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Growth Temperature-Induced Alterations in the Thermotropic Properties of *Nerium oleander* Membrane Lipids[1]

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ABSTRACT

The temperature boundary for phase separation of membrane lipids extracted from *Nerium oleander* leaves was determined by analysis of spin label motion using electron spin resonance spectroscopy and by analysis of polarization of fluorescence from the probe, *trans*-parinaric acid. A discontinuity of the temperature coefficient for spin label motion, and for *trans*-parinaric acid fluorescence was detected at 7°C and −3°C with membrane lipids from plants grown at 45°C/32°C (day/night) and 20°C/15°C, respectively. This change was associated with a sharp increase in the polarization of fluorescence from *trans*-parinaric acid indicating that significant domains of solid lipid form below 7°C or −3°C in these preparations but not above these temperatures. In addition, spin label motion indicated that the lipids of plants grown at low temperatures are more fluid than those of plants grown at higher temperatures.

A change in the molecular ordering of lipids was also detected by analysis of the separation of the hyperfine extrema of electron spin resonance spectra. This occurred at 2°C and 33°C with lipids from the high and low temperature grown plants, respectively. According to previous interpretation of spin label data the change at 29°C (or 33°C) would have indicated the temperature for the initiation of the phase separation process, and the change at 7°C (or −3°C) its completion. Because of the present results, however, this interpretation needs to be modified.

Differences in the physical properties of membrane lipids of plants grown at the hot or cool temperatures correlate with differences in the physiological characteristics of plants and with changes in the fatty acid composition of the corresponding membrane lipids. Environmentally induced modification of membrane lipids could thus account, in part, for the apparently beneficial adjustments of physiological properties of this plant when grown in these regimes.

The evergreen higher plant *Nerium oleander* (oleander) acclimates to both hot summer and cool winter conditions and grows actively in these contrasting regimes (2). The temperature optimum for photosynthesis is between 35 and 40°C for plants grown in a 45°C/32°C (day/night) regime and between 25 and 30°C for plants grown at 20°C/15°C (1). This ability to acclimate to the prevailing temperature is probably an important adaptive mechanism for this and other desert evergreen species, such as *Larrea divaricata* (9) and *Atriplex lentiformis* (12), which experience large seasonal differences in environmental temperature. The variation in fatty acid composition of *A. lentiformis* with growth temperature (11) suggests that changes in the physical properties of membranes might be the basis for the acclimation.

To characterize the oleander membrane lipids, we have utilized ESR[4] spectroscopy and measurements of fluorescence intensity and polarization with the probe, *trans*-parinaric acid (25). Both techniques revealed the same temperature for the separation of solid and fluid phase lipids but indicate a need to reinterpret some previous ESR studies of plant membrane lipids. Furthermore, the results show that the physical properties of *N. oleander* membrane lipids change during thermal acclimation.

MATERIALS AND METHODS

Rooted cuttings of a single individual *N. oleander* were grown in controlled environment chambers with a 14 h photoperiod at about 30 nE cm^{-2} s^{-1}, at either 20°C/15°C or 45°C/32°C, day/night temperature (2). Plants grown at the higher temperature received approximately 1,000 µl/l CO_2. Enrichment with CO_2 is required to permit net growth of *N. oleander* at this temperature.

Lipids were extracted from young, fully expanded leaves with chloroform:methanol (2:1, v/v) containing butylated hydroxytoluene (0.01%, w/v). The extract was partitioned against 0.55 M KCl followed by water (4). Polar lipids for ESR studies were separated by chromatography on a column of Bio-Sil A (Bio-Rad), washed sequentially with chloroform, 10% (v/v) acetone in chloroform, and methanol. The methanol fraction contained galacto- and phospholipids, as verified by TLC. The presence of pigments in this polar lipid fraction precluded its use in fluorescence measurements. Phospholipids for the fluorescence studies were prepared as above except the column was eluted with chloroform, acetone, and methanol. The methanol fraction contained the phospholipids. Liposomes were prepared by gentle sonication of the polar or phospholipids in 0.1 M Tris acetate buffer (pH 7.2) containing 0.005 M Na_2 EDTA.

For ESR spectroscopy, liposomes were made to 50% (v/v) with ethylene glycol. The liposomes were labeled with spin probes having the general structure

$$CH_3-(CH_2)_m-\underset{\text{(oxazolidine ring: }C(CH_3)_2\text{, O, N–O)}}{C}-(CH_2)_n-\overset{O}{\overset{\|}{C}}-(-OH)\quad I_{(m,n)}$$
$$(-OCH_3)\quad II_{(m,n)}$$

and abbreviated $I_{(m,n)}$ for the acid form and $II_{(m,n)}$ for the methyl

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[4] Abbreviation: ESR, electron spin resonance.

ester form. The determinants *m* and *n* refer to the position of the oxazolidine group relative to the terminal methyl and carboxy (or carbomethoxy) groups, respectively. These labels were synthesized by the general procedure of Keana *et al.* (5), and intercalated into the lipid samples at the rate of 1 mol label/150 mol lipid. The empirical motion parameter, τ_0, was calculated using the relationship

$$\tau_0 = 6.45 \times 10^{-10}[(h_0/h_{-1})^{1/2} + h_0/h_{+1})^{1/2} - 2)]W_0$$

where h_{+1}, h_0 and h_{-1} refer to the height of the low-field, mid-field and high-field lines, respectively, and W_0 is the width in gauss of the mid-field line of the first-derivative ESR spectrum (24). Separation of the hyperfine extrema ($2T_{\parallel}$) was determined from direct measurement of spectra as described in (21). Spectra were recorded using a Varian E112 spectrometer. Temperature of the sample was controlled by regulating the temperature of dry N_2 gas which flowed through the cell housing with a Model TCM 20 control unit (Deltron Pty, Ltd. Sydney, Australia). Flow rate was adjusted such that the vertical temperature gradient within the sample tube was less than 0.2°C. The sample temperature was measured continuously 5 mm above the cavity of the spectrometer with a copper-constantan thermocouple, and was stable to within ± 0.005°C.

Fluorescence measurements (Perkin-Elmer MPF-3L spectrofluorimeter) were made on samples of liposomes containing 1,200 μg lipid (approximately 1.6 μmol) and 4 nmol of *trans*-parinaric acid in 3 ml buffer, containing 25% (v/v) ethylene glycol. The thermo-regulated sample cuvette was stirred and the temperature was monitored with a copper-constantan thermocouple. The excitation beam (320 nm) was passed through a polarizing prism (Karl Lambrecht, Chicago). A film polarizer (Edmund Scientific) was oriented either parallel ($I_{\parallel}$) or perpendicular ($I_{\perp}$) to the excitation polarizer and the intensity of fluorescence at 420 nm (20 nm half band width) measured. The ratio, $I_{\parallel}/I_{\perp}$, was calculated at each temperature. All temperature scans were in the ascending direction, and measurements were made on three replicate samples.

The fatty acid composition was determined on the polar lipids fraction. Lipids were hydrolyzed in NaOH/methanol, methylated using BF_3 as a catalyst, and the individual fatty acids were determined by integration of the peak areas obtained by GLC separation according to procedures similar to (11).

RESULTS

Figure 1 shows the effect of temperature on the motion of spin label $II_{(5,10)}$ intercalated into liposomes of polar lipids from 45 and 20°C-grown oleander plants. The motion is expressed as τ_0 and is inversely related to the relative viscosity of the lipid bilayer (24). For any measurement temperature, tumbling motion of the probe is faster in the lipids from plants grown at the lower temperatures than in those from plants grown at the higher temperature. In addition, a change in the temperature coefficient of motion, which is indicative of a change in the molecular ordering of the host lipids, was observed at 7°C for the lipids from the 45°C/32°C-grown plants and at −3°C for the lipids from the 20°C/15°C-grown plants. A change in the separation of the hyperfine extrema of ESR spectra as a function of temperature (using the probe $I_{(12,3)}$, Fig. 2) was detected at 29°C with lipids from the plant grown at the high temperature indicating a change in the ordering of the acyl chains of these lipids at this temperature. A similar change was observed at about 33°C with lipids from plants grown at the lower temperature.

The fluorescence polarization ratio of *trans*-parinaric in the phospholipids as a function of temperature is shown in Figure 3. A slope change was observed at 7 and −3°C for the lipids from warm- and cool-grown plants, respectively. These coincide with

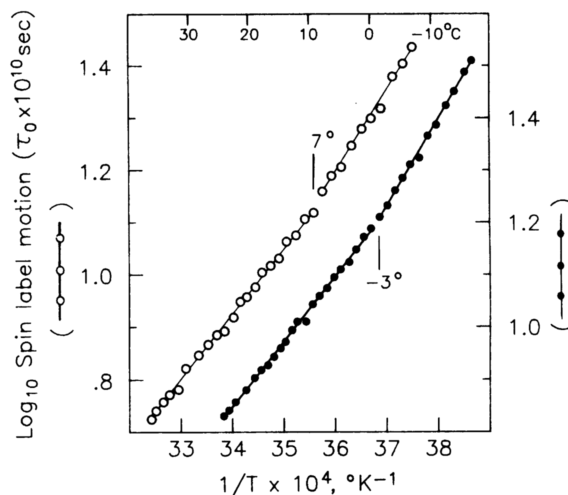

FIG. 1. The effect of temperature on the motion of the spin label $II_{(5,10)}$, associated with polar lipids from *N. oleander*. Sample temperature was increased at the rate of 0.5°C/min. The data were analyzed by regression analysis and the change in the temperature coefficient of motion for the 45/32°C grown plants (○, left axis) at 7°C and for the 20/15°C grown plants (●, right axis) at −3°C represents the intersection of two straight lines which give a minimum for the sum of the residual sums of squares of the two lines (for details see Pollard [13]).

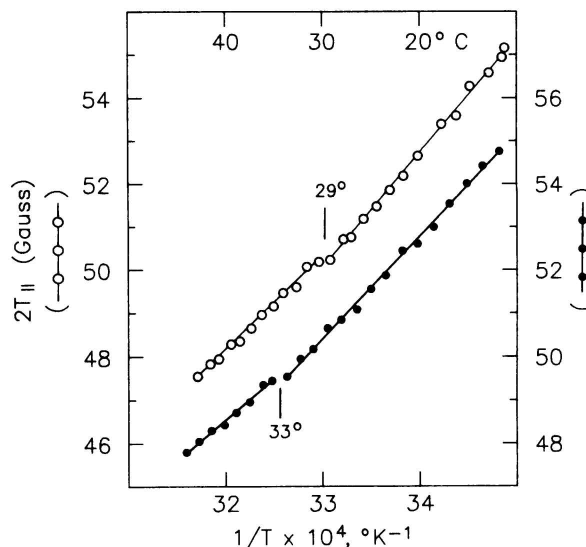

FIG. 2. The change in the separation of the hyperfine extrema ($2T_{\parallel}$) for $II_{(12,3)}$ in the polar lipids of the 45/32°C (○ , left axis) and 20/15°C (●, right axis) grown plants as a function of temperature. The intersect of the two straight lines was determined as described in Figure 1.

the lower temperature change detected with the ESR probes. In the lipids from the warm-grown plants, the polarization ratio reached a maximum at about −7°C; measurements were not continued to temperatures low enough to observe if a maximum occurred with the lipids of the cool-grown plant. Figure 4 shows plots of fluorescence intensity (with the polarizers parallel) as a function of temperature. Changes in slope were observed at 6 and −3°C for the lipids from warm- and cool-grown plants respectively.

The proportion of unsaturated fatty acids in the polar lipids increased in the plants grown at the lower temperature (Table I). The greatest changes occurred in oleic ($C_{18:1}$) and linolenic ($C_{18:3}$) acids.

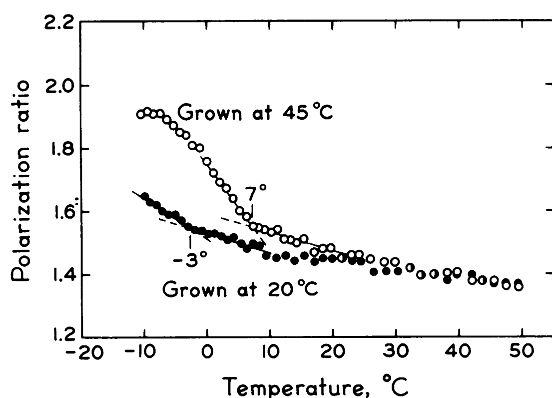

FIG. 3. Fluorescence polarization ratio of *trans*-parinaric acid associated with phospholipids from *N. oleander*. Lipids were prepared and fluorescence measured as described in the text. The polarization ratio is defined as $I_{\parallel}/I_{\perp}$ (fluorescence intensity with emission polarizer oriented parallel and perpendicular, respectively, to the polarized excitation beam).

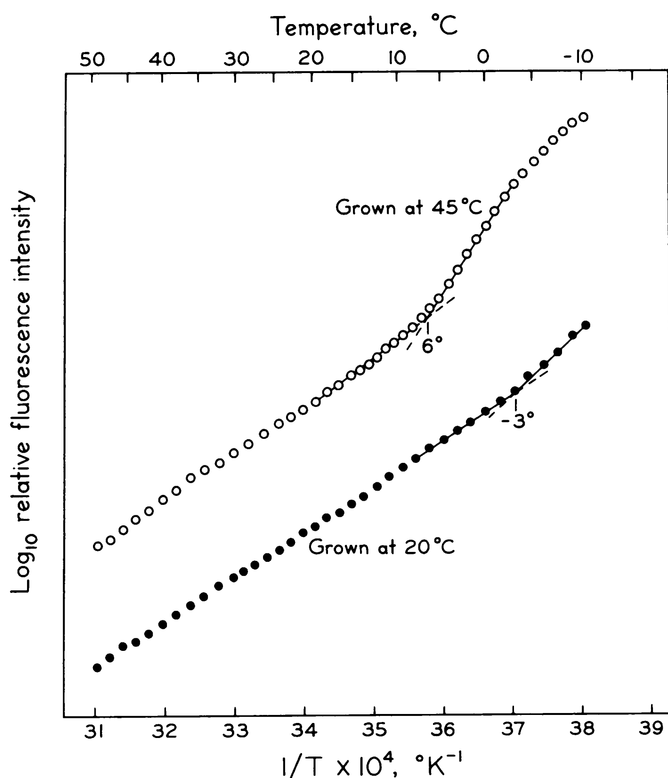

FIG. 4. Fluorescence intensity of *trans*-parinaric acid associated with phospholipids from *N. oleander*. The intensity was measured with the emission polarizer oriented parallel to the polarized excitation beam ($I_{\parallel}$).

Table I. *Fatty Acid Composition of Total Polar Lipids From Leaves of Nerium oleander Grown at 20/15°C or 45/32°C*

All data are the mean of three determinations on single polar lipid samples; $\sigma \simeq \pm 0.05 \times$ mol %.

Growth Temperature	Fatty Acid					
	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
°C	*mol %*					
20/15	16.0	0.8	2.2	3.7	13.7	63.2
45/32	19.0	1.0	4.7	11.2	16.3	47.8

DISCUSSION

Interpretation of temperature-induced changes in the molecular ordering of membrane lipids in terms of a lipid phase transition is based on studies using binary mixtures of phospholipids and relatively simple bacterial membrane systems (7, 22). In these systems, a transition with lateral phase separation occurs when a lipid mixture in the fluid phase is cooled. The beginning of this transition has been denoted T_f, and its completion at some lower temperature, denoted T_s (27). Between T_f and T_s the membrane lipids are a mixture of solid and fluid phase lipids (27). Successive changes in the temperature coefficient for spin label motion occur in membrane lipids of rat liver mitochondria (19) and these have been interpreted in terms of the phase boundaries T_f and T_s which were observed with mixtures of phospholipids or with bacterial membrane systems (22, 27). This interpretation has also been applied to studies of spin label motion in the more complex lipid mixtures of plant membranes (18). If, as was previously assumed (18, 19), the first change in temperature coefficient for spin label motion as the temperature is lowered, T_f, corresponds to the initiation of phase separation, and the second change, T_s, to the completion of the solidification process then the polar lipids of warm- and cool-grown oleander plants should be mostly solid below 7 and −3°C respectively (Fig. 1).

On the basis of the fluorescence polarization changes (Fig. 3) there is reason to question the above assignment of T_f and T_s in plant membrane lipids. Studies of model lipid systems with *trans*-parinaric acid indicate that this probe is sensitive to the formation of a few percent solid in a lipid mixture (26). The polarization ratio in a fluid lipid system is 1.4 to 1.5 and in a mostly solid lipid system is 2.2 to 2.6 (26). The polarization ratio of oleander phospholipids at temperatures above about 10°C is less than 1.6 (Fig. 3), indicating little or no solid phase lipid is present above this temperature. The increase in polarization ratio, which begins at 7 and −3°C as the temperature is lowered (Fig. 3), is thus interpreted as indicative of the formation of domains of solid phase lipid as the temperature declines. Thus, T_f is 7 and −3°C for the lipid preparations from the high and low temperature-grown oleander plants, respectively. The spin probe II (5, 10) showed this transition at the same temperatures (Fig. 1). However, as noted above, this point would previously have been interpreted as T_s, the completion rather than the initiation of a transition.

The temperature for the completion of the solidification process T_s was not determined for these lipid preparations. *Trans*-parinaric reaches its maximum fluorescence polarization with about 50% of ordered lipid in model systems (26). Thus, while the polarization ratio of the probe in phospholipids from leaves of plants grown at 45°C appears to have peaked by −10°C (Fig. 3), it is not likely that the phase transition was complete at this temperature. Motion of methyl stearate-type spin labels is severely restricted at these low temperatures, and it is also not possible to use this method to determine the temperature for completion of lipid solidification.

The spin probe I (12.3) indicated temperature-induced changes in the molecular ordering of polar lipids at temperatures well above the temperature for the initiation of the transition, T_f, (Fig. 2). In other studies of plant lipids, such changes in molecular order have been detected at temperatures between 25 and 35°C (15, 18). With oleander lipids this change was detected at 29°C in warm-grown plants and at 33°C in cool-grown plants. Fluorescence measurements showed no indication of phase separation at these temperatures. The physical change indicated by the spin label at these high temperatures is not known. If it is a transition involving solid phase lipids, the quantity of lipid must be very small (<5%), inasmuch as there was no change in the polarization ratio of *trans*-parinaric acid and it would appear to be independent of the transition which begins at the lower temperature (7 or −3°C). Sklar *et al.* (26), found that *trans*-parinaric acid detected some changes in structural order at temperatures 10°C or more

above that of T_f in model lipid systems, and they suggest that this may be due to clustering of immiscible lipids (26). Furthermore, clustering is reported to occur in vesicles of phosphatidylcholine containing unsaturated fatty acids at temperatures above the liquid-gel transition temperature (6).

Interpretation of results obtained with both the spin and fluorescent probes provides a new insight into the phase properties of plant lipids. Major solidification of membrane lipids does not begin to occur in the leaf polar lipids of *N. oleander* plants until 7 or −3°C, depending upon growth conditions. This solidification was detected with spin label probes using preparations that contained the total polar lipids of the leaves (mostly galacto- and phospholipids) and with the fluorescence probe using only the phospholipids of the lipid extracts. Using the phospholipids with the spin label probes we found similar transition temperatures (data not shown) but the transitions were more distinct than shown in Figure 1. The presence of pigments in the total polar lipid fraction interferred with use of the fluorescent probe with this fraction. The similar values of T_f determined with these preparations may indicate that properties of the phospholipids determine the phase separation temperature of these membrane lipids.

The phase separation temperatures determined for membrane lipids of high- and low-temperature-grown oleander leaves are similar to the minimum summer and winter temperatures which these plants would experience in their natural habitat. The possible consequences of exposing *N. oleander* to temperatures below the temperature of the phase separation have not been examined. In studies with other plants, physiological events such as changes in the activation energy of succinate oxidation by mitochondria (14, 18) or $NADP^+$ photoreduction by chloroplasts (23), have previously been correlated with this lower change, now interpreted as T_f. It has been suggested that this change in lipids marks the lower temperature limit for normal physiological performance of the plant (8). Apparently, some features of membrane performance in higher plants are lost when phase separation begins (15).

The lipids of the cool-grown oleander are more fluid (*i.e.* τ_0 less) at any temperature, than those of the warm-grown plants (Fig. 1). The temperature at which the same fluidity is reached in the two preparations differs by about 8°C degrees. That is, the fluidity of the lipids of the high-temperature-grown plants at 18°C is similar to that of lipids from the low-temperature-grown plant at 10°C. The changes in T_f and influidity are not as large as the change in growth temperature (25°C day/17°C night). While this partial compensation is in contrast to the complete adaptation to fluidity seen with bacterial membranes (24), it nevertheless correlates with the physiological adaptation of oleander leaves. Warm-grown oleander plants suffer irreversible inhibition of photosynthesis at temperatures about 10°C higher than cool-grown plants; at 53°C and at 43°C, respectively (17). The spin label II (5, 10) was used to probe the fluidity of chloroplast membrane polar lipids. Over the temperature range 35 to 55°C the lipids from cool-grown plants were more fluid (16), and the fluidity of the two preparations was approximately equal at the corresponding temperatures for denaturation of the intact membranes: 53 and 43°C (17). This finding indicates a possible regulatory role of membrane lipids in the adaptation of plants to contrasting thermal regimes (1, 2).

Shifts in the physical properites of membrane lipids can result from changes in the chain length or unsaturation of fatty acids or in head group composition of the complex lipids (3). We have yet to explore all possibilities, but the increase in the proportion of the more unsaturated fatty acids in cool-grown plants (Table I) is consistent with the lower transition temperature (Figs. 1 and 4) of the lipids of these plants. This result, however, does not imply that a change in transition temperature necessarily correlates with a change in fatty acid composition of the bulk membrane lipids (1). In terms of properties pertinent to the adaptation of plants to their thermal habitat, direct measurements of the physical properties of membrane lipids could be more informative than extrapolations based on lipid chemical composition. The probe *trans*-parinaric acid would seem to be well suited for such measurements.

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