward the interior of the membrane and makes contact with the aqueous phase with the hydrophilic region in the vicinity of the phytol ester link.

It is well known that the cyclopentanon ring of chlorophyll shows a high chemical reactivity. The previous observation (Steinemann *et al.*, 1971) that chlorophyll *a* in a lipid bilayer may be chemically attacked by a membrane-insoluble reagent added to the aqueous phase is therefore not in disagreement with the proposed orientation.

It is interesting to note that chlorophyll *b* which has an additional polar group at some distance from the phytol ester link ($-$CHO instead of $-CH_3$ in position 3*c* of the porphyrin ring) shows a markedly different β_R value as compared with chlorophyll *a*. Though it cannot be excluded that this comes from a difference in the orientation of the red transition moment within the porphyrin plane, it is tempting to assume that the presence of the additional polar group leads to a change in the orientation of the chlorophyll *b* molecule with respect to the membrane.

The conclusions drawn above may be compared with the results of monolayer experiments as well as with dichroism studies of chloroplast membranes. From force-area measurements, Bellamy, Gaines and Tweet (1963) conclude that the porphyrin plane of chlorophyll *a* in the monolayer is tilted. Summarizing the results of previous studies and their own findings, they propose that the chlorophyll molecule is anchored to the water phase by its oxygen-containing groups and that both the porphyrin ring and the phytol chain are directed upward from the water. The same conclusion is reached by Trosper, Park and Sauer (1968). From the angular dependence of the fluorescence of chlorophyll *a* in monolayers, Tweet, Gaines and Bellamy (1964) calculate the orientation of the blue and red transition moments. Under the assumption that the angle between the porphyrin plane and the surface is $>60°$ (derived from force-area measurements) they obtain $\chi_B \simeq 28°$, $\chi_R \lesssim 20°$, where χ_B and χ_R are the angles between the blue and red transition moments and the surface, measured in the porphyrin plane.

There are numerous reports on dichroism studies of oriented chloroplasts or isolated chloroplast lamellae; for a recent survey of the literature, *see* Morita and Miyazaki (1971) and Breton and Roux (1971). A noticable dichroism has been observed only at 695 nm; at all other wavelengths the dichroism was very weak or absent. From this finding, most authors concluded that only chlorophyll 695 (a chlorophyll in a special environment) shows a preferred orientation with respect to the chloroplast lamella, and that all other chlorophyll is randomly oriented. Kreutz (1968), however, pointed out that the absence of dichroism can well be explained if both the red and blue transition moments are inclined at angles $\beta_R \simeq \beta_B \simeq 35°$, so that $\cos^2 \beta_R \simeq \cos^2 \beta_B \simeq 2/3$ [*compare* Eq. (16)]; this would imply that the porphyrin ring forms an angle of $\gamma \simeq 55°$ with the chloroplast lamella. It is interesting to note that the values of chlorophyll *a* in the artificial bilayer membranes are not too far from 35°, the range being 23 to 29° for β_B and 34 to 36° for β_R with three different lipids. It is therefore possible that chlorophyll *a* has a similar conformation in the thylakoid membrane and in the lipid bilayer. Our finding that the dichroism of chlorophyll *a* in the red absorption band is very weak may also be compared with the recent polarized absorption studies of Breton and Roux (1971). Using a highly sensitive method, these authors were able to measure the dichroism of oriented chloroplasts in the whole range between 350 and 750 nm and found that the dichroism in the near red absorption band ($\lambda = 673$ nm) was much weaker than in the blue Soret band.

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Appendix

Calculation of $\overline{\cos^2 \psi}$

For the calculation of the angle ψ between the electrical vector and the linear transition moment, we introduce two cartesian coordinate systems x, y, z and x', y', $z' = z$ with common origin (Fig. 9). We assume that the membrane lies in the $x'z'$ plane. The light beam is oriented parallel to the y axis with the electrical vector oscillating in the xz plane. The transition moment is represented by a vector μ of unity length which makes an angle β with the $x'z'$ plane. The components of μ with respect to the $x'y'z'$ system are then given by

$$\mu_{x'} = \cos\beta \cos\varphi, \tag{A.1}$$

$$\mu_{y'} = \sin\beta, \tag{A.2}$$

$$\mu_{z'} = \cos\beta \sin\varphi \qquad \text{(A.3}$$

where φ is the azimuthal angle (Fig. 8). The components of μ in the xyz system are equal to

$$\mu_x = \mu_{x'} \cos\alpha - \mu_{y'} \sin\alpha, \tag{A.4}$$

$$\mu_y = \mu_{x'} \sin\alpha + \mu_{y'} \cos, \tag{A.5}$$

$$\mu_z = \mu_{z'}. \tag{A.6}$$

The amplitude of the electrical field in the light beam is represented by a vector E of unity length with the components

$$E_x = \cos\vartheta, \tag{A.7}$$

$$E_y = 0, \tag{A.8}$$

$$E_z = \sin\vartheta. \tag{A.9}$$

ϑ is the angle between E and the plane of incidence which is perpendicular to the xz plane and parallel to the y axis. cos ψ is then given by

$$\cos\psi = \mu_x E_x + \mu_y E_y + \mu_z E_z. \tag{A.10}$$

The mean value of $\cos^2\psi$ is obtained by averaging over the azimuthal angle φ:

$$\overline{\cos^2\psi}=\frac{1}{2\pi}\int_0^{2\pi}\cos^2\psi\,d\varphi. \tag{A.11}$$

The result of the integration is represented in Eqs. (7) through (9).

If the orientation of the transition moment is completely arbitrary, as for a pigment molecule in solution, the averaging has to be performed over a sphere of unity radius and surface element $\sin\psi\,d\psi\,d\varphi$:

$$\overline{\cos^2\psi}=\frac{1}{4\pi}\int_{\varphi=0}^{2\pi}\int_{\psi=0}^{\pi}\cos^2\psi\sin\psi\,d\psi\,d\varphi=\tfrac{1}{3}. \tag{A.12}$$

In certain cases the angle between transition moment and membrane will not have a fixed value β, but rather show a distribution about a mean value $\bar\beta$. For this reason we finally calculate $\overline{\cos^2\psi}$ under the assumption that β is homogeneously distributed between $\bar\beta-\Delta$ and $\bar\beta+\Delta$:

$$\overline{\cos^2\psi}=\frac{\int_{\varphi=0}^{2\pi}\int_{\bar\beta-\Delta}^{\bar\beta+\Delta}\cos^2\psi\cos\beta\,d\beta\,d\varphi}{\int_{\varphi=0}^{2\pi}\int_{\bar\beta-\Delta}^{\bar\beta+\Delta}\cos\beta\,d\beta\,d\varphi}. \tag{A.13}$$

$\overline{\cos^2\psi}$ may then be used to calculate the dichroism δ in the same way as done before for the derivation of Eq. (16). This gives

$$\delta=\frac{3}{2}\sigma_m N\frac{\sin^2\alpha}{\cos\alpha}\cdot\frac{1-\cos^2\bar\beta\sin^2\Delta-3\sin^2\bar\beta\cos^2\Delta}{3-\cos^2\bar\beta\sin^2\Delta-3\sin^2\bar\beta\cos^2\Delta}. \tag{A.14}$$

In the limit $\Delta\to 0$ Eq. (A.14) reduces to Eq. (14); on the other hand, for $\bar\beta=\pi/4$ and $\Delta=\pi/4$ (completely random distribution), $\delta=0$. Eq. (A.14) may be solved for $\bar\beta$ to give

$$\cos^2\bar\beta=\frac{1-3p-3(1-p)\cos^2\Delta}{(1-p)(1-4\cos^2\Delta)} \tag{A.15}$$

$$p\equiv\frac{2}{3}\frac{\cos\alpha}{\sin^2\alpha}\frac{\delta}{\sigma_m N}. \tag{A.16}$$

For small Δ the following approximation is obtained from Eq. (A.15):

$$\beta-\bar\beta\approx\frac{\Delta^2}{6\sqrt{2}}\frac{1-9p}{\sqrt{1-3p}} \tag{A.17}$$

where β is the value of $\bar\beta$ for $\Delta=0$ (β, $\bar\beta$, and Δ expressed in radians).

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