Segregation of Chlorophyll a Incorporated into Lipid Bilayers†

A. G. Lee

ABSTRACT: Absorption and fluorescence spectra are reported for chlorophyll a incorporated into a number of aqueous phospholipid dispersions. Absorption spectra show that in dipalmitoylphosphatidylcholine bilayers, monomeric and oligomeric forms of chlorophyll a are present in both the gel and liquid crystalline phases. The formation of aggregates of chlorophyll a is reflected in the fluorescence spectra by a marked concentration quenching. In bilayers containing small proportions of chlorophyll a, a marked increase in aggregation occurs at the transition temperatures that can be detected calorimetrically. At higher concentrations (>1 chlorophyll:100 lipid), the "pretransition" is abolished in the phosphatidylcholines, and the main transition is broadened, consistent with an orientation for the chlorophyll a with the chlorine ring in the head group region and the phytol chain in the fatty acid chain region of the bilayer. In mixtures of saturated and unsaturated lipids, there is no preferential segregation of the chlorophyll a into the unsaturated lipid.

Despite its obvious importance for any study of photosynthesis, relatively little is known about the organization of chlorophylls incorporated into lipid bilayers. This is in marked contrast to the very considerable amount of information available concerning the state of the chlorophylls in organic solvents (Vernon and Seely, 1966; Cotton et al., 1974) and in monolayers (Ke, 1966). However, in the light of the marked aggregation of chlorophyll a found in nonpolar solvents and the similarity between the absorption spectra of many of these aggregates and chlorophyll a in photosynthetic membranes (Cotton et al., 1974), it has often been suggested that aggregates of chlorophyll a are present in photosynthetic membranes.

Recent studies have established some of the characteristics of component segregation within lipid bilayers (Lee, 1975). In particular, studies of phospholipid–phospholipid (Shimshick and McConnell, 1973) and phospholipid–steroid mixtures (Trauble and Sackmann, 1972) have established the presence of segregated pools of lipid and steroid in bilayers at temperatures below that of the gel to liquid crystalline phase transition of one or more of the lipid components of the mixture. However, phospholipids in the liquid crystalline phase seem to be completely miscible in all proportions (Shimshick and McConnell, 1973), and the spin-labeled steroid analog studied by Trauble and Sackmann (1972) is completely miscible with lipid in the liquid crystalline phase at least up to a steroid/lipid molar ratio of 1:4.

Since the lipids of most photosynthetic plants contain a relatively high proportion of polyunsaturated fatty acids (Galliard, 1973), the photosynthetic membrane will be predominantly in the liquid crystalline phase at ambient temperature. Aggregation of chlorophyll a within the membrane could then only occur either because of specific chlorophyll–chlorophyll interaction, or because the chlorophyll was in some way tightly coupled to the protein component of the membrane.

In this paper are reported experiments which show that aggregates of chlorophyll a are present in lipid bilayers, both when the lipid is in the gel and in the liquid crystalline phases, although the proportion of aggregated chlorophyll is increased at the expense of monomer when the lipid is in the gel state. The formation of aggregates of chlorophyll a in lipid bilayers in the liquid crystalline state shows that chlorophyll–chlorophyll interactions alone are sufficient to explain their formation, and thus the formation of "antenna" chlorophyll in the photosynthetic membrane.

Materials and Methods

Dipalmitoylphosphatidylcholine was obtained from Koch-Light, dioleoylphosphatidylcholine from P-L Biochemicals, and dimyristoylphosphatidylethanolamine from Fluka. Chlorophyll a was purified by column chromatography on powdered sugar columns by the method of Strain and Svec (1966). Chlorophyll a concentrations were estimated from absorption spectra, using the extinction coefficients given by Strain and Svec (1966).

Lipids plus chlorophyll a (1.6×10^{-9} mol) dissolved in chloroform were mixed in 10-ml stoppered flasks and evaporated to dryness under a stream of nitrogen. Buffer (4 ml; 0.01 *M* Tris-HCl (pH 7.2)–0.1 *M* NaCl) was added and the mixture shaken on a Vortex mixer. The optical density at 670 nm was less than 0.1 for all samples. Absorption spectra were recorded on a Cary 14 spectrophotometer. Fluorescence measurements were made on an Aminco Bowman SPF fluorimeter. Samples were continuously stirred during the fluorescence experiments, and temperatures were measured by a thermocouple. Fluorescence of chlorophyll a was excited at 420 nm and recorded at 670 nm. Over the lipid/chlorophyll a ratios used in these experiments (5000:1 to 30:1), the absorption intensity at 670 nm did not change significantly, so that changes in fluorescence intensity correspond to changes in fluorescence quantum yield.

Results

Absorption spectra of chlorophyll a in dioleoylphosphatidylcholine and dipalmitoylphosphatidylcholine bilayers at 45°C and at chlorophyll/lipid molar ratios of 1:800 and 1:8, respectively, are shown in Figure 1a and b. The maximum of the chlorophyll a α peak appears at 668–672 nm, depending on concentration, and the spectra are virtually independent of temperature in the range 10–50°C. With in-

† From the Department of Physiology and Biochemistry, University of Southampton, Southampton S09 3TU. *Received May 8, 1975.*

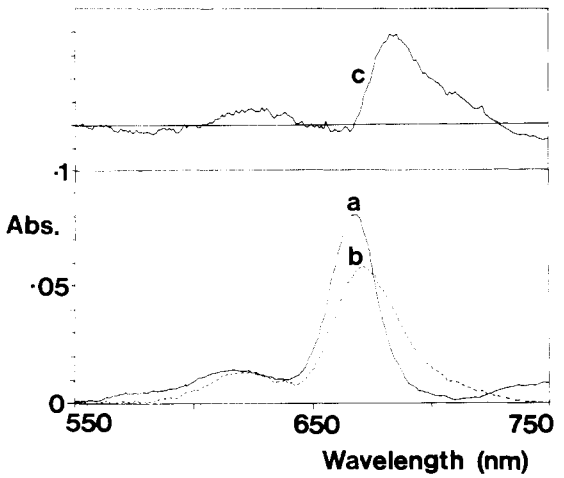


FIGURE 1: Absorption spectrum of chlorophyll a (1.6×10^{-9} mol) in liposomes of: (a) dioleoylphosphatidylcholine at a chlorophyll/lipid molar ratio of 1:800, (b) dipalmitoylphosphatidylcholine at a chlorophyll/lipid molar ratio of 1:8 (absorption scale × 2), both dispersed in 4 ml. Tris buffer at 45°C. The difference spectrum showing aggregated chlorophyll a formed at high concentration, produced by subtracting the spectrum of Figure 1a from that of Figure 1b, after normalizing the latter spectrum to the maximum peak height of the former, is shown in c.

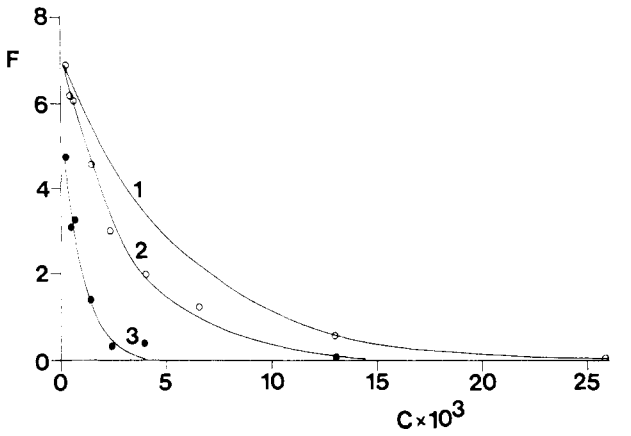


FIGURE 2: Fluorescence intensity (arbitrary units) at 670 nm vs. lipid/chlorophyll molar ratio 1:c. The amount of chlorophyll a was kept constant at 1.6×10^{-9} mol. ○ at 50°C; ● at 20°C. Curves 1, 2, and 3 are calculated from a random distribution model (see text): curve 1, interaction distance 60 Å, lipid cross-sectional area 70 Å^2; curve 2, interaction distance 86 Å, lipid cross-sectional area 70 Å^2; curve 3, interaction distance 119 Å, lipid cross-sectional area 40 Å^2.

creasing concentration, there is a marked increase in peak width at half-height, from ca. 30 nm (up to a chlorophyll/lipid molar ratio of 1:80), to 37 nm at a molar ratio of 1:40 and to 43 nm at a molar ratio of 1:8. The increase in peak width cannot be attributed to an artifact such as light scattering, since in these experiments the amount of chlorophyll is kept constant, and the chlorophyll/lipid molar ratio is increased by decreasing the amount of lipid: this means that the samples which show the greatest peak width are those with the least amount of lipid, and which therefore scatter light the least. The increase in width seems rather to be due to the appearance of a shoulder at ca. 685 nm at the higher concentrations. This is shown most clearly by the difference spectrum (Figure 1c). Deconvolution studies of spectra of chlorophyll a in nonpolar organic solvents have previously shown the presence of a major band at ca. 680 nm, due to aggregated chlorophyll (Cotton et al., 1974).

Fluorescence Spectra in Dipalmitoylphosphatidylcholine. In bilayers of dipalmitoylphosphatidylcholine, chlorophyll a has a fluorescence maximum at 670 nm, whereas in diethyl ether the fluorescence maximum is at 667 nm. In the lipid bilayer, it is necessary to determine whether or not there are more than one fluorescent chlorophyll a species present, since the absorption spectra of these systems show the presence of chlorophyll aggregates. This can be done since the intensity of fluorescence emitted at a certain wavelength by a mixture of several fluorescent forms with different positions of absorption bands will depend on the wavelength of excitation. Within experimental error, fluorescence spectra normalized to equal intensity at 670 nm are identical with excitation wavelengths between 390 and 440 nm, and at temperatures between 20 and 50°C. This suggests therefore that there is only one fluorescent form of chlorophyll a present in bilayers of dipalmitoylphosphatidylcholine, both in the gel and liquid crystalline states.

Figure 2 shows the effect on fluorescence intensity of changing the lipid/chlorophyll a molar ratio, while keeping the amount of chlorophyll a constant. In the absence of any concentration quenching, the fluorescence intensity in this experiment would remain constant: in fact, the fluorescence intensity is much greater for those liposomes containing a high proportion of lipid. Analogous concentration quenching has been observed for many fluorescent dyes, and attributed to deactivating collisions between excited molecules and molecules in the ground state (Seliger and McElroy, 1965). In that case, the fluorescence intensity N should follow the Stern–Volmer relation

$$N = \frac{N_0}{1 + \tau_0 q} \tag{1}$$

where N_0 is the number of quanta absorbed per second, τ_0 is the natural lifetime of the excited state, and q is the number of effective quenching collisions per second. Since q should be proportional to the concentration, eq 1 can be rewritten as

$$N_0/N = 1 + c/c^* \tag{2}$$

where c is the concentration of fluorophor and c^* is the concentration at which the fluorescence yield has fallen to one-half. If collisional quenching were important for chlorophyll a in these lipid bilayers, a plot of the reciprocal of fluorescence intensity against chlorophyll/lipid ratio would be a straight line. This is not so for the data plotted in Figure 2.

An alternative explanation that has been proposed for the concentration quenching of dyes is the formation of ground state dimers or polymers which are nonfluorescent (Rabinowitch and Epstein, 1941). There are two forms that such quenching could take in our case. In the first, chlorophyll a molecules are randomly distributed within the lipid bilayer, and two chlorophyll a molecules closer than some given interaction distance form a nonfluorescent pair. In the second, the distribution of chlorophyll a within the lipid bilayer is nonrandom, and nonfluorescent dimers or oligomers are formed by specific chlorophyll–chlorophyll interaction.

If the distribution of chlorophyll molecules is random, then the probability that the nearest neighbor chlorophyll molecule to one particular chlorophyll molecule in the bilayer plane is between r and $r + dr$ can be written as $w(r)dr$, where $w(r)$ is the probability density. This is equal to the probability that there is no chlorophyll closer than r, times the probability that another chlorophyll molecule ex-

ists in the circular ring between r and $r + dr$. Thus the probability density is given by

$$w(r) = \left[1 - \int_0^r w(r)dr\right]2\pi rn \quad (3)$$

Integration then leads to

$$w(r) = 2\pi rn \exp(-\pi r^2 n) \quad (4)$$

Here n is the number of chlorophyll a molecules in unit area. For simplicity in the calculations, the area occupied by a chlorophyll a molecule in the bilayer was taken to be equal to that of a lipid molecule: with the very low proportions of chlorophyll used in these experiments, the fact that the cross-sectional area of a phytol chain is ca. one-half of that of a lipid molecule will have no significant effect. Figure 2 shows attempts to fit the experimental data to this random distribution model, the calculations being normalized to the observed fluorescence intensity at a chlorophyll/lipid molar ratio of 2×10^{-4}:1.

In the liquid crystalline phase, a lipid cross-sectional area of 70 Å^2 was assumed (Lee, 1975), and the best fit to the data was then obtained with an interaction distance of 86 Å, although the calculated fluorescence for bilayers containing little lipid was then rather too low (curve 2, Figure 2). The data for the high chlorophyll/lipid ratios at 50°C were better fitted with an interaction distance of 60 Å (curve 1, Figure 2) but then the fluorescence intensities calculated at low chlorophyll/lipid ratios were too high. Decreasing the area occupied per lipid molecule increases the calculated proportion of nonfluorescent chlorophyll at any given chlorophyll/lipid ratio and interaction distance, without, however, changing the shape of the fluorescence–molar ratio curve. In the gel state, the surface area of a lipid molecule is ca. 40 Å^2 (Lee, 1975), and curve 3 of Figure 2 shows that a good fit to the fluorescence data at 20°C in the more dilute bilayers can then be obtained with an interaction distance of 119 Å.

All of these interaction distances seem to be unreasonably large to represent the formation of nonfluorescent pairs of chlorophyll a molecules. The area of the porphyrin group of a chlorophyll is ca. 225 Å^2, and if, as has been suggested, the porphyrin ring is tilted at an angle of ca. 50° to the bilayer plane (Hoff, 1974), then the area occupied at the bilayer surface is ca. 144 Å^2. This means that the centers of two porphyrin rings must be closer than ca. 16 Å to allow overlap, and in crystals of ethyl chlorophyllide a dihydrate, the Mg–Mg distance between adjacent molecules can be estimated from the published data (Strouse, 1974) to be less than 10 Å. Since the interaction distances calculated from the random distribution model are considerably greater than those expected from the size of the chlorophyll a molecule, it seems that the distribution of chlorophyll a molecules is not random, but rather that nonfluorescent dimers or oligomers are formed.

If only monomeric and dimeric chlorophyll a are present in the bilayer, then if [Chl], [Chl$_2$], and m represent the mole fractions of monomer, dimer, and total chlorophyll, it follows that

$$m = [\text{Chl}] + 2[\text{Chl}_2] \quad (5)$$

If the equilibrium constant for dimerization is K, then

$$K = \frac{[\text{Chl}_2]}{[\text{Chl}]^2} = \frac{(m - [\text{Chl}])}{(2[\text{Chl}]^2)} \quad (6)$$

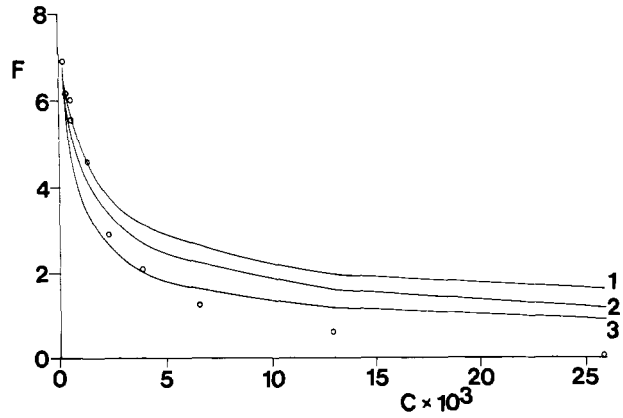


FIGURE 3: Attempted fit of fluorescence vs. concentration data to a dimer model. Data as in Figure 4. Curve 1, K = 500 (mole fraction)$^{-1}$; curve 2, K = 1000 (mole fraction)$^{-1}$; curve 3, K = 3800 (mole fraction)$^{-1}$.

Thus,

$$[\text{Chl}] = (\tfrac{1}{4}K)[(8Km + 1)^{1/2} - 1] \quad (7)$$

If the dimer is nonfluorescent, then the fluorescence intensity at any given chlorophyll/lipid ratio is proportional to the amount of monomeric chlorophyll present. Figure 3 shows attempts to fit the fluorescence data for chlorophyll a in dipalmitoylphosphatidylcholine at 50°C to such a model of dimer formation: again, the calculations were normalized to the observed fluorescence intensity at a chlorophyll/lipid molar ratio of 2×10^{-4}:1. In the most dilute bilayers a reasonable fit can be obtained with an equilibrium constant for dimerization of K = 500 (mole fraction)$^{-1}$: assuming a density of 1 for the lipid (Levine, 1973), this corresponds to $K \simeq 600$ l. mol^{-1}. At chlorophyll/lipid molar ratios of greater than 1.3×10^{-3}:1, however, the fit is very poor. Increasing the equilibrium constant improves the fit at higher chlorophyll/lipid molar ratios, but then predicts a lower than observed fluorescence at low chlorophyll/lipid molar ratios. It seems therefore that although dimers may be formed at low chlorophyll concentrations, aggregates of more than two chlorophylls are certainly present at chlorophyll/lipid molar ratios greater than ca. 2×10^{-3}:1, when the lipid is in the liquid crystalline state. When the lipid is in the gel state, aggregation beyond the dimer would occur at even lower chlorophyll/lipid ratios.

It is also possible that some of the fluorescence quenching observed at higher chlorophyll concentrations could be due to nonradiative energy transfer to the nonfluorescent dimers of chlorophyll. However, estimated values for the rate of nonradiative transfer suggest that this can only be a very minor effect. The pairwise energy transfer rate τ by dipole–dipole resonance interaction is given by the Förster theory as (Seliger and McElroy, 1965)

$$\tau = \frac{1}{\tau_0}\left(\frac{R_0}{R}\right)^6 \quad (8)$$

where τ_0 is the natural fluorescence lifetime, R is the separation between the pair of chlorophyll molecules, and R_0 is the critical interaction distance. The values of R_0 and τ_0 have been estimated as 65–70 Å and 15 nsec, respectively (Duysens, 1964). At a chlorophyll/lipid molar ratio of 2.3×10^{-3}:1, the average separation of chlorophyll molecules is 170 Å, and the pairwise transfer rate is then 2×10^5 sec^{-1}, so that the probability of excitation energy transfer to a chlorophyll dimer, even if it were the nearest neighbor,

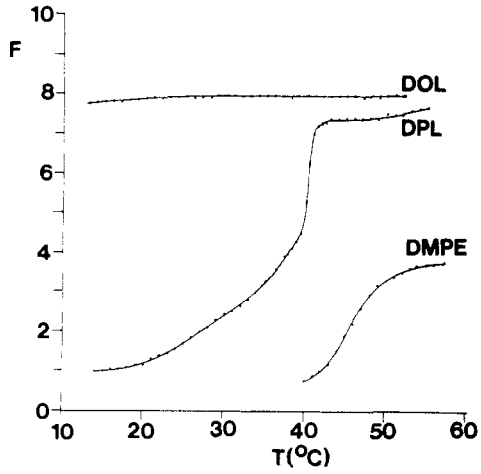


FIGURE 4: Fluorescence intensity of chlorophyll a vs. temperature, in liposomes of dioleoylphosphatidylcholine (DOL), dipalmitoylphosphatidylcholine (DPL), and dimyristoylphosphatidylethanolamine (DMPE), at a chlorophyll/lipid molar ratio of 1:430.

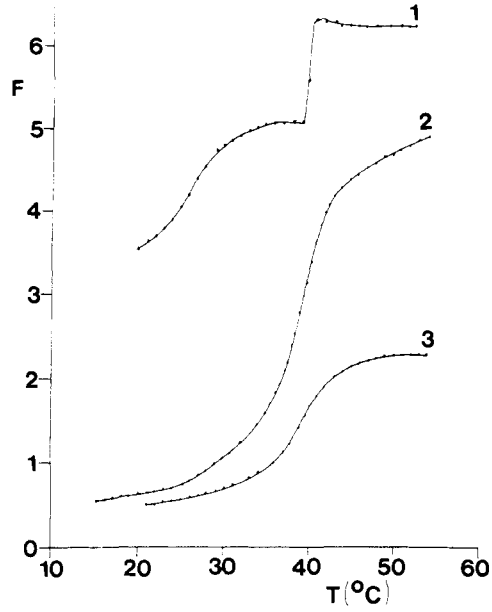
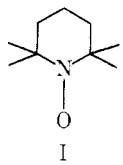


FIGURE 5: Fluorescence intensity of chlorophyll a vs. temperature in liposomes of dipalmitoylphosphatidylcholine, at chlorophyll/lipid molar ratios of (1) 1:1560; (2) 1:77 (sensitivity increased by a factor of 20); (3) 1:39 (sensitivity increased by a factor of 45).

would be very small. At a chlorophyll/lipid molar ratio of 2.6×10^{-2}:1, the pairwise transfer rate has increased to 3×10^{8} sec^{-1}, but even then the probability of transfer to a nonfluorescent chlorophyll dimer will be very small.

Effect of Temperature on Fluorescence. Although temperature has no effect on the wavelength of fluorescence emission, there is a marked decrease in fluorescence intensity when the temperature drops below that of the liquid crystalline to gel phase transition. This is shown by the data in Figure 4, for chlorophyll a present at a chlorophyll/lipid molar ratio of 1:430 in bilayers of dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, and dimyristoylphosphatidylethanolamine. The temperature of the gel to liquid crystalline phase transition for dioleoylphosphatidylcholine is −22°C (Phillips et al., 1972), and there is relatively little effect of temperature on the fluorescence of chlorophyll a in this lipid. There is a slight decrease in fluorescence intensity below ca. 25°C, which could correspond to the reported onset of cluster formation in dioleoylphosphatidylcholine at ca. 30°C (Lee et al., 1974): although the change in fluorescence intensity below 25°C is only slight, it is in contrast to the usual observation of an increase in fluorescence as the temperature is lowered, due to a decrease in the rates of collisional quenching and other processes (Guilbault, 1973).

In bilayers of dipalmitoylphosphatidylcholine at a chlorophyll/lipid molar ratio of 1:430, there is a marked drop in fluorescence intensity at 40.5°C. This agrees well with the phase transition temperature of 41.75°C found by calorimetry (Hinz and Sturtevant, 1972) and agrees exactly with that detected by the change in the partitioning of the small molecule Tempo[1] (I) (Metcalfe et al., 1972; Shimshick and McConnell, 1973). The reduction of fluorescence intensity for chlorophyll a at this temperature can be interpreted as a partial exclusion of the freely dispersed, monomeric chlorophyll from the crystalline lipid region, with the formation of segregated regions of nonfluorescent (aggregated) chlorophyll. Two observations show that this decrease in fluorescence is not due to extrusion of chlorophyll out of the lipid bilayer with the formation of a nonfluorescent chlorophyll colloid in the aqueous phase. Firstly, the changes of fluorescence intensity with temperature are fully reversible. Secondly, no fluorescence develops even after several hours when lipid dispersions (sonicated or unsonicated) are mixed with (nonfluorescent) chlorophyll a dispersions in water.



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Immediately below this sharp transition, there is still an appreciable amount of monomeric, fluorescent chlorophyll in the lipid bilayer. This is almost totally eliminated by a second transition occurring at lower temperature, with a greater width than the primary transition. The temperature of this secondary transition in dipalmitoylphosphatidylcholine is ca. 29°C, again corresponding well to that which is observed in Tempo partition studies (29.5°C), and to the "pretransition" observed calorimetrically (34°C).

The fluorescence intensity in dimyristoylphosphatidylethanolamine at any given lipid/chlorophyll ratio is about a half that observed in dipalmitoylphosphatidylcholine when both are in the liquid crystalline phase. It has also been observed that the amount of Tempo partitioning into dipalmitoylphosphatidylethanolamine is half that partitioning into dipalmitoylphosphatidylcholine (Shimshick and McConnell, 1973). The phase transition for dimyristoylphosphatidylethanolamine is broader than that observed for dipalmitoylphosphatidylcholine, and is centered at ca. 46°C. The phase transition temperature detected calorimetrically is 48°C (Ladbrooke and Chapman, 1969).

Although the chlorophyll a fluorescence accurately monitors the phase transition when the chlorophyll is present at very low concentrations in the bilayer, at higher concentrations it appears to broaden the transition and shift it to lower temperatures. As shown in Figure 5, as the ratio of chlorophyll a to dipalmitoylphosphatidylcholine increases, the pretransition at 29°C also becomes less distinct. At a chlorophyll/lipid ratio of 1:77, the transition detected by chlorophyll a fluorescence has broadened, and occurs over the range 42–31°C. Nicholls et al. (1974) have reported

[1] Abbreviation used is: Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

that incorporation of chlorophyll b into liposomes of dipalmitoylphosphatidylcholine also broadens the phase transition.

Chlorophyll a in Lipid Mixtures. It appears that at chlorophyll/lipid molar ratios less than ca. 1:100, chlorophyll a fluorescence accurately detects the temperature of the lipid phase transition. Figure 6 shows fluorescence intensity as a function of temperature for chlorophyll a in mixtures of dioleoylphosphatidylcholine and dipalmitoylphosphatidylcholine at a chlorophyll/lipid molar ratio of 1:790. With increasing amounts of dioleoylphosphatidylcholine, the apparent "transition" broadens and shifts to lower temperature. The overall shape of the fluorescence-temperature plots are again remarkably similar to the Tempo partition-temperature plots for similar mixtures (Lee et al., 1974).

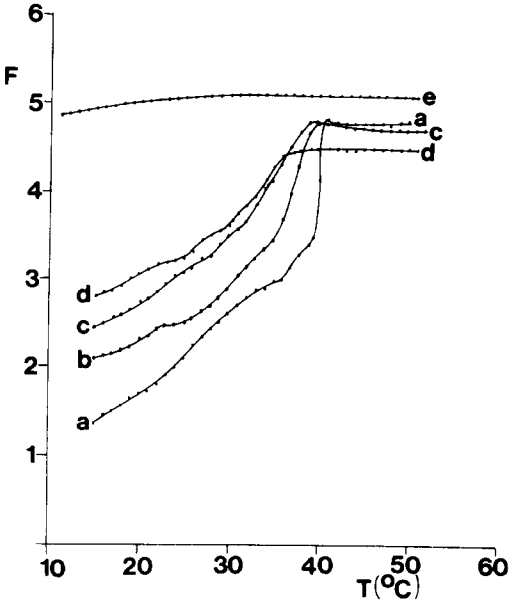

FIGURE 6: Fluorescence intensity of chlorophyll a vs. temperature at a chlorophyll/lipid molar ratio of 1:790, in (a) dipalmitoylphosphatidylcholine; (b) 97:3, dipalmitoylphosphatidylcholine (DPL)/dioleoylphosphatidylcholine (DOL); (c) 90:10, DPL/DOL; (d) 80:20, DPL/DOL; (e) DOL. The data for 97:3 DPL/DOL are very similar to that for 90:10 DPL/DOL above 42°C.

Discussion

The organization of chlorophyll a within the photosynthetic membrane is of profound importance in the process of photosynthesis. It has been estimated that there are some 300 chlorophyll a molecules per reaction center in chloroplasts, and chlorophyll is present at a concentration of about 0.1 *M* in the photosynthetic membrane (Duysens, 1964). It is assumed that a small proportion of this chlorophyll is associated with protein but that the bulk of the chlorophyll is "antenna" chlorophyll, organized for efficient energy transfer to the reactive center. The distribution of the bulk chlorophyll appears not to be homogeneous, since absorption spectra of chlorophyll a in photosynthetic membranes are complex and can be analyzed into several components (Cotton et al., 1974). The experiments reported in this paper are aimed at understanding the nature of antenna chlorophyll. The aggregation of the antenna chlorophyll could be caused by interaction with protein in the membrane. On the other hand, it could be due to specific chlorophyll-chlorophyll interactions within the lipid matrix of the membrane, in which case similar aggregation would be observed in simple lipid bilayers. In fact, the absorption and fluorescence experiments reported here show that chlorophyll aggregation does occur in lipid bilayers, in the absence of any protein.

Chlorophyll a and Lipids in the Liquid Crystalline Phase. Unfortunately, the location of chlorophyll within the phospholipid bilayer has not yet been conclusively established. Hoff (1974) has suggested that the plane of the porphyrin ring in chlorophyll a makes an angle of 55°C with the bilayer surface, but the porphyrin ring could project into the aqueous phase at this angle or it could be bent backwards so that it is in the fatty acid chain region. At present there appears to be no firm basis for distinguishing between these possibilities. It is interesting, however, that the molecular area of chlorophyll a at a paraffin-water interface is ca. 40 $Å^2$ at a surface pressure of 20 dyn/cm (Karan and Brody, 1974), this surface pressure in general seeming to produce a state of film molecular packing similar to that found in a liposome (Lee, 1975). With an area of the porphyrin ring of ca. 225 $Å^2$, and a molecular area of ca. 35–55 $Å^2$ measured for a monolayer of free phytol at a pressure of 25 dyn/cm (Bellamy et al., 1963), it seems rather unlikely that the chlorophyll adopts a folded configuration in these monolayers, with both the porphyrin ring and the phytol chain being directed into the paraffin phase. A more likely configuration would be with the phytol chain directed into the paraffin phase and the porphyrin ring in the aqueous phase, with the ester linkages of the molecule at the paraffin-water interface.

Whatever the location of the chlorophyll within the bilayer, the question of the state of aggregation remains. As shown here, absorption spectra of chlorophyll a in lipid bilayers in the liquid crystalline phase show several components, reminiscent of those observed in organic solvents and in photosynthetic membranes (Cotton et al., 1974). The position of the red band in oligomeric species will depend on the interactions between the dihydropyrrole rings in the aggregate. The fact that no absorption band is seen at 740 nm as for chlorophyll a colloids (Ballschmiter and Katz, 1969) presumably reflects the structural constraint imposed on the chlorophyll by the lipid bilayer.

While the appearance of an absorption band at 685 nm for chlorophyll a in dipalmitoylphosphatidylcholine supports the idea that both monomeric and oligomeric forms of chlorophyll a are present, the spectra do not allow any more than a qualitative interpretation. In contrast, fluorescence spectroscopy is, in general, very sensitive to the formation of dimers or higher aggregates (Guilbault, 1973). The studies reported here show that there is a very marked concentration quenching for chlorophyll a in lipid bilayers. This fluorescence quenching cannot be fitted to a collisional deactivation model, or to a model in which chlorophyll a molecules are randomly distributed throughout the bilayer, with pairs of chlorophyll molecules closer than some interaction distance being nonfluorescent. Rather, the data are consistent with the formation of nonfluorescent dimers and oligomers in lipid bilayers, both in the gel and the liquid crystalline state. Fluorescence quenching in the dipalmitoylphosphatidylcholine liposomes at 50°C with relatively small proportions of chlorophyll can be fitted to dimer formation, with an equilibrium constant of ca. 600 l. mol^{-1}. It seems likely that the fluorescent chlorophyll a species is monomeric Chl·H_2O, and that nonfluorescent species are formed by aggregation to give dimers and oligomers. A possible structure for the oligomer is that given by Strouse (1974), based on a structure determination of ethyl chlorophyllide a dihy-

drate with intermolecular hydrogen bonds linking the ketone oxygen of one chlorophyll to the coordinated water molecule of another, to form a one-dimensional chain. The relative proportions of monomeric and oligomeric chlorophyll a present in the lipid bilayers seem to depend on the fluidity or packing density of the lipid. Thus the proportion of monomeric chlorophyll appears to be slightly higher in dioleoylphosphatidylcholine than in dipalmitoylphosphatidylcholine. This is in agreement with the looser packing and greater fluidity of the former lipid (Lee, 1975), and is also in agreement with the greater partitioning of the small molecule Tempo (Lee et al., 1974). These effects are much more marked in dimyristoylphosphatidylethanolamine, where the amount of monomeric chlorophyll is about half that in the phosphatidylcholines. Again this is in agreement with studies which show that the packing density is greater in phosphatidylethanolamines (Shimschick and McConnell, 1973; Michaelson et al., 1974). This can probably be attributed to a strong interaction between the phosphatidylethanolamine head groups, with the positively charged amine group of one molecule interacting electrostatically with the negatively charged phosphate group of an adjacent molecule.

Thermal Effects. For chlorophyll a in dioleoylphosphatidylcholine, there is a slight, but experimentally significant, decrease in fluorescence intensity below ca. 25°C, perhaps corresponding to the onset of cluster formation at ca. 30°C as detected by the decrease in partitioning of the spin-label Tempo at this temperature (Lee et al., 1974). The lipid in such clusters would have a higher mean molecular density and a lower fluidity, so that the "solubility" of chlorophyll a would be less, and hence the amount of monomeric, fluorescent chlorophyll would decrease relative to the amount of oligomeric, nonfluorescent chlorophyll. In dipalmitoylphosphatidylcholine, much more marked effects are seen, because of the gel to liquid crystalline phase transition found calorimetrically to be at 41.75°C, with a "pretransition" at 34.0°C (Hinz and Sturtevant, 1972). Both of these transitions are reflected by a decrease in fluorescence intensity for chlorophyll a incorporated into the lipid at low molar ratios. At higher chlorophyll/lipid molar ratios (>1:100), the pretransition becomes indistinct, and the main transition broadens and shifts to lower temperature. A similar effect has been observed calorimetrically with chlorophyll b (Nicholls et al., 1974). The pretransition is probably associated with a change in configuration for the choline head group, from a folded configuration in the gel phase to a straightened form in the liquid crystalline phase (Lee, 1975). The marked effect of chlorophyll a on this transition at very low chlorophyll concentrations would be consistent with a location for the chlorophyll with the chlorine ring in the head group region, projecting out into the aqueous phase, with the ester linkage in the glycerol backbone region of the bilayer.

In mixtures of dioleoylphosphatidylcholine and dipalmitoylphosphatidylcholine, the chlorophyll a fluorescence monitors the average "fluidity" of the bilayer as detected by Tempo partitioning (Lee et al., 1974). In this it is unlike cholesterol (De Kruyff et al., 1974) or spin-labeled fatty acids (Oldfield et al., 1972), which preferentially associate with the unsaturated lipid in mixed bilayers when both liquid crystalline, unsaturated lipid, and crystalline, saturated lipid are present. If in these lipid mixtures, the chlorophyll were to segregate into pools of fluid unsaturated lipid, then the chlorophyll concentration in these pools would be high, and so concentration quenching would be more marked than in pure dipalmitoylphosphatidylcholine. Since this is not observed, there can be no preferential association of the chlorophyll with either of the liquid crystalline or crystalline lipids.

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