Absorption and Picosecond Fluorescence Characteristics of Chlorophyll Vesicles as a Function of Temperature

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With chlorophyll *a*-dipalmitoylphosphatidylcholine-liposomes, the absorption increases at 706 and 450 nm, and decreases at 660 and 420 nm, as the temperature is lowered. As the temperature is increased opposite changes are observed. A lipid phase change occurs at 34°. The pigment to lipid ratio is 1 to 5 in the liposome.

With chlorophyll *a*-soy bean lecithin-liposomes the absorption increases at 706, 680 and 440 nm, and decreases at 650 and 430 nm, as the temperature is lowered. As the temperature is increased, opposite changes are observed. A lipid phase change occurs at 26–27 °C. The pigment to lipid ratio is 1 to 13. The spectral change at 706 nm is identified with aggregated chlorophyll. The concentration of chlorophyll aggregate increases as the temperature is lowered, and decreases as the temperature is raised.

Fluorescence decay from chlorophyll *a*-soy bean lecithin-liposomes is biphasic. The lifetimes of freshly prepared liposomes are 121 ± 4 ps and 1400 ± 200 ps. The relative contribution of the fast and slow fluorescence components are modified by temperature. Heating results in an increase in both lifetimes, and an increase in fluorescence from the long lived component. These changes are interpreted as resulting from a decrease in energy transfer and concentration quenching. The origin of the biphasic fluorescence and spectral transformations in liposomes, and the possible relation between *in vitro* and *in vivo* picosecond fluorescence is discussed.

Introduction

Varying the temperature of chloroplasts or intact algae results in changes in absorbance in specific spectral regions. Brody and Singhal [1] reported that in chloroplasts, as the temperature is increased, there are decreases in absorbance at 675 nm (region of absorbance by antennae chlorophyll) and at 500 nm (region of absorption by carotene), accompanied by an increase in absorbance at 690 nm (region of absorbance by photosystem II chlorophyll). As temperature is decreased opposite changes in spectra are observed (*i.e.* 690 nm decreases, and 675 and 500 nm increase). Similar temperature dependent spectral changes are also observed at these wavelengths in *Porphyridium cruentum* and *Anacystis nidulans* [2]. Fluorescence from chlorophyll has been used as an endogenous probe to detect phase change of lipids *in vivo* [3]. The spectral properties of pigments are presumably modified as the lipids undergo a temperature induced change of phase.

Fluorescence from chlorophyll-liposomes has been used to investigate phase changes in lipids [4]. Nicholls *et al.* [5] reported temperature dependent spectral shifts with chlorophyll *b*-dipalmitoylphosphatidylcholine-liposomes. Their liposomes contained a pigment to lipid ratio of 1 to 52. In the present work, a higher pigment to lipid ratio is used. Since chlorophyll *a* is the ubiquitous photosensitizer in higher plants and algae, it, rather than chlorophyll *b*, is considered in this study. Chlorophyll *a*-liposome systems are used to help distinguish between the temperature dependent spectral changes arising from chlorophyll-lipid interactions, and those arising from chlorophyll-protein interactions. In liposomes, only interactions between chlorophylls, and between chlorophyll and lipid need be considered in interpreting temperature dependent spectral changes in absorption, or in fluorescence decay.

Materials and methods

Liposomes were formed as described previously by Brody [6]. To obtain the desired lipid to chlorophyll ratio 30 mg of lipid and an appropriate amount of chlorophyll were dissolved in 10 ml of chloroform. A film of the mixture was formed by placing 0.10 ml of the mixture in a small round bottom flask and evaporating the solvent in a gentle stream of pure nitrogen. The dried films were either

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used immediately to form liposomes or stored in a deep freezer (−20 °C) and used at a later date. To form liposomes, buffer (4 ml of 10 mM phosphate, pH 7.6) was added to the flask containing the film. The flask was immersed in an ice bath and sonicated in a stream of nitrogen for 20 min, with a Bronson sonifier at low power (a dial setting of 3).

The mole ratio of dipalmitoylphosphatidylcholine to chlorophyll used to form liposomes was 5 to 1. The mole ratio of soy bean lecithin to chlorophyll used to form liposomes was 13 to 1.

A Beckman spectrophotometer (model DW-2) was used to measure absorption spectra. The absorbance (O.D.) of the red absorption band of the liposome samples was about 0.5. Difference spectra were measured with the spectrometer set for a full scale absorbance of 0.1 or 0.05. Spectral changes were determined by measuring the difference absorption spectrum between two identical liposome suspensions. The reference sample was held at room temperature (26 °C) while the temperature of the sample was varied between 4° and 44 °C. The difference in absorption spectra between a reference sample held at room temperature (26 °C) and the sample at some other temperature (t °C) is indicated as OD (t °, λ) − OD (26 °, λ).

When both samples are at the same temperature (26 °C) the spectrophotometer records a small difference in absorption. This base line is subtracted from the difference spectrum by measuring the changes in absorbance relative to the absorbance at a wavelength where there is little or no absorption by chlorophyll (*e.g.* 560 and 760 nm). Usually a wavelength longer than 750 nm is satisfactory, however, with liposomes formed from chlorophyll and dipalmitoylphosphatidylcholine the absorption spectrum extends far into the red. At 760 nm there still appears to be absorption. Furthermore, as will be shown in the results absorbance at 760 nm, relative to the absorbance at 560 nm, is modified by the variation in temperature used in these experiments. Consequently, the minimum of absorption observed at 560 nm is used as the reference point from which to measure changes in absorption and correct the spectra for the base line, OD (t °, 560) −OD (26 °, 560), *i.e.* ΔOD (t °, λ) = OD (t °, λ) −OD (26 °, λ) −OD (t °, 560) + OD (26 °, 560).

The sample cuvette was cooled or heated by circulating water, from a constant temperature bath, through the cuvette holder. Temperature of the sample was measured with a calibrated copper-constantan thermocouple immersed in the suspension of liposomes. The rates of cooling and heating were 1.6 ° and 1.3 °C/min, respectively.

The spectral changes in particular regions of the spectrum are identified with specific pigment systems. For this work the change in absorbance at 704 nm (ΔOD (704)) is identified with aggregated chlorophyll *a*; the ΔOD (673) and ΔOD (440) are identified with monomeric chlorophyll.

The instrumentation used in the present work to resolve picosecond fluorescence was previously described in detail [7, 8]. A train of pulses was generated by a mode-locked Nd^{3+} glass laser. A frequency doubler gave 530 nm pulses. An electro-optic shutter selected a single, 6 ps pulse. Fluorescence from the sample passed through a filter into an Imacon 600 streak camera (John Hadland P.I. Ltd., England). The fluorescence streak trace was detected by a 500 channel optical multichannel analyzer (OMA 1205 A and B, Princeton Applied Research, N.J.) and stored in its digital memory. Data in the digital memory were transferred directly into a Data General computer where data reduction was carried out (summation of curves, curve fitting and graphing of results). Excitation intensity was less than 2×10^{14} photos cm^{-2} to avoid annihilation effects. A least squares type computer program was used to find the

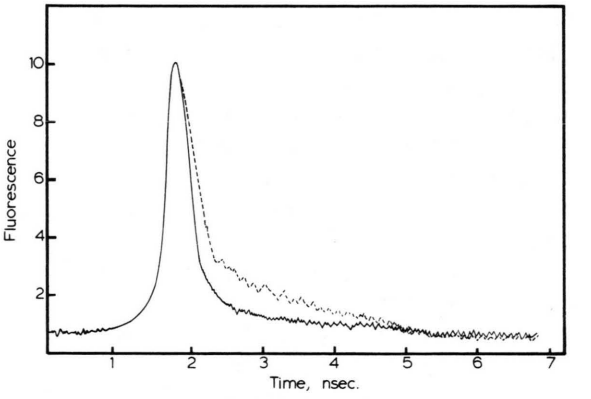

Fig. 1. Fluorescence as a function of time from chlorophyll *a*-soy bean lecithin-liposomes. The fluorescence decay at 23 °C from freshly formed liposomes is shown by a solid (——) line. The fluorescence decay from liposomes formed from films of chlorophyll *a* and soy bean lecithin that were stored for 5 days at −20 °C, is shown as a broken line (- - - - -). Fluorescence is sensitized with a 6 psec laser pulese. The ratio of chlorophyll to lecithin used to form the liposomes is 1 to 13. The liposomes were suspended in 10 mM phosphate buffer, pH 7.6.

best fit for the summed fluorescence decay to the equation $y(t) = A_1 \exp -t/\tau_1 + A_2 \exp -t/\tau_2$, where τ is the fluorescence lifetime and A_1 is the relative fluorescence intensity of the slow component and A_2 the relative intensity of the fast component. This analysis is not meant to show that the fluorescence decay is composed of only two exponential decays. Each fluorescence decay reported in this paper represents the average of 9 to 12 experiments. As in previous picosecond fluorescence studies [7, 8] no corrections are made for the fact that polarized flight is used to excite fluorescence.

The lipid L – α dipalmitoylphosphatidylcholine was obtained from Sigma Chem. Co. (St. Louis, Mo.), soy bean lecithin was obtained from Applied Physics (State College, Pa.). Both were used without further purification. Chlorophyll *a* was isolated from spinach and purified as described previously [9]. DCMU is 3-(3,4-dichlorophenyl)-1,1-dimethyl urea.

Table I. Fluorescence properties of chlorophyll a-soy bean lecithin-liposomes.

Sample	Temp. [°C]	A_1/A_2	τ_1,ps	τ_2,ps
Fresh liposomes	23	.048±.009	1400±200	121± 4
	23–39	.19 ±.05	1548±140	208±15
	23–39–0	.21 ±0.1	1621± 80	178±10
5 Day old film*	23	.53 ±.02	776±30	216±20
	23–39	.37 ±.01	758±20	103± 8
	23–39–0	.28 ±.02	898±20	114± 8
	23–0	.29 ±.05	932±60	147±30

A_1 is the relative intensity of the slow fluorescence component.
A_2 is the relative intentsity of the fast fluorescence component.
* Liposomes were formed from chlorophyll-lipid films stored 5 days in darkness, at –20 °C

Results

A. Picosecond fluorescence

Fluorescence decay from chlorophyll *a*-soy bean lecithin-liposomes can not be described by a single exponential. The fluorescence decay appears to be biphasic (Fig. 1). For freshly prepared liposomes the lifetime of the slow fluorescence component at 23 °C is 1400 ± 200 ps, the lifetime of the fast component is 121 ± 4 ps and the ratio A_1/A_2 is 0.048 ± 0.009 (Table I). Increasing the temperature from 23 ° to 39 °C results in a rather large increase in the relative intensity of the slow fluorescence component A_1. The lifetimes of both the slow and the fast fluorescence components are also increased. Subsequent cooling from 39 °C to 0 °C results in only minor changes (see Table I).

The lifetimes and relative intensities of the fluorescence components from chlorophyll-liposomes are very sensitive to the age of the films used to form liposomes. The lifetime of the slow fluorescence component from fresh liposomes is significantly longer, than the lifetime from liposomes formed from chlorophyll-lipid films stored for 5 days at –20 °C (the latter are referred to as '5-day old film' liposomes). Furthermore, the fluorescence contribution of the slow component from '5 day old film' liposomes is larger than from fresh liposomes (see Fig. 1 and Table I). Heating or cooling the '5 day old film' liposomes results in a decrease in the relative contribution of the slow component and modifies the fluorescence lifetimes. The fluorescence lifetimes and their relative intensities, under various conditions, are listed in Table I.

B. Difference spectroscopy with dipalmitoylphosphatidylcholine liposomes

The red absorption band of the chlorophyll liposome is rather broad, extending into the red region of the spectrum, well beyond 700 nm (Fig. 2). Maxima are observed at 674 and 440 nm.

Cooling results in progressive spectral changes. At 4 °C the difference spectrum has maxima at 692 and 448 nm, plus minima at 656 and 430 nm. The graph ΔOD (4 °, λ) is shown in Fig. 2. The maximum percent change in absorbance (ΔOD/OD) is 0.7% at the red absorption maximum, and 0.4% at the blue maximum.

Heating results in an opposite set of progressive spectral changes. At 41 °C the difference spectrum has minima at 696 and 440 nm, plus a maximum at 668 nm. The graph of ΔOD (41 °, λ) is shown in Fig. 2. The maximum percent change in absorbance is 4% at the red absorption maximum, and 3.5% at the blue absorption maximum.

There appears to be a fast and slow time dependent change in the spectra. The fast change takes

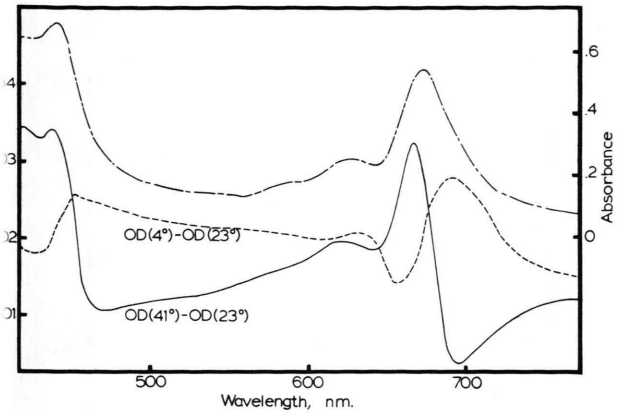

Fig. 2. Absorption spectrum of chlorophyll *a*-dipalmitoylphosphatidylcholine-liposomes is shown by the upper curve (- · · ·). Absorbance is given by the scale on the right. The difference in absorption spectra between two samples, one at 41 °C and the other at 23 °C, is indicated as OD (41 °) – OD (23 °), (and a solid line). Scale is on the left. The difference in absorption spectra between two identical samples, one at 4 °C and the other at 23 °C, is indicated as OD (4 °) – OD (23 °), (and a broken line). Divide the absorbance indicated on the left scale by 4, to obtain the correct change in absorbance at 4 °C. The pigment to lipid ratio is 1 to 5 in liposomes, and the liposomes are suspended in 10 mM phosphate buffer, pH 7.6.

less than one minute (the time required to run a complete spectrum). The slow spectral change takes several minutes.

Graphs of ΔOD (λ) verses temperature are shown in Fig. 3. Changes in spectra are measured relative to the absorbance at 560 nm. While there are no maxima, there are changes in the slope at about 34 °C (*i.e.* the second derivatives of ΔOD (λ) with respect to temperature have minima at around 34 °C). When changes in absorbance are measured relative to the absorbance at 760 nm, maxima are observed for ΔOD (683) and ΔOD (446) at about the same temperature (*i.e.* 34 °C). These spectral characteristics at about 34 °C are interpreted as signaling the lipid phase change. Only the heating cycle from 4 ° to 41 °C is shown in Fig. 3. Neither the cooling cycle from 26 ° to 4 °C nor the cooling cycle from 41 ° to 4 °C follows the same path. There is a "hysteresis-like" effect. A similar effect is observed with intact cells (a red and blue-green alga) [2] and chloroplasts [1].

The baseline used to correct the spectra is observed to change with temperature. On cooling, there is a small decrease in the absorbance at 760 nm relative to the absorbance at 560 nm. On heating the opposite is observed.

C. *Difference spectroscopy with soy bean lecithin*

The absorption spectrum of the chlorophyll-liposome has maxima at 673 and 440 nm (Fig. 4). Cooling from 23 ° to 7 °C gives rise to changes in the difference spectra. There are maxima at 704, 673, and 450 nm, plus minima or decreases in absorbance at 660 and 434 nm. The graph of ΔOD (7 °, λ) is shown in Fig. 4. The maximum percent change in absorbance at the red and blue absorption maxima are 1.4% and 2.0%, respectively.

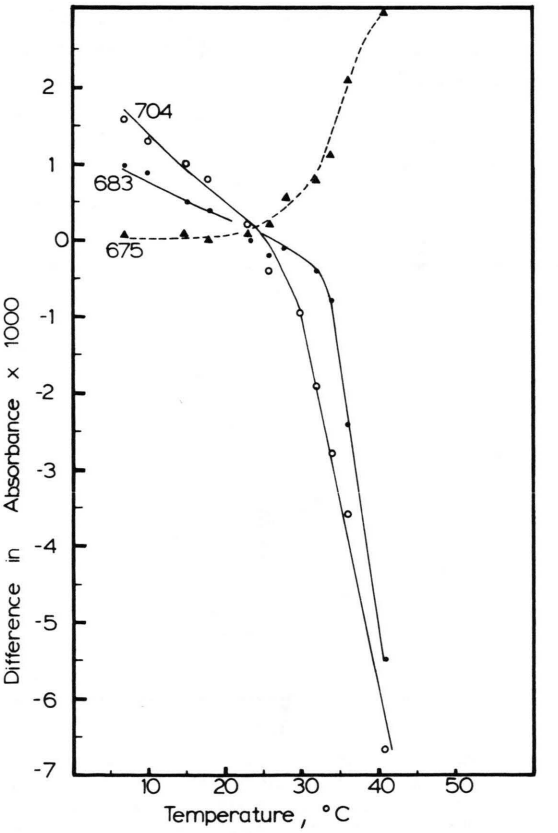

Fig. 3. Change in absorbance as a function of temperature with chlorophyll *a*-dipalmitoylphosphatidylcholine-liposomes. The change at 704 nm is indicated by open circles (–○–○–); the change at 683 nm is indicated by solid circles (–●–●–); the change at 675 nm is indicated by a broken line and triangles (△–△–△). Pigment-lipid ratio and solution are the same as in Fig. 2.

Heating results in opposite spectral changes, as shown by the graph of ΔOD (39 °, λ) in Fig. 4. At 39 °C there are minima at 704, 680, 673 and 448 nm, plus a maximum at 657 nm. The red absorption maximum undergoes a small blue shift on heating. The blue absorption maximum undergoes a red shift. The maximum percent change in absorbance at the red and blue absorption maxima are 1.3% and 2.3%, respectively.

The decreases in absorbance at 673 and 448 nm (Fig. 4) corresponds to the main absorption bands of chlorophyll in the liposome. The long wavelength band at 686 nm observed in Fig. 4 probably corresponds to an absorption band of an aggregated form of chlorophyll that is not resolved in the absorption spectrum.

Graphs of absorbance ΔOD (λ) versus temperature at 676, 657 and 434 nm show maxima at 26–27 °C (Fig. 5). A maximum is observed at this temperature, whether changes in absorbance are measured relative to absorbance at 760 or at 560 nm. The temperature maximum in Fig. 5 is interpreted as signaling the lipid phase change.

The ΔOD (704) decreases upon heating and increases on cooling. In contrast to the other wavelengths in Fig. 5, the ΔOD (704) does not have a maximum or minimum as a function of temperature.

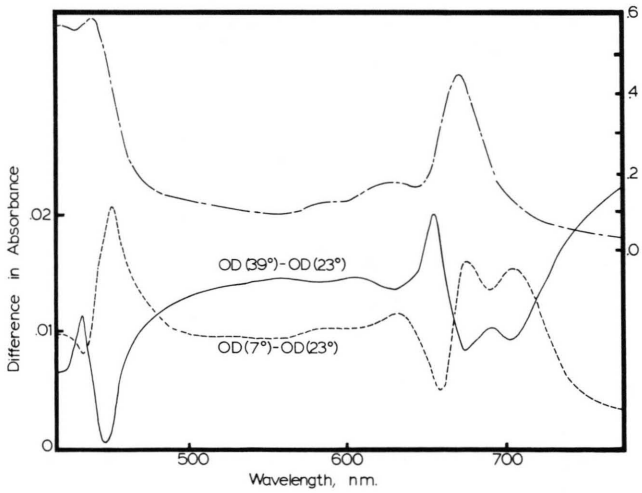

Fig. 4. Absorption spectrum of chlorophyll *a*-soy bean lecithin-liposomes is shown by the upper curve. Absorbance is given by the scale on the right. The difference in absorption spectra between two identical samples, one at 7 °C and the other at 23 °C, is indicated as OD (7 °) – OD (23 °), (and a broken line). Absorbance is given by the scale on the left. The difference in absorption spectra between two samples, one at 39 °C and the other at 23 °C is indicated as OD (39 °) – OD (23 °), (and a solid line). The ratio of pigment to lipid is 1 to 13, and the liposomes are suspended in 10 mM phosphate buffer, pH 7.6.

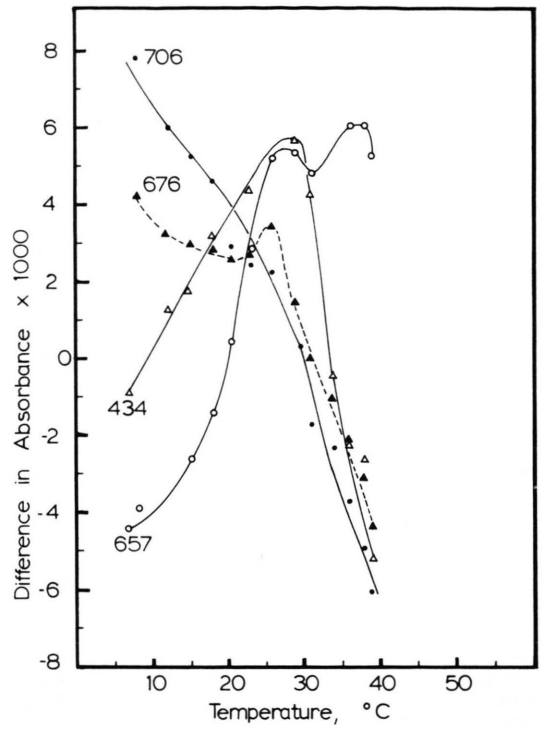

Fig. 5. Change of absorbance of chlorophyll *a*-soy bean lecithin-liposomes on heating from 7 °C to 40 °C. The change at 706 nm is indicated by solid circles (–●–●–), the change at 676 nm is indicated by solid triangles (–▲–▲–), the change at 434 nm is indicated by open triangles (–△–△–), the change at 657 nm is indicated by open circles (–○–○–). The pigment-lipid ratio and solution is the same as in Fig. 4.

As the temperature is changed it is observed that the baseline changes. On heating there is an increase in the difference between the absorbance at 760 nm and the absorbance at 560 nm. On cooling there is a decrease in the difference between the absorbance at 760 and at 560 nm. At 26–27 °C there is a relatively large increase in absorbance at 760 nm, relative to the absorbance at 560 nm. This change in baseline is much larger than that observed with the chlorophyll-dipalmitoylphosphatidylcholine-liposomes (described in the section above), at the same wavelengths. The exact temperature of the maxima in Fig. 5 may be shifted slightly because of this change in the baseline. The large change in baseline coupled with the fact that the exact spectral dependence of the change in baseline is not determined, makes it difficult to quantitatively compare changes in difference spectra over a wide spectral range.

Discussion

It is observed that protein-free, liposome systems exhibit biphasic fluorescence decay from chlorophyll, similar to that reported from chlorophyll in chloroplasts. This fact invites a reconsideration of earlier interpretations of the mechanism underlying the biphasic fluorescence decay from chloroplasts. When the concentration of chlorophyll to lecithin in vesicles is kept low, to prevent self quenching of fluorescence, the fluorescence from chlorophyll has a single exponential decay with a lifetime of 5.1 nsec [10]. This lifetime is similar to the fluorescence lifetime of chlorophyll in a solution of anhydrous diethyl ether, which is 5.1 nsec [10, 11]

The fluorescence (F_M level) from chlorophyll in chloroplasts is biphasic with lifetimes reported to be 413 ps and 1463 ps [12]. Beddard *et al.* [12, 13] discussed the possible origins of the biphasic fluorescence *in vivo*. They suggest it may result from an intrinsic time dependence in the probability of emission. This can give information on the mechanism of energy transfer. The biphasic fluorescence can also arise from inhomogeneity of the emitting species. For example, there may be different quenching probabilities in different physical regions of the chloroplast or photosynthetic unit, thus giving structural information. They suggested that concentration quenching *in vivo*, shortens the fluorescence lifetime of chlorophyll to 1500 ps (from the 5 nsec observed in solution). Energy transfer decreases the lifetime still further.

The short fluorescence lifetime is interpreted as arising from exciton quenching by 'open' traps in photosynthetic units. The fluorescence is from chlorophyll *a* antenna of photosystem I, and light harvesting photosystem II pigment combinations that are strongly coupled to photosystem I antenna chlorophyll. The long fluorescence lifetime is interpreted as arising from excitation in photosynthetic units with 'closed' traps. The fluorescence is ascribed to light harvesting photosystem II pigment combinations not coupled with antenna of photosystem I [14]. The proportion of closed traps to open traps gives the ratio of the intensities of the long to short lived fluorescence components [12].

With fresh liposomes, the lifetimes of the biphasic fluorescence are about 1400 and 120 psec. These lifetimes are similar to those reported from chloroplasts in the presence of DCMU. One can not invoke the concept of 'open' and 'closed' reaction or fluorescence centers (as done *in vivo*) to account for the biphasic fluorescence from liposomes. Furthermore, the biphasic characteristic of fluorescence from chlorophyll in liposomes does not depend on different chlorophyll–protein interactions, as no protein is present. The biphasic fluorescence can only arise from interactions of chlorophyll with its environment (*i.e.* neighboring chlorophylls, lipids, water). A modification of chlorophyll-lipid interaction with temperature is indicated by the temperature dependent changes in the lifetimes and relative intensities of the two fluorescence components (Table I).

The biphasic fluorescence in liposomes may reflect fluorescence from different forms of chlorophyll (monomers and aggregates), or fluorescence from a single form of chlorophyll in two different chemical environments. By analogy with the arguments put forth by Beddard *et al.* [12, 13] the biphasic fluorescence from liposomes may arise as follows. The nonexponential fluorescence decay indicates that the chlorophylls have distributed themselves in at least two different regions of the liposome. Some aspects of the asymmetric nature of the liposome is discussed below. The fast fluorescence lifetime may result from energy transfer from monomeric chlorophyll to non-fluorescent, aggregated chlorophyll. The long fluorescence liftetime may result from concentration quenching.

The change in A_1/A_2, and lifetimes may occur from temperature dependent changes in fluorescence yields and energy transfer (to non-fluorescent pigments). These changes could come about from lipid phase change and ensuing change in distribution of pigments in the liposome (see below). As indicated by increases in A_1/A_2 and both life times, it would appear that heating fresh liposomes results indecreases in both concentration quenching and energy transfer. From spectral measurements, it appears that heating results in a decrease in concentration of aggregated chlorophyll (non-fluorescent chlorophyll) (Figs. 2 and 4). A decrease in concentration of nonfluorescent aggregates, which act as an energy trap, would tend to increase the fluorescence lifetime.

A lipid phase transition in *Anacystis nidulans* results in a lateral and vertical displacement of the integral membrane protein complexes [15]. The result is to redistribute and concentrate lipoproteins in the lipid membrane. Lateral displacement occurs by exclusion of membrane protein from liquid crystal do-

mains. A similar redistribution of aggregated and monomeric chlorophyll (nonfluorescent and fluorescent forms, respectively) might occur in liposomes as a function of temperature. Pigment aggregates may be partly squeezed out of liquid crystal regions by increased lateral pressure on the aggregated chlorophyll, exerted by the expanding regions of lipids in the gel phase. The redistribution may simply be determined by the different solubility and orientation properties of chlorophyll in the different states of the lipid. Vertical displacement could also change the microenvironment of the chlorophyll aggregates, thereby altering their configuration and consequently their spectral properties.

In general, when chlorophyll-lipid films are stored for a length of time before being used to form liposomes, it can result in pheophytinization or oxidation of lipids [6]. It should be noted, however, that in the present work no spectral evidence of pheophytin was detected with the '5 day old film' liposomes.

Based on the data in Fig. 5 there appears to be a phase transition in the chlorophyll-soy bean lecithin-liposome at 26–27 °C. The transition is manifested by maxima in the curves for OD (676, 567, 434) verses temperature (Fig. 5), and change in scattering properties of the liposome.

There is a phase transition in the chlorophyll-dipalmitoylphosphatidylcholine liposomes at about 34 °C. The transition is indicated by a sharp change in absorbance of the band at 685 nm (Fig. 3). Liposomes formed with the pure lipid have a phase change at 41 °C [16]. It has been shown that the presence of increasing concentration of pigment in the lipid, lowers and broadens the temperature of the lipid phase change [4].

A phase change of lipids could give rise to several effects, *e.g.* change in solubility of pigment, change in surface pressure, change in pigment-lipid interaction and perhaps diameter of the liposome. A change in the diameter of the liposome could have many ramifications. With the small diameter liposomes (250 Å) used in this work, there is a (15%) difference in area between the outer and inner shell of the bimolecular sphere. This difference in area can lead to asymmetry in molecular packing. A change in size of the sphere could alter the asymmetry. The asymmetry and heterogeneity of the liposomes could result in a wide range of pigment–pigment, pigment–water, pigment–lipid interactions. Pigment interaction determines the spectral properties. The broad, absorption bands of the liposomes is indicative of the existence of a wide range of weak and strong pigment interactions (Figs. 2 and 4). Any change in asymmetry of the liposome could modify the pigment interactions. A temperature induced change in size of the sphere would modify the asymmetry and consequently the pigment interactions.

Some of the spectral changes shown in Fig. 2 could be brought about by a temperature dependent change in the index of refraction of the lipid. The position of the absorption maxima of a pigment in solution is a function of the index of refraction and dielectric constant of the solvent. Since the chlorophyll is dissolved in or associated with the lipid phase, any temperature dependent change in index of refraction would result in a shift in absorption spectrum.

Liposomes are formed at low temperature (0 °C), where lipids are in the gel phase. Interaction between chlorophyll and lipid in the liposome is limited or determined by the phase of the lipid. Once the lipid undergoes a phase change new chlorophyll interactions (with lipid or chlorophyll) are possible.

In the liquid crystal phase, the spectral changes appear to occur as rapidly as the temperature is changed and the spectrum can be recorded. There is greater mobility of the pigment of lipid in the liquid crystal phase, than in the gel or solid-like phase. The time dependence for the change in absorption spectrum in the gel phase may be related to the time required for the pigments to reorient in the lipid bilayer.

The temperature induced spectral changes are partially irreversible. This would indicate that some of the temperature induced changes in chlorophyll interactions (with lipids, water and other chlorophylls) are irreversible.

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