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TIME-DEPENDENT DECAY AND ANISOTROPY OF FLUORESCENCE FROM DIPHENYLHEXATRIENE EMBEDDED IN THE CHLOROPLAST THYLAKOID MEMBRANE

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The hydrophobic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene has been incorporated into the membranes of isolated thylakoids, separated granal and stromal lamellae and aqueous dispersions of extracted thylakoid galactolipids. Time-resolved fluorescence decays have been recorded on a nanosecond scale using single-photon counting in order to assess the motional properties of the probe. All the experimental systems used showed biphasic decay kinetics and the anisotropies of the decays have been interpreted in terms of a model for wobbling diffusion confined to a cone. The analysis has given information about dynamic and structural restraints of the lipid acyl chains. In the intact thylakoid membrane the degree of order of the fatty acid acyl chains is higher and their rate of motion slower than for isolated lipids. Even so, the dynamic and structural parameters indicate that the thylakoids can be considered as a relatively fluid membrane system when compared with many other biological membranes, a property which is probably required to facilitate efficient long-range diffusion of lipophilic mobile electron-transport components. It is suggested that the optimization of thylakoid fluidity is linked to regulation of the membrane protein/lipid ratio which is also likely to be responsible for the higher fluidity of stromal membranes relative to those of the grana.

Introduction

There is every reason to believe that the fluidity of the chloroplast thylakoid plays an important role in controlling electron-transport processes which take place within this membrane [1,2]. For this reason we have been using the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene, to estimate the fluidity of the thylakoids under different conditions [3–5]. This probe is a rod-shaped polyene hydrocarbon with a stable all-*trans* configuration which readily partitions into the lipid matrix of membranes [6]. The transition dipole of its longest absorption band and its fluorescence lie close to parallel to the long axis of the molecule so that the fluorescence depolarization reflects almost exclusively the angular displacement of this axis. Diphenylhexatriene has a thickness which approximates to one acyl chain of a lipid molecule so that each probe molecule presumably displaces one acyl chain in the bilayer structure and is in motion around an axis which is perpendicular to the plane of the membrane.

In our earlier publications [3–5] we have measured the steady-state polarization of the diphenylhexatriene emission. As long as care is taken to avoid artefacts due to energy transfer from diphenylhexatriene to chlorophyll [3], the approach has been useful but unfortunately gave no information about the heterogeneity of the system and could not be used to estimate microviscosities using the Perrin equation [7] as originally assumed [6]. A

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Abbreviation: PS, photosystem.

much more satisfactory approach is to obtain time-resolved fluorescence measurements so as to estimate lifetimes and anisotropy values. This information is extremely useful and can be analysed to obtain parameters relating both to dynamic friction (microviscosity) and to static orientation constraint (lipid ordering). The results and analysis of this type of experiment for thylakoid membranes are reported in this paper.

Theory

With steady-state fluorescence, the polarization (p_s) and anisotropy (r_s) are determined by measuring the intensity of emission through an analyzer oriented parallel ($F_{\parallel}$) and perpendicular ($F_{\perp}$) to the direction of polarization of the excitation light using the following expressions:

$$p_s = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}} \qquad r_s = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + 2F_{\perp}} \tag{1}$$

where:

$$r_s = \frac{2p_s}{3 - p_s} \tag{2}$$

The parameters p_s and r_s are related to the time-dependent polarization (p_t) and anisotropy (r_t) observed after giving a very short excitation pulse. For example for r_s:

$$r_s = \int_0^{\infty} r_t F_T(t)\, dt \Big/ \int_0^{\infty} F_T(t)\, dt \tag{3}$$

in which the total fluorescence intensity $F_T(t) = F_{\parallel}(t) + 2F_{\perp}(t)$ has been introduced and the assumption made that the decay follows the simple exponential low $F(t) = A \exp(-t/\tau)$ where τ is the fluorescence lifetime.

After giving the excitation pulse, the initial fluorescence anisotropy (r_0) will be large but due to the rotation (wobbling) of the probe the time-dependent anisotropy will decrease exponentially towards zero:

$$r(t) = r_0 \exp(-t/\phi) \tag{4}$$

where t is time and ϕ the rotational correlation time for the reorientations of the long molecular axis [9].

In the case of diphenylhexatriene, Eqn. 4 does hold for certain reference oils but for biological membranes a time-limiting anisotropy value (r_∞) is reached because the probe does not assume all possible orientations with equal probability after a prolonged time period [8–13]. That is to say, the final distribution of the emitting dipole is anisotropic and r_∞ is a measure of the structural order of the membrane. Thus, a modified form of Eqn. 4 is required:

$$r(t) = (r_0 - r_\infty) \exp(-t/\phi) + r_\infty \tag{5}$$

Eqns. 3 and 5 can be combined to yield:

$$r_s = \frac{r_0 - r_\infty}{1 + \tau/\phi} + r_\infty \tag{6}$$

Note that Eqn. 6 is a modified Perrin equation; if $r_\infty = 0$ it reduces to:

$$r_s = \frac{r_0}{1 + \tau/\phi} \tag{7}$$

which is the classical Perrin expression.

The rotational correlation time (ϕ) obtained from Eqn. 6 is inversely related to the wobbling diffusion coefficient (D_w) of the probe molecule within its cone of rotation. The relationship includes a constant dependent on the average half-angle of rotation θ_c. The viscosity in the cone η_c, which reflects the dynamic friction against the wobbling motion [13], can be estimated using the equation:

$$\eta_c = \frac{kT}{6D_w V_e f} \tag{8}$$

where V_e and f denote the effective volume and shape factor of the probe, respectively, k is Boltzmann's constant and T absolute temperature.

The value r_∞ is a measure of the order of the lipid acyl chains within the lipid matrix. It is normally assumed that the diphenylhexatriene probe rotates in a cone which is axially symmetrical to the normal of the membrane [8–14]. Thus, the average half-angle θ_c of this cone of wobble reflects the degree of molecular packing of the lipids and can be related to r_∞ as:

$$\frac{r_\infty}{r_0} = \left[\tfrac{1}{2} \cos\theta_c (1 + \cos\theta_c)\right]^2 \tag{9}$$

The r_∞/r_0 ratio has been called the 'degree of orientational constraint' by Kinosita et al. [8] and can be related to an order parameter (S) by the following:

$$S^2 = \frac{r_\infty}{r_0} \quad (10)$$

Materials and Methods

Fresh pea chloroplast thylakoids were isolated as previously described [15] and their chlorophyll level determined by the method of Arnon [16]. Thylakoid membrane fragments, (stromal lamellae and granal stack membranes) were prepared by Yeda pressure cell treatment as described previously [5]. The incorporation of diphenylhexatriene into the thylakoid membranes and the measurement of steady-state fluorescence anisotropy values were achieved as reported previously [3,5]. Chloroplast lipids, monogalactosyldiacylglycerol and digalactosyldiacylglycerol were prepared in large quantities by high-pressure liquid chromatography (HPLC) and further purified using thin-layer chromatography to remove trace amounts of carotenoid impurities. The lipid samples were then analysed by gas chromatography as described previously [5]. No break-down of the lipids was present as judged by the high levels of unsaturation of the lipid acyl chains. The thylakoid lipids were mixed in chloroform solution and then dried down by rotary evaporation before being dispersed in nitrogen-gassed aqueous media by sonication in a Kerry bath sonicator at 25°C for 20 min. The aqueous dispersion of lipids was labelled with diphenylhexatriene to give a probe-to-lipid ratio of about 1 : 500 (molar ratio). All lipid samples were stored at −40°C in chloroform solution under nitrogen.

Time-resolved fluorescence decays were recorded at 25°C by using the single-photon counting technique with a nanosecond fluorescence spectrophotometer (Applied Photophysics Ltd.). Excitation light at 360 nm was provided by a thyratron-gated nitrogen-filled flash-lamp set to run at 50 kHz and at a voltage of 5 kV in experiments with intact thylakoid membranes and 20 kHz and 4 kV in experiments with chloroplast lipid dispersions. Fluorescence decays were recorded at 460 nm with a counting frequency which was 2% of the lamp flash frequency; under these conditions 99% of the photons detected are single photons. Corrections for scattering and intrinsic fluorescence from the thylakoid suspension were made by subtracting counts from an unlabelled sample over an equal time period. The background fluorescence and scattering account for about 10% of the total counts for labelled thylakoid membranes and less than 3% for chloroplast lipid dispersions. In experiments to determine the decay of anisotropy of diphenylhexatriene fluorescence in thylakoid membranes, the data were collected over several hours and the samples were changed every 15 min by taking a fresh sample from a concentrated stock solution stored on ice. The lamp profile was also recorded at regular intervals to correct for any drifts in the lamp intensity and pulse width over the course of the experiment.

The intensity of the fluorescence from diphenylhexatriene in experiments involving thylakoid membranes was low, since very dilute suspensions were used to minimize the reabsorption of the diphenylhexatriene fluorescence by in vivo chlorophyll and carotenoids. The intensity of the flash-lamp was therefore adjusted to be as high as possible by using a nitrogen-filled lamp with a broad electrode gap; relatively broad pulses were obtained with a full-width at half-maximum of about 6 ns. The problem in the deconvolution procedures for anisotropy decays becomes more difficult as the duration of the lamp pulse becomes longer, thus it was necessary to take a value of 0.39 as the time-zero anisotropy (r_0) value for diphenylhexatriene, as has often been the procedure for this type of experiment [10–14].

The lamp profile (plus instrument response function), and the resulting convoluted fluorescence decay were recorded on a multi-channel analyzer, and then double- or single-exponential deconvolution procedures were performed with a Vector Graphic micro-computer using a non-linear regression-type analysis with four- or two-parameter fitting.

Anisotropy decay data were obtained from fluorescence decay profiles by essentially the same method as those of Kawato et al. [11] and Chen et al. [9]. The lamp pulse was passed through a Glan-Thompson polarizing prism and the fluores-

cence from the sample was detected at 90° through a second prism oriented parallel or perpendicular to the polarizing prism. The recorded decay profiles with polarizer and analyzer parallel ($F_{\parallel}(t)$) and perpendicular ($F_{\perp}(t)$) were then used to create the total ($F_T(t)$) and difference ($F_D(t)$) fluorescence decay data:

$$F_T(t) = F_{\parallel}(t) + 2F_{\perp}(t)G \tag{11}$$

$$F_D(t) = F_{\parallel}(t) - F_{\perp}(t)G \tag{12}$$

where G is a correction factor for partially polarized incident excitation light, and was calculated so that:

$$r_s = \Sigma F_D(t)/\Sigma F_T(t) \tag{13}$$

The total fluorescence decay data $F_T(t)$ were analyzed by a double-exponential deconvolution procedure and the best-fit exponential (A_i) and lifetime (τ_i) parameters obtained for the decay were then used in a second deconvolution procedure to analyze the difference decay data $F_D(t)$. Thus, the fluorescence and anisotropy decays were assumed to have the following forms:

$$F_T^{\delta}(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \tag{14}$$

$$r^{\delta}(t) = (r_0 - r_{\infty}) \exp(-t/\phi) + r_{\infty} \tag{15}$$

where the superscript δ represents the response to an infinitely short (delta-function) pulse of light, ϕ is the rotational correlation time of the probe, and r_0 and r_{∞} the time-zero and limiting anisotropy values at long times, respectively. Therefore, the parameters A_i, τ_i, r_{∞} and ϕ were determined so that when convoluted with the lamp profile, the calculated total and difference decays best fitted the recorded total and difference decays.

Results and Discussion

A typical fluorescence decay profile of diphenylhexatriene incorporated into isolated thylakoid membranes and the plot of the best fit after deconvolution are shown in Fig. 1. The result of the analyses of these types of signals for isolated thylakoids, separated granal and stromal membranes and for dispersions of either digalactosyldiacylglycerol or a 2:1 mixture of monogalactosyldiacylglycerol and digalactosyldiacylglycerol are shown in Table I. In all cases the decays were best fitted by assuming double exponentials with lifetimes of τ_1 and τ_2 but for convenience an average lifetime $\langle \tau \rangle$ is also given.

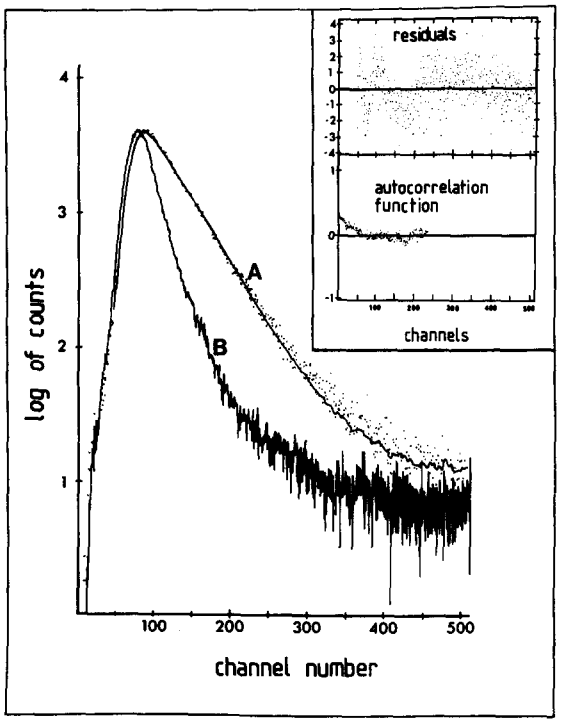

Fig. 1. Trace A is the data points (dots) of the fluorescence decay of diphenylhexatriene in isolated pea thylakoid membranes at 25°C. The solid line is the computed best fit after deconvolution of the lamp profile (trace B) assuming a double-exponential approximation. The best-fit parameters for this experiment were $\alpha_1 = 0.9$, $\tau_1 = 0.3$ ns, $\alpha_2 = 0.1$, $\tau_2 = 6.7$ ns and the modified residuals and autocorrelation function are shown in the inset. The concentration of diphenylhexatriene was $7.5 \cdot 10^{-7}$ M and that of chlorophyll $10 \cdot 10^{-6}$ M. The time axis corresponds to 0.163 ns per channel. Other conditions as in Materials and Methods.

Stubbs et al. [17] have shown that in general the fluorescence lifetime of diphenylhexatriene becomes shorter as the degree of unsaturation of the fatty acid acyl chains in its environment is increased. For saturated lecithin the diphenylhexatriene lifetime is of the order of 10 ns [17] and thus the relatively shorter lifetimes for diphenylhexatriene in the thylakoid membrane and lipid systems used in our study are expected, since these

TABLE I

FLUORESCENCE LIFETIMES OF DIPHENYLHEXATRIENE IN THYLAKOID MEMBRANE SYSTEMS AT 25°C

$\langle\tau\rangle$ = average fluorescence lifetime = $\Sigma_i(A_i\tau_i^2)/\Sigma_i(A_i\tau_i)$ (from Ref. 9); $\alpha_i = A_i/\Sigma_i A_i$; χ^2 for non-linear regression fit and standard errors are given in brackets. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.

Membrane system	No. of experiments	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)	χ^2
Intact thylakoids	6	0.79 (0.04)	1.1 (0.4)	0.21 (0.04)	7.1 (0.3)	5.1 (0.1)	1.5–4.9
Granal stack membranes	4	0.75 (0.04)	1.4 (0.1)	0.25 (0.04)	7.1 (0.3)	5.0 (0.3)	1.4–4.5
Stromal lamellae membranes	7	0.56 (0.05)	1.7 (0.2)	0.44 (0.05)	7.6 (0.1)	6.3 (0.2)	1.3–4.3
MGDG/DGDG lipid dispersion (2:1 mixture)	2	0.33	1.2	0.67	6.4	5.9	2.4–2.5
DGDG dispersion	2	0.68	0.7	0.32	5.5	4.4	2.0–2.6

all contained a high level of unsaturated fatty acids, particularly linolenic acid [18]. Shortening of the lifetime of the probe in the thylakoid membrane could, however, also be due to the presence of the photosynthetic pigments. The emission spectrum of diphenylhexatriene indicates that Förster energy transfer would be possible to both carotenoids and chlorophyll. Attempts to detect energy transfer from diphenylhexatriene to the in vivo pigments have been made by measuring the steady-state excitation spectra of room-temperature chlorophyll fluorescence [3,19]. No energy transfer was detected even when the diphenylhexatriene level was raised to about a 10-times greater level than that used for the life-time experiments. These chlorophyll fluorescence measurements do not exclude the possibility that energy transfer occurs to some of the carotenoids in the membrane which show no fluorescence properties and do not efficiently transfer energy to chlorophyll. The apparent absence of energy transfer from diphenylhexatriene to the in vivo photosynthetic pigments may reflect the inability of the lipophilic probe to partition closely to the protein complexes which contain chlorophyll and carotenoid [20], thus avoiding a significant dipole-dipole interaction. Others [12,13] have suggested, however, that the shortening of the diphenylhexatriene lifetime in certain biological membranes is due to energy transfer to an acceptor chromatophore protein (e.g., bacteriorhodopsin, hemes, etc.) but no firm experimental evidence for this suggestion has been given.

The meaning of the biphasic nature of the decay signals is unclear but presumably reflect heterogeneity in the distribution of the probe even when a pure lipid system was used. Double-exponential decays are often found for pure lipid systems [9,20] and it is well known that isolated thylakoid galactolipids form complex non-homogeneous structures when dispersed in aqueous media which vary with different lipid types and mixtures [22].

The calculated total and difference fluorescence decays of diphenylhexatriene and its time-dependent fluorescence anisotropy measured with isolated pea thylakoid membranes are shown in Fig. 2. A summary of the dynamic (ϕ, D_w and η_c) and structural restraint (r_∞, θ_c and S) parameters obtained from analysing the anisotropy decays for intact thylakoids and aqueous dispersions of extracted lipids is presented in Table II. Because of the probable heterogeneity in the distribution and motional properties of diphenylhexatriene in the various experimental systems, these quantities should be considered as (weighted) averages. By

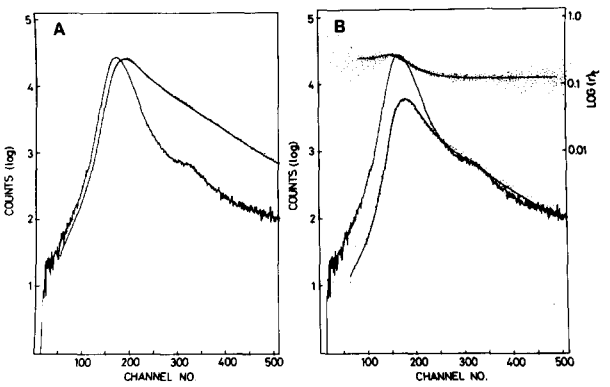

Fig. 2. Analysis of fluorescence anisotropy decays of diphenylhexatriene in isolated pea thylakoid membranes at 25°C. (A) Total fluorescence decay, $F_T(t)$, calculated from Eqn. 11 (dots) and the best fit of the data points after deconvolution assuming a double-exponential decay of the form shown in Eqn. 14. (B) Difference fluorescence decay, $F_D(t)$, calculated from Eqn. 12 (dots) and the best fit of the data points assuming total fluorescence and anisotropy decays of the form shown in Eqns. 14 and 15. Also shown on a logarithmic scale are the time-dependent anisotropy values (dots), r_t, calculated from the relationship $r_t = F_D(t)/F_T(t)$ using the experimental data points. The solid line through the r_t values has been obtained from dividing the best-fit $F_D(t)$ curve by the best-fit $F_T(t)$ curve. The time axis corresponds to 0.083 ns per channel. Other conditions as for Fig. 1.

assuming $r_0 = 0.39$ [10–14] and using Eqn. 6 it has been possible to calculate r_s from the various parameters. The calculated r_s can be compared with measured steady-state anisotropies and a good agreement was obtained in all cases as shown in Table II. Both the dynamic and structural parameters show a difference between the intact membrane and the isolated lipids. The order parameter (S) is higher for the thylakoid membrane than the pure lipids, indicating that the acyl chains are more restrained. This is also clearly expressed by the differences in the cone angles (θ_c). The increased ordering of the fatty acids is probably due to the presence of integral protein in the thylakoid membrane, a conclusion also reached by Hiller and Raison [23] from their ESR spectroscopy studies.

The time-resolved fluorescence anisotropy technique also gives information about the dynamic state of the lipids and can be used to estimate microviscosities. The diffusion coefficient of wobbling of diphenylhexatriene within a cone of half-angle θ_c can be estimated using the following expression [12,24]:

$$D_w\phi(r_0 - r_\infty)/r_0 = -x^2(1+x)^2[\ln(1+x)/2 + (1+x)/2]/[2(1-x)] + (1-x)(6x+8x-x^2-12x^3-7x^4)/24$$

where $x = \cos\theta_c$.

The calculated D_w value of the thylakoid membrane is lower than the values for the pure lipid systems as is the rotational correlation time. The η_c values are a measure of the viscosity within the cone of rotation which is higher for the natural membrane. Therefore, overall it can be concluded that the 'fluidity' of the lipid matrix of the thylakoid is lower than that of the extracted lipids,

TABLE II

MOTIONAL PARAMETERS OF DIPHENYLHEXATRIENE INCORPORATED INTO THYLAKOID MEMBRANE AND LIPID SYSTEMS AT 25°C

η_c was calculated using eqn. 10 where $V_c f$ has been taken as $17 \cdot 10^{-23}$ cm^3 as determined by Kawato et al. [10] for liquid paraffin. Abbreviations as in Table I.

Membrane	r_s	r_s (calcd.)	r_∞	ϕ (ns)	θ_c^0	D_w (ns^{-1})	η_c (P)	S
Intact thylakoids	0.18	0.18	0.12	1.4	48	0.12	0.34	0.55
DGDG dispersion	0.11	0.11	0.01	1.5	77	0.20	0.20	0.16
MGDG/DGDG (2:1 dispersion)	0.13	0.13	0.05	1.5	61	0.16	0.25	0.36

i.e., within the intact membrane the degree of order of the acyl chains is higher and their rate of motion slower. Nevertheless, when compared with other biological membranes [13], the values given in Table II indicate that this chloroplast membrane is a relatively fluid system. For example, at 35°C the human erythrocyte membrane has a D_w value of 0.1 ns^{-1}, $\theta_c = 37.4°$ and a calculated η_c of 0.42 P. The purple membrane of *Halobacterium halobium* (strain R_1M_1) is even more rigid at 35°C than the red blood cell membrane having $D_w = 0.048$ ns^{-1}, $\theta_c = 30.5°$ and $\eta_c = 0.87$ P. The values obtained for the thylakoid membrane are similar to those found for the rat liver mitochondrial membrane at 35°C where $D_w = 0.146$ ns^{-1}, $\theta_c = 53.1°$ and $\eta_c = 0.29$ P. The higher fluidity of the mitochondrial membrane probably reflects its low cholesterol content compared with the erythrocyte membrane while the rigidity of the purple membrane is almost certainly due to its very high protein-to-lipid ratio and unusual lipid composition [13]. In the case of the thylakoid membrane its high fluidity is probably due to the absence of sterols and the very high degree of unsaturation of the acyl chains [18,29]. Our measurements were made at 25°C so that it can be anticipated that at 35°C the thylakoid is likely to be even more fluid than rat liver mitochondrial membranes at the same temperature.

The observed large values of the cone angle θ_c and the magnitude of the other dynamic parameters for the isolated chloroplast lipids are similar to other pure lipid systems which are in the liquid-crystalline state and contain unsaturated acyl chains [9–12,17].

Our previous studies of thylakoid membrane fragments using steady-state fluorescence polarization of diphenylhexatriene and ESR of spin labels indicated that the stromal lamellae is more fluid than grana lamellae [5]. Preliminary time-dependent anisotropy studies of diphenylhexatriene fluorescence supports this conclusion (Ford and Barber, unpublished data). For example, the cone angle of diphenylhexatriene rotation in the granal membrane has been estimated to be 45° while in stromal membranes it is 60°C. Analyses of granal and stromal membranes indicated that the difference in fluidity of the two types of membrane fragments was not due to significant changes in the saturation of the acyl chains or of the lipid classes but related more to the protein/lipid ratios [5].

The results presented in this paper indicate the usefulness of time-dependent fluorescence anisotropy measurements for investigating the physical state of the lipid matrix of thylakoid membranes. The significance of these types of measurements is becoming more evident as our knowledge of the spatial relationship between electron-transport components increases. The concept that PS II and PS I can be separated by large distances [1,2,25,26] places importance on the lipid matrix as the 'medium' for long-range diffusion of mobile lipophilic electron carriers (e.g., plastoquinone). For this reason, maintaining an optimal fluidity of the thylakoid membrane under different growth conditions is probably a basic regulatory process in response to changes in environment. In animal membranes, sterols may function to control the degree of lipid acyl chain ordering [27] while in prokaryotic organisms the same regulation may involve tri- and tetraterpenes [28]. In thylakoids, however, the regulatory mechanism could be via changes in protein/lipid ratio, since this membrane contains little or no sterols [28]. Certainly in the case of pea, which is a cold-tolerant plant, we have found that adaptation to growth at low temperature is reflected by no large change in the unsaturation level of the thylakoid lipids or in the lipid ratio [30].

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