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Optical Properties of Artificial Chlorophyll Membranes

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Summary. The composition and structure of lipid bilayer membranes containing chlorophyll *a* have been studied with photometric and fluorometric methods. A sensitive double-beam spectrophotometer is described by which the pigment concentration in the bilayer can be determined. Up to 3×10^{13} chlorophyll molecules per cm^2 can be incorporated into the membrane, corresponding to a mean distance of 20 Å between the porphyrin rings. At high chlorophyll concentrations, the absorption peaks are shifted toward longer wavelengths, indicating an interaction between porphyrin rings in the film. Parallel to the spectral shifts, a large decrease in the fluorescence quantum yield and a depolarization of the fluorescence are observed. These findings suggest that transfer of excitation energy takes place between neighboring chlorophyll molecules in the membrane. When an oxidating agent ($K_2S_2O_8$) is added to *one* external phase, exactly half of the chlorophyll in the film is destroyed. This observation suggests that the chlorophyll molecules are localized in the membrane surfaces with the phytyl chains inserted into the hydrocarbon core of the membrane and the porphyrin rings facing the aqueous solution.

In a previous communication (Alamuti & Läuger, 1970), we reported on a fluorometric study of lipid bilayer membranes containing chlorophyll *a*. These membranes are formed in aqueous phase from a mixture of chlorophyll *a* and dioleoyllecithin and have a final thickness of about 50 Å. Artificial membranes of this kind are valuable models for the study of photochemical reactions of chlorophyll and may be regarded as a primitive approximation of the much more complicated structure of the thylakoid membrane in the chloroplasts of green plants (Ting, Huemoeller, Lalitha, Diana & Tien, 1968; Tien, 1968*a*, *b*; Trissl & Läuger, 1970). In many aspects, these studies are an extension of previous work on chlorophyll monolayers at the air-water interphase (for a survey of the Literature, *see* Ke, 1966). In this paper we present a more detailed study of the composition and structure of artificial chlorophyll membranes in aqueous phase.

Materials and Methods

Membrane Formation

Dioleoylphosphatidylcholine (=dioleoyllecithin) was synthesized by acylation of glycerylphosphorylcholine (Robles & van den Berg, 1969). The purity of the product was checked by thin layer chromatography. Chlorophyll *a* was obtained from Fluka and used without further purification. The spectrum of this product agreed with the spectrum reported in the literature (Holt & Jacobs, 1954).

Lipid bilayer membranes were formed in the usual way (Läuger, Lesslauer, Marti & Richter, 1967) on Teflon frames with circular apertures of 6 to 8 mm in diameter. The solvent was *n*-nonane or *n*-decane. The time required for the whole area to become "black" was somewhat shorter in the case of *n*-nonane (about 20 min), but the membranes were more stable in *n*-decane (usually several hours). The optical properties of the black film were found to be independent of whether *n*-nonane or *n*-decane was used as a solvent. If not otherwise indicated, the lecithin concentration in the film-forming solution was 5 mg/ml (~6 mM). To this solution, various amounts of chlorophyll *a* were added. The aqueous phase contained 0.1 M KCl, pH ≃6.5, in all experiments.

Double Beam Spectrophotometer for the Measurement of the Optical Absorption of Lipid Bilayer Membranes

The spectrophotometer was a modified version of the instrument described by Nail, Moser and Urbach (1956) and by Frieser and Brandt (1965).

Optical Part. The light source was an Osram 50-W halogen-tungsten lamp combined with a Hewlett-Packard Mod. 6966 A d.c. power supply (stabilization better than 10^{-4}). The lamp house was water-cooled and was equipped with a heat-absorbing filter between the light bulb and the entrance slit of the monochromator. After passing the monochromator (Jarrel Ash, dispersion 33 Å/mm, slit width 0.1 mm), the light beam was split with a rotating mirror, one half of which was reflecting and the other half transmitting (*see* Fig. 1). The motor of the rotating mirror was driven at 70 cps by a sine generator followed by a power amplifier. The frequency of the generator was con-

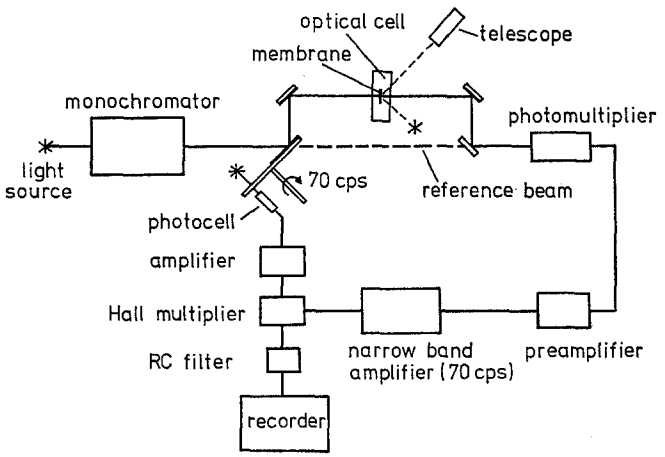

Fig. 1. Double-beam spectrophotometer for the measurement of the optical absorption of bilayer membranes

trolled to 5×10^{-5} by a tuning fork oscillator (Sourian, Boulogne). A Teflon support for the membrane was mounted inside the optical cell which was filled with an aqueous buffer solution. The measuring beam was focused onto the membrane and had a size of 1.5×1.5 mm in the plane of the membrane (membrane diameter 6 mm). Both the measuring beam and the reference beam were reflected onto the cathode of the photomultiplier (EMJ 9558 B) by a semitransparent mirror. The thinning process of the membrane could be followed by an additional light source and a laboratory telescope, each arranged at an angle of 45° with respect to the measuring beam.

Electronic Part. The high-voltage power supply for the photomultiplier was a John Fluke Mod. 405 B (stabilization better than 5×10^{-5}). In order 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 through a coupling capacitator into the main amplifier. The latter was tuned to the frequency of the rotating mirror (70 cps) by means of a TT filter network in the feedback loop. In addition to the main signal, the output of the tuned amplifier contained noise with frequencies near 70 cps. For a further increase of the signal-to-noise ratio, a Hall multiplier (Siemens MB 26 EJ 38/MU) combined with an RC filter was used. For this purpose, a reference signal was derived from the rotating mirror by means of an additional small light source and a photodiode. Before entering the Hall multiplier, the reference signal was amplified with a narrow-band amplifier tuned to 70 cps. The phase of the reference signal could be adjusted by varying the position of the photoelectric pickup relative to the mirror. The Hall multiplier plus RC filter further reduced noise signals of frequencies near 70 cps and also rejected 70 cps noise which was out of phase with the main signal. The time constant τ of the RC filter could be varied between 0.4 and 16 sec; as a compromise between noise level and response time, $\tau = 6$ sec was used in most cases.

Adjustment and Calibration. Prior to the formation of the membrane, the two beams were optically balanced to give zero output signal. For the coarse balance, a set of grey filters with graded transmittances was used. The fine adjustment was done by partially screening the reference beam with a blackened wire mounted on a micrometer screw. In order to minimize the error caused by a slow drift of the zero point, the bilayer membrane was destroyed by an electrical impulse at the end of the experiment, and the extinction was determined from the difference of the signal before and after the destruction.

The photometer was calibrated with very dilute chlorophyll *a* solutions of known extinction E. The output signal of the instrument was found to be a linear function of E up to at least $E = 10^{-2}$. The calibration was repeated after each experiment. For simplicity, this was done with another extinction standard consisting of a glass plate which was tilted by an angle of 10° in the measuring beam. The extinction change corresponding to this angle difference was previously determined to be $\Delta E = 2.80 \times 10^{-3}$. The sensitivity of the instrument (i.e., the smallest extinction which could be detected) was $E \simeq 5 \times 10^{-5}$. However, without special precautions, the sensitivity was not limited by the electronic and optical stability of the photometer but by the light scattering owing to microscopic lipid particles suspended in the sample cell. The sensitivity in an actual bilayer experiment was therefore only $E \simeq 2 \times 10^{-4}$. Attempts are now being made to reduce the scattering noise by continuous filtering of the solution after membrane formation.

Fluorescence Measurements

The fluorescence experiments were carried out with the same arrangement as described previously (Alamuti & Läuger, 1970). For the measurement of the fluorescence spectrum, a series of narrow-band interference filters (Balzers) were used with half-widths

of 10 nm; the mean spacing between successive filters was 15 nm. For the polarization experiments, Polaroid filters could be introduced between the exciting light source and the membrane, as well as between the membrane and the photomultiplier.

Results and Discussion

Optical Absorption of Chlorophyll Membranes

The time-course of the extinction E at the peak wavelength of the Soret band (432 nm) is shown in Fig. 2. At time $t=0$, a small amount of a lecithin/chlorophyll *a* solution is brought onto the hole of the Teflon support, and a lamella is formed which starts with a thickness of several microns. Correspondingly, the extinction is very high at the beginning, but decreases as the lamella becomes gradually thinner. The expansion of the black film always starts at the lower rim of the hole and proceeds very regularly. When the black area reaches the cross section of the measuring beam, an inflection in the E(t) curve is observed (point A). As soon as the total illuminated area is in the "black" state (point B), the extinction becomes constant.

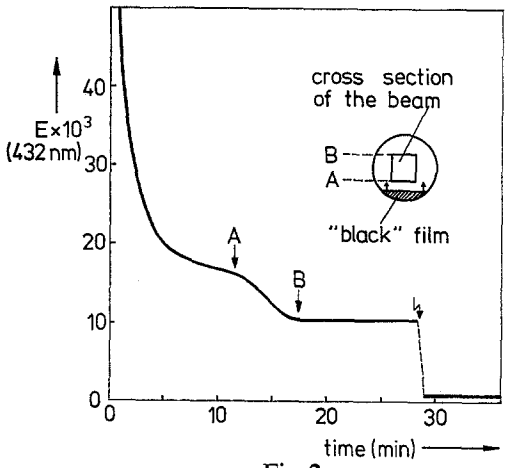

Fig. 2

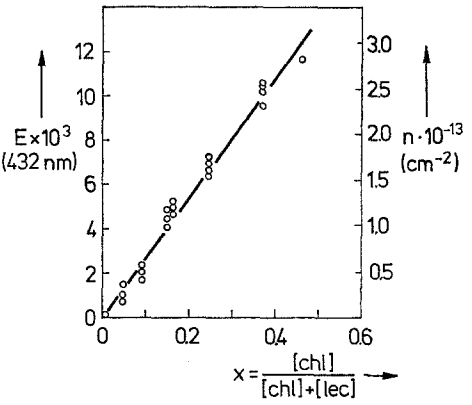

Fig. 3

Fig. 2. Extinction of a chlorophyll membrane as a function of time. The film-forming solution contained 2.4 μmoles chlorophyll *a* + 6.5 μmoles dioleoyllecithin per ml *n*-nonane. The lamella is formed at $t=0$. The black film reaches the cross section of the photometer beam at point A. At point B, the total illuminated area is in the "black" state. At the end of the experiment, the membrane is destroyed by an electrical impulse

Fig. 3. Extinction of the black film as a function of the molar fraction, x, of chlorophyll *a* in the film-forming solution. In each case, the solution contained 5 mg dioleoyllecithin (6.5 μmoles) per ml *n*-nonane. Each point corresponds to a different membrane. The right-hand ordinate indicates the number n of chlorophyll molecules per cm^2 of each interface. For the calculation of n, the value $\varepsilon_{432} = 1.3 \times 10^5\ M^{-1}\ cm^{-1}$ for the extinction coefficient has been used

When the membrane is destroyed by an electrical impulse at the end of th experiment, the photometer signal drops to zero (apart from a small zer point drift). As is to be expected, the $E(t)$ curve is completely analogous t the time course of the fluorescence of a chlorophyll/lecithin membran (Alamuti & Läuger, 1970).

Fig. 3 shows the extinction E of the black film as a function of th molar fraction $x \equiv [\text{chl}]/([\text{chl}]+[\text{lec}])$ of chlorophyll in the film-formin solution. The extinction is a linear function of x up to $x \simeq 0.45$ (at highe values of x, the films become unstable).

In principle, the extinction E of a thin film is dependent not only or absorption but also on reflection. However, the reflection coefficient of a lipid bilayer membrane is only about 6×10^{-5} (Cherry & Chapman, 1969) This gives a contribution of $6 \times 10^{-5}/2.30$ to E, which may be neglected From the measured extinction E, we may therefore calculate the number N of chlorophyll molecules per cm^2 of the black film:

$$N = \frac{N_A E}{10^3 \varepsilon} \tag{1}$$

where ε = molar extinction coefficient and N_A = Avogadro's number. Later in this paper, we present evidence that the chlorophyll molecules are localized in the two membrane-solution interfaces. We may therefore calculate directly the number, $n = N/2$, of chlorophyll molecules per cm^2 of each interface. With $\varepsilon_{432} = 1.3 \times 10^5\ M^{-1}\ cm^{-1}$ (Holt & Jacobs, 1954), we obtain the relation $n = 2.32 \times 10^{15}\ E$. For instance, at a molar fraction of $x \simeq 0.4$ in the film-forming solution, we find $n = 2.6 \times 10^{13}\ cm^{-2}$. In a hexagonal lattice, this would correspond to a center-to-center distance of 21 Å between neighboring porphyrin rings.

It should be pointed out, however, that the calculation of n is only approximate, because the value of the extinction coefficient ε is not precisely known. There are various reasons for this uncertainty. First, the special environment in the membrane-solution interface may influence the absorption properties of the chlorophyll molecule. In fact, it is found that the peak of the Soret band is slightly shifted when the density of the chlorophyll molecules in the interface is increased (*see* below). Second, the porphyrin ring may have a preferential orientation in the interface leading to an anisotropy in the optical absorption. The extinction coefficient is then, in general, different from the extinction coefficient of chlorophyll in solution where the transition moment has a random orientation. We plan to study this problem more closely by polarization measurements.

To investigate further the relation between the composition of the bulk solution and the number of chlorophyll molecules incorporated in the black film, we performed a second series of experiments in which the concentration, [chl], of chlorophyll in the solution was varied at a constant chlorophyll : lecithin ratio. The result is summarized in Fig. 4. Interestingly, it is found that the concentration of chlorophyll in the film becomes constant above [chl] $\simeq$ 1 mM. In other words, the chlorophyll concentration in the film is independent of the amount of solvent in the bulk lipid solution as long as the solution is not too diluted.

The spectrum of the bilayer membrane at two different chlorophyll : lecithin ratios is shown in Fig. 5. For comparison, the solution spectrum of chlorophyll *a* in *n*-nonane is also plotted. At a low concentration of chlorophyll in the membrane, the extinction maxima coincide with the peaks in the solution spectrum. However, at a high chlorophyll concentration in the film, both absorption peaks are shifted toward longer wavelengths. Similar (but larger) shifts are observed with crystalline chlorophyll and with chlorophyll *in vivo*. In the case of the crystal, the spectral shift is explained by a strong interaction between the closely spaced porphyrin rings. As the mean center-to-center distance between porphyrin rings is only about 20 Å at the higher chlorophyll concentrations, such an interaction is also feasible in the artificial membrane.

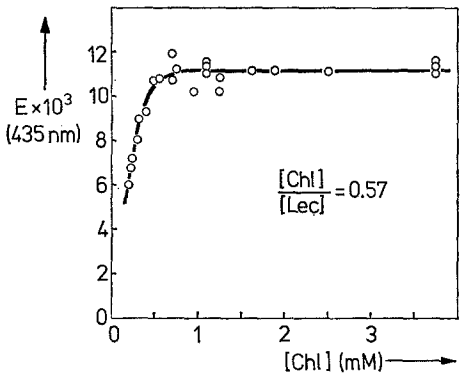

Fig. 4

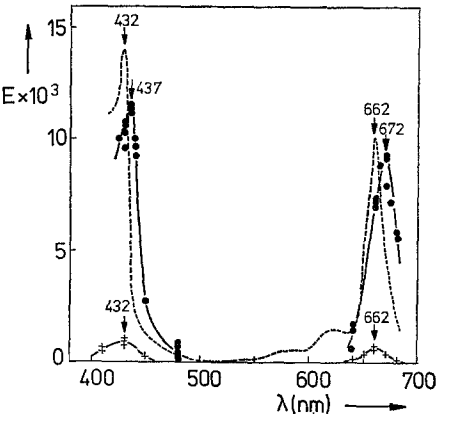

Fig. 5

Fig. 4. Extinction of the black film as a function of the chlorophyll concentration in the film-forming solution at a constant molar ratio of chlorophyll : lecithin equal to 0.57. Each point represents a different membrane

Fig. 5. Spectrum of the bilayer membrane at two different values of the molar fraction, $x \equiv [\text{chl}]/([\text{chl}]+[\text{lec}])$, of chlorophyll *a* in the film-forming solution (each point obtained from a different membrane). The lecithin concentration was 6.5 mM. •, $x=0.37$; +, $x=0.050$; ---- 1.1×10^{-7} M chlorophyll *a* in *n*-nonane

Fluorescence Measurements

The fluorescence intensity of the bilayer membrane as a function of th chlorophyll : lecithin ratio has been studied previously (Alamuti & Läuger 1970). It was found that the fluorescence increases up to [chl] : [lec] $\simeq 0.$ ($x \simeq 0.23$), and thereafter decreases. The decrease of the emission at highe chlorophyll concentrations has tentatively been explained by a self-quench ing of fluorescence. This conclusion is confirmed by the present photometri results (*see* below). At low values of [chl], where self-quenching is negligible the number of chlorophyll molecules per cm^2 of the film could be deter mined by comparison with the emission of a dilute chlorophyll solution o known concentration. For instance, at $x=0.05$, the interfacial density wa $n=N/2 \simeq 0.8 \times 10^{12}$ cm^{-2}. The corresponding photometric value at the same molar fraction x is $n \simeq 3.3 \times 10^{12}$ cm^{-2}. Thus, both methods lead to the same order of magnitude for the chlorophyll concentration in the membrane. The difference between the photometric and the fluorometric values of n possibly arises from a preferential orientation of the porphyrin ring in the interface, as discussed above.

As n is a linear function of x in the concentration range studied (Fig. 3), the relative quantum yield, Φ/Φ_0, of the fluorescence may be evaluated. The photomultiplier signal J at molar fraction x may be represented by:

$$J(x)=k\,n(x)\,\Phi(x)=k'\,x\,\Phi(x) \tag{2}$$

where k and k' are constants which contain the intensity of the exciting light, the sensitivity of the phototube, and geometrical factors, The quantum yield $\Phi(x)$ has the property that:

$$\lim_{x\to 0} \Phi(x)=\Phi_0=\text{constant}. \tag{3}$$

If we assume that self-quenching is negligible at $x^*=0.05$, then $J(x^*)= k'x^*\,\Phi_0$, and

$$\frac{\Phi}{\Phi_0}=\frac{x^*\,J(x)}{x\,J(x^*)}. \tag{4}$$

Φ/Φ_0 as a function of x is plotted in Fig. 6. It is seen that the fluorescence quantum yield steeply decreases for $x>0.2$, corresponding to $n>10^{13}$ cm^{-2} or a center-to-center distance of porphyrin rings in the interface <30 Å. This result is in accordance with the observations of Tweet, Gaines and Bellamy (1964), and of Trosper, Park and Sauer (1968). These authors studied the fluorescence of mixed monolayers of chlorophyll *a* and oleyl alcohol at the air-water interface and also found a sharp decrease of the quantum yield at approximately the same chlorophyll concentrations.

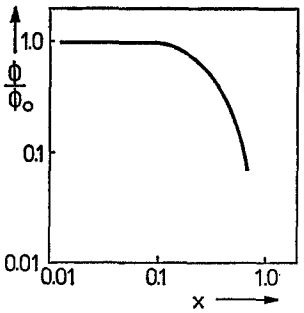

Fig. 6. Relative quantum yield, Φ/Φ_0, of the fluorescence of chlorophyll *a* in the membrane as a function of the molar fraction x

Self-quenching of fluorescence may occur in several different ways. The simplest mechanism is the so-called static quenching. This term implies that an equilibrium exists between fluorescent monomers and nonfluorescent dimers (or higher aggregates). When the pigment concentration is increased, more and more pigment molecules are present in the form of nonfluorescent aggregates. In many cases, however, quenching is greatly enhanced by energy transfer, whereby the excitation migrates among the assembly of pigment molecules and may finally reach a nonfluorescent molecule. It is well known that this process takes place with high efficiency in the chloroplasts of green plants (Robinson, 1966). The most probable mechanism for energy migration is the inductive resonance transfer or Förster mechanism (Förster, 1951) in which distances between pigment molecules of the order of 50 Å are usually sufficient for a rapid energy transfer. For instance, the critical distance at which emission and intermolecular energy transfer are equally probable is 80 Å for chlorophyll *a* in solution (Förster, 1951, p. 177). In contrast to static quenching, energy migration leads to a depolarization of the fluorescence. It is therefore possible to discriminate between the two cases by polarization measurements. The Table shows the result of an ex-

Table. *Degree of fluoroscence polarization p as a function of the molar fraction x of chlorophyll a in the film-forming solution* [a]

x	0.05	0.09	0.5
p	0.065 ± 0.003	0.060 ± 0.003	0.002 ± 0.001

[a] The exciting light is linearly polarized with the electric vector parallel to the plane of the membrane. p is defined as $p \equiv \frac{J_{\parallel} - J_{\perp}}{J_{\parallel} + J_{\perp}}$, where $J_{\parallel}$ and $J_{\perp}$ are the photomultiplier signals when the analyzer is parallel or perpendicular to the polarizer, respectively. (The signals remain unchanged within the limits of error if both filters are rotated by 90°.)

periment in which the exciting light is linearly polarized with a Polaroi filter, and the fluorescence radiation is analyzed with a second polarizatio filter. At low chlorophyll concentration where quenching is small, the degre of fluorescence polarization p is found to be 0.065. At high chlorophyl concentration ($x=0.5$), however, the polarization is practically zero. Thi result indicates that energy transfer takes place in the membrane at th higher chlorophyll concentrations. A similar conclusion has been drawı by Trosper *et al.* (1968) from their monolayer studies. In the case of mixed monolayer of chlorophyll *a* and oleyl alcohol, they found a limitin value of $p_0 \simeq 0.05$ in the absence of quenching, and also a substantial de crease of p in the concentration range where quenching occurs. It is inter esting to note that the observed value of the limiting polarization ($p_0 \simeq 0.065$ is much lower than would be expected for chlorophyll *a* in a completel rigid medium (Trosper *et al.*, 1968). This result suggests that the porphyri ring in the membrane has sufficient freedom for a reorientation within th lifetime of the excited state ($\sim 10^{-8}$ s).

The fluorescence spectrum of the chlorophyll membrane has been meas ured with a series of narrow-band interference filters and is shown in Fig. 7 The procedure for the construction of the spectral curve from the experi mental data was the same as described by Tweet *et al.* (1964). The maximun emission occurs near 680 nm. The fluorescence peak of chlorophyll *a* ir

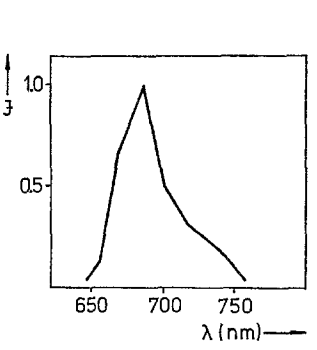

Fig. 7

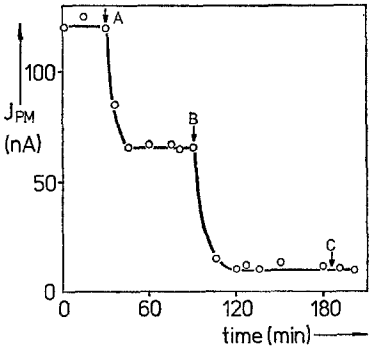

Fig. 8

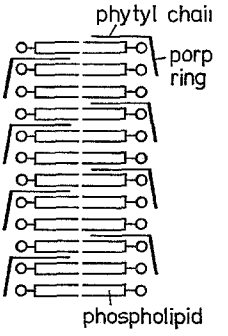

Fig. 9

Fig. 7. Fluorescence intensity J (in arbitrary units) as a function of wave length λ. The molar fraction of chlorophyll *a* in the film-forming solution was $x=0.09$

Fig. 8. Fluorescence of the bilayer during oxidation of chlorophyll *a* with $K_2S_2O_8$. Ordinate: photomultiplier current (*nA*). The membrane has been formed in 0.1 M KCl and is in its final black state at time $t=0$. At point A, 2.5 mM $K_2S_2O_8$ is added to one external phase. At point B, the same concentration of $K_2S_2O_8$ is added to the other side. The membrane is destroyed by an electrical impulse at the end of the experiment (point C)

Fig. 9. Proposed structure of the chlorophyll bilayer

monolayers (Tweet *et al.*, 1964) and *in vivo* (French, 1960) is observed at approximately the same wavelength, whereas that for chlorophyll *a* in solution is located at 670 nm.

By the photometric and fluorometric measurements described above, the number of chlorophyll molecules in the bilayer membrane could be determined. These measurements are not sufficient, however, to decide how the chlorophyll molecule is built into the bilayer structure. Two alternatives are conceivable: (1) the chlorophyll is statistically distributed in the hydrocarbon core of the membrane; or (2) the chlorophyll molecule is localized in the two membrane-solution interfaces. It is possible to distinguish these two cases if a nonpermeating reagent which alters the optical properties of chlorophyll is added to *one* external phase. A suitable reagent is potassium peroxodisulfate ($K_2S_2O_8$) by which chlorophyll is oxidized (if $K_2S_2O_8$ is added to a methanolic solution of chlorophyll *a*, the green color slowly disappears). Furthermore, it may be inferred from conductance data that the membrane is practically impermeable for $S_2O_8^{--}$. The result of the experiment is shown in Fig. 8. At time $t=0$, the membrane is in its final black state in which the fluorescence intensity is constant. At point *A*, a small amount of a concentrated $K_2S_2O_8$ solution is added to one side. As a result, the fluorescence intensity drops to almost half its original value and thereafter remains constant. If, after some time, the same concentration of the oxidizing agent is added to the other side, the photomultiplier signal goes down to the dark current.

From the observation that exactly half of the chlorophyll in the film reacts after the addition of the oxidant to one side, the solubility model (alternative 1) can be excluded. The experiment therefore indicates that the chlorophyll is localized in the membrane interfaces. This conclusion is in accordance with the amphiphilic character of the chlorophyll molecule. It may be assumed that the hydrophobic phytyl chain of the molecule is inserted into the hydrocarbon-like interior of the membrane, whereas the slightly hydrophilic porphyrin ring is in contact with the aqueous phase. The resulting membrane structure is depicted schematically in Fig. 9; a similar structure has already been proposed by Ting *et al.* (1968). It should be emphasized, however, that it is not yet possible to specify the angle between the porphyrin ring and the membrane surface on the basis of experimental evidence. For a discussion of this problem in the case of the thylakoid membrane, *see* Kreutz (1968).

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