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Free fatty acid effects on leakage, phase properties and fusion of fully hydrated model membranes

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Free fatty acids accumulate in the membranes of plant cells following several lethal environmental stresses, and their accumulation has been correlated with the appearance of gel phase domains, increased microviscosity, and enhanced leakage of cytoplasmic solutes Saturated and unsaturated free fatty acids which accumulate as a result of phospholipid de-esterification have been shown previously to have distinctly different effects on the phase properties of complex lipid mixtures In the simple model membrane composed of dipalmitoylphosphatidylcholine (DPPC), the presence of free fatty acid, sodium palmitate or linoleate, enhanced leakage of trapped carboxyfluorescein at temperatures below the phase transition of pure DPPC, 41°C. In the case of sodium linoleate, this was accompanied by a broadening of the phase transition which agreed well with the enhanced leakage. However, in the case of sodium palmitate, the phase transition was shifted upwards and broadened, which did not explain the enhanced leakage at low temperatures. The increased leakage in this latter case was shown by resonance energy transfer to be the result of vesicle fusion. Thus, the presence of relatively low concentrations of saturated free fatty acid in the bilayer, enhanced leakage, altered the phase properties and promoted aggregation and fusion of vesicles, which is consistent with the symptoms of stress injury in plant membranes

Introduction

Plant cell membranes are directly altered by a variety of environmental stresses including freezing [1–4], desiccation [5], anoxia [6,7], air pollutants [8] and seed aging [9] In many of these cases the plasmalemma is thought to be the primary site affected by the stress [3], but the nature of this effect has remained elusive A range of responses have been observed which are common to all of these stresses, and suggest some degree of similarity in the nature of the damage These include an increased leakage of cytoplasmic solutes, a general degradation of membrane components, the formation of osmiophilic (presumably lipid) bodies in electron micrographs, the appearance of IMP-free membrane areas and hexagonal phases in freeze-fracture micrographs, the appearance of gel phase domains in X-ray diffraction patterns, and a general increase in microviscosity of the bilayer as shown using the fluorescent probe, DPH Although not as extensively documented as the above physical changes, the only substantial change in the lipid composition of microsomal membranes from plant cells is the presence of elevated levels of free fatty acids

Since the composition of this free fatty acid fraction is similar to that of the total membrane fatty acids, and since their accumulation coincides with of loss of lipid-phosphorus [2,5,8], it has been suggested that the free fatty acids arise from deesterification reactions in the membrane leading to the loss of the phosphate containing head group into the aqueous medium and the retention of the acyl chains in the bilayer [10,11]

Under conditions of normal cellular metabolism, enzymes such as lipoxygenase might be expected to catabolize the free fatty acids and effectively remove them from the bilayer However, in plant tissues frozen at subzero temperatures, dried to below 20% moisture, or encased in a sheet of ice, normal metabolism does not occur, and the free fatty acids may accumulate to the point of disrupting membrane function [11]

Abbreviations IMP, intramembrane particle, CF, carboxyfluorescein

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It has not been established whether the accumulation of a complex mixture of free fatty acids in a plant cell membrane can account for all of the membrane dysfunction observed following stress, or whether there are alternative processes involved The addition of free fatty acids to liposomes composed of complex lipid mixtures can simulate the changes in phase properties and microviscosity observed following desiccation stress, but the saturated and unsaturated free fatty acids acted in a distinctly different manner [12] The addition of saturated free fatty acids, palmitate and stearate, to liposomes prepared from the total lipid extract from microsomal membranes of soybean seeds increased the gel to liquid-crystalline phase transition temperature and increased microviscosity, as determined by wide-angle X-ray diffraction and fluorescence depolarization, respectively Conversely, the addition of the unsaturated free fatty acids, oleic, linoleic, and linolenic, did not influence the X-ray determined phase transition and decreased microviscosity (i e , increased fluidity) A mixture of both types as observed in plant membranes, largely followed the effect of the saturated free fatty acids

The objective of the present study was to characterize the interaction of two free fatty acids, palmitate and linoleate, with a well characterized phospholipid, DPPC, in the fully hydrated bilayer A related study [13], describes the effects of free fatty acids on membrane properties in the dry state which might be expected to occur in nature during desiccation or freezing of plants

Materials and Methods

Leakage of trapped carboxyfluorescein Unilamellar vesicles of DPPC (Avanti Polar Lipids, Birmingham, AL) were formed in the presence of purified 5,6-carboxyfluorescein (CF) by sonication in at bath sonicator (Lab Industries, Hicksville, NY) at 55 °C The suspension was passed through a Sephadex G-50 fine column to remove the CF outside the vesicles Aliquots (10–20 μl) of the samples were dispersed in 2 5 ml of 10 mM Tes buffer (pH 7 5), in a temperature controlled cell and fluorescence readings were taken through the temperature ranges shown in the figures. Fluorescence was measured using a Perkin Elmer LS-5 spectrofluorometer with excitation at 460 nm and emission at 550 nm At the end of the temperature scan, the vesicles were lysed by the addition of 20 μl of 2% (v/v) Triton X-100 and the final fluorescence was read The retention of CF at any temperature was calculated as

$$\% \text{ retention} = \frac{F_i - F_i}{F_f - F_i} \times 100$$

where F_i is the initial fluorescence before the temperature scan, F_i is the fluorescence reading at any temperature, and F_f is the final fluorescence after the temperature scan and lysis.

Infrared spectroscopy Multilamellar vesicles of DPPC with the addition of various concentrations of sodium palmitate or sodium linoleate (Sigma Chemical Co , St Louis, MO) were formed by drying 10 mg mixtures of the lipids from chloroform followed by high vacuum overnight The samples were hydrated with 300 μl of water at approx 55 °C and vortexed to a uniform suspension In some experiments unilamellar vesicles were prepared by bath sonicating the above suspension to clarity Infrared spectra were taken with a Perkin-Elmer Fourier transform infrared (FTIR) spectrometer (Model 1750) and data were acquired and analyzed with a Perkin-Elmer 7500 data station using Perkin-Elmer software Each spectrum is the average of 10 scans over the infrared region 3000 cm^{-1} to 900 cm^{-1} at each temperature Spectra were taken at approx 3 C° intervals from 28 to 55 °C The regions of interest in the spectra were extracted, converted to absorbance and expanded by a software routine to enhance the significant peaks The gel to liquid-crystalline phase transition was monitored by shifts in the CH_2 symmetric and asymmetric stretch at 2850 and 2923 cm^{-1}, respectively

Resonance energy transfer These studies were conducted essentially as previously described [14–16] The donor probe cholesteryl anthracene-9-carboxylate (CA9C) was obtained from Molecular Probes Inc , Eugene, OR, and the acceptor probe 7-nitro-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) was obtained from Avanti Polar Lipids Birmingham, AL Both were maintained in stock solutions in chloroform at −20 °C Donor and acceptor liposomes were made by adding 2 mol% of CA9C or NBD-PE to 20 mg of DPPC in chloroform and drying under a stream of nitrogen The lipids were resolubilized to a known concentration with chloroform and an aliquot was removed from both donor and acceptor tubes and combined in a third tube to produce mock-fused lipid All samples were redried with nitrogen followed by vacuum, rehydrated with 10 mM Tes buffer, and bath sonicated to form small unilamellar liposomes Lipid mixing between aliquots of donor and acceptor liposomes as a result of changes in temperature was determined by the relative amount of quenching of the donor fluorescence Zero probe intermixing was determined by unquenched donor fluorescence and 100% probe intermixing was determined by the donor fluorescence of the mock-fused vesicles Donor and acceptor liposomes were mixed in a temperature controlled fluorometer cell and fluorescence was recorded during a temperature scan using a Perkin Elmer LS-5 with excitation at 370 nm and emission at 450 nm

Results and Discussion

Leakage studies

Retention of CF inside DPPC unilamellar vesicles was used as an indicator of the relative effects of

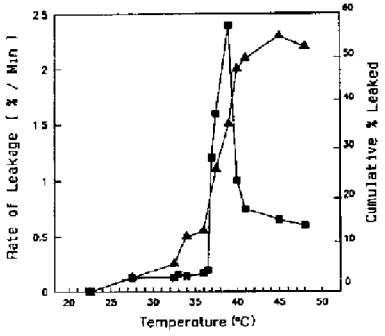

Fig 1 Leakage and rate of leakage of trapped carboxyfluorescein from sonicated vesicles of DPPC as a function of temperature Cumulative leakage (▲) and rate of leakage (■) were calculated from separate experiments

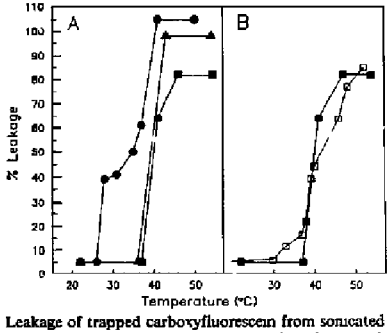

Fig. 2 Leakage of trapped carboxyfluorescein from sonicated vesicles of DPPC and DPPC with the addition of free fatty acids as the vesicles are taken through a temperature scan (A) Sodium palmitate at molar concentration is of 0% (■) 2 2% (▲) and 6 5% (●) (B) Sodium linoleate at molar concentration is of 0 (■) and 5% (□)

palmitate and linoleate on the permeability and integrity of the lipid bilayer as it is taken through its thermotropic phase transition In sonicated DPPC liposomes, leakage of CF increased sharply between 38 and 42°C until approx 50% of the originally trapped CF was released (Fig 1) In a separate experiment, sonicated DPPC vesicles were taken to a prescribed temperature and held there for up to 30 min while fluorescence readings were taken at short intervals At any particular temperature, the rate of leakage was constant over the 30 min period These data (Fig 1) show that the rate of leakage as well as the total leakage increase sharply at the onset of the thermotropic phase transition and that the rate of leakage falls sharply at the end of the transition From the beginning to the end of the phase transition, both the rate and the cumulative leakage parallel each other Since it is far more convenient to measure cumulative leakage than rate of leakage, we used the former values as a measure of phase transitions in subsequent experiments

Addition of 2 2 mol% palmitate to DPPC vesicles had a minimal effect on this leakage, although it did increase the total amount leaked (Fig. 2) However, addition of 6 5 mol% palmitate increased leakage of trapped CF at temperatures as low as 27°C, by 41°C all of the trapped CF had leaked (Fig. 2A) On the other hand, addition of sodium linoleate broadened the temperature range over which the leakage occurred without changing the midpoint of the transition (Fig 2B)

Infrared spectroscopy

These changes in the temperature profile for the leakage of trapped CF at first suggested that the gel to liquid-crystalline phase transition of DPPC which typically occurs at 41°C [17] had been shifted to lower temperatures by the inclusion of sodium palmitate or broadened slightly by the inclusion of sodium linoleate

This assumption was not confirmed using FTIR to follow the shifts in wavenumber of the symmetric and asymmetric stretch of the CH_2 groups of the acyl chains as the liposomes underwent their thermotropic phase transition

Unilamellar vesicles of DPPC, without the addition of any free fatty acid, exhibited a transition at approx. 38°C as shown by the shift in wavenumber from 2850 to 2853 cm^{-1} (Fig 3) Similarly, the CH_2 asymmetric stretch shifted from 2919 to 2923 cm^{-1} over the same narrow temperature range (Fig 3) In multilamellar vesicles, the gel to liquid-crystalline transition is sharper and at somewhat higher temperature (41°C, Fig 4) Above and below the transition in both multilamellar and unilamellar vesicles, there were no changes in the

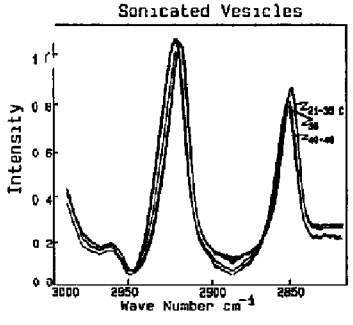

Fig 3 A series of infrared absorption spectra of sonicated vesicles of pure DPPC at various temperatures The spectral region shown includes the symmetric CH_2 stretch at 2850 cm^{-1} and the asymmetric CH_2 stretch at 2920 cm^{-1} As the DPPC goes through its gel to liquid-crystalline phase transition, these bands increase in wavenumber, broaden slightly, and the peak height ratios change These shifts can be used to follow the phase transition

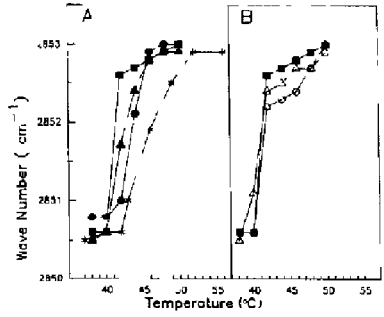

Fig 4 Phase transitions of pure DPPC multilamellar vesicles and DPPC vesicles made with the inclusion of free fatty acids (A) Sodium palmitate at molar concentrations of 0% (■), 2% (▲) 5% (●), and 10% (•) (B) Sodium linoleate at molar concentrations of 0% (■) 2% (△) and 5% (○) Phase transitions were determined from shifts in the wavenumber of the symmetric CH_2 stretch The addition of palmitate broadens the transition and shifts the transition temperature upwards while linoleate broadens the transition slightly without affecting the midpoint of the transition

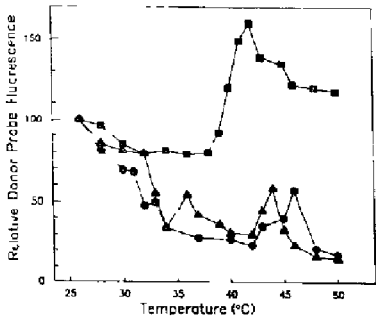

Fig 6 Probe intermixing measured by resonance energy transfer which estimates aggregation and fusion of vesicles as a function of temperature Decreased relative donor probe fluorescence indicates increased probe intermixing The addition of 5 (▲) or 10 (●) mol% sodium palmitate to the vesicles greatly increases aggregation and fusion of vesicles The apparent sharp decrease of probe intermixing at the phase transition is attributed to greater mobility of the probes which leads to an apparent decrease in their proximity in the bilayer

wavenumber of the CH_2 stretches, and scans were very reproducible (e g , Fig 3)

Addition of increasing amounts of sodium palmitate to multilamellar vesicles of DPPC broadened the phase transition and increased the midpoint temperature (Fig. 4A) Sodium linoleate, on the other hand, only slightly broadened the transition and the midpoint of the transition remained at 41°C (Fig 4B) In unilamellar vesicles broadening of the phase transition occurred [18] (Fig 5) In addition, an increase in the transition temperature due to the addition of 5% palmitate was seen in the unilamellar vesicles as well as multilamellar vesicles (Fig 5)

Thus, the results from both the leakage experiments and FTIR suggest that sodium linoleate acts as a simple

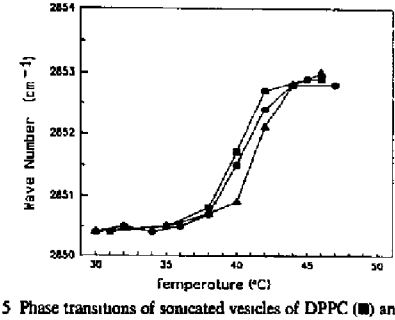

Fig 5 Phase transitions of sonicated vesicles of DPPC (■) and DPPC with either 5 mol% sodium palmitate (▲) or sodium linoleate (●) The transition is broadened due to the changes in packing brought about by the change from multilamellar to unilamellar vesicles. The presence of 5% palmitate also causes an upward shift in the phase transition temperature

impurity in the DPPC vesicles and slightly broadens the thermotropic gel to liquid-crystalline phase transitions (Figs 1B, 3B) On the other hand the effects of sodium palmitate on the DPPC bilayer cannot be so easily explained As shown in Fig 4A, sodium palmitate both broadens the phase transition of DPPC and causes a significant increase in the transition temperature to above 41°C, while CF leakage from DPPC vesicles begins several degrees below the beginning of the phase transition when the bilayer is in gel phase (Fig 2A)

Vesicle fusion

An alternative explanation for the enhanced leakage below the phase transition of DPPC promoted by sodium palmitate is that this free fatty acid is fusigenic and that part of the trapped CF was lost during vesicle fusion While fusion can occur without leakage of vesicle contents, leakage is a common result of this event [19] Resonance energy transfer was used as a means of estimating the amount of aggregation and fusion among the DPPC vesicles A decrease in relative donor fluorescence occurs when the donor and acceptor probes located in separate populations of the liposomes are brought into close proximity during aggregation and fusion of the vesicles (Fig. 6) As the temperature of the pure DPPC vesicles was increased above 26°C, fluorescence slowly decreased, indicating that the vesicles were aggregating and possibly fusing. As the temperature approached the gel to liquid-crystalline transition, fluorescence markedly increased We suggest that this increased fluorescence in the donor as the lipids pass through their thermotropic transition is due to the following At the transition temperature, the mobility of the donor and acceptor probes in the bilayer would be

expected to increase as fluidity rises As a result, their distribution becomes more random, and their apparent proximity decreases It is interesting to note in passing that this dramatic change in fluorescence of the donor probe can in itself be used to indicate the phase transition As the temperature was increased beyond the transition, fluorescence decreased again, at a greater rate than before, suggesting increasing aggregation and fusion of the vesicles Addition of 5 and 10 mol% sodium palmitate altered both the extent of aggregation and fusion when compared to pure DPPC vesicles and also shifted the phase transition temperature of the vesicles containing palmitate If the sharp upward break in the fluorescence temperature profile is taken as the phase transition, addition of 5 mol% palmitate increased the apparent onset of the transition from 39 to 42°C which is in agreement with the FTIR results (cf Fig 4A) The extent of aggregation and fusion was indicated by the value of the relative donor fluorescence at a given temperature As the temperature was increased from 26°C the decline in fluorescence was more pronounced in those vesicles containing sodium palmitate, and at 33°C there was a substantial increase in the rate and extent of aggregation and fusion of these vesicles

Conclusions

Different free fatty acids apparently alter the physical properties of a phospholipid bilayer in different ways For example, linoleate acts as an impurity in multilamellar and sonicated vesicles of DPPC, and broadens the phase transition In contrast, a second free fatty acid palmitate, has much more striking effects on DPPC vesicles, causing, in addition to a significant broadening of the gel to liquid-crystalline phase transition, an increase in the transition temperature This increase in transition temperature indicates that the palmitate has a rigidifying effect on the DPPC bilayer, probably by increasing van der Waals interactions between the acyl chains of the DPPC and the palmitate In addition, the presence of the palmitate increases the apparent aggregation and fusion of DPPC vesicles, both below and above the phase transition temperature This fusion is reflected by increased leakage of a trapped solute at all temperatures Thus, in this simple model lipid system, many of the physical changes observed in the cell membranes from environmentally stressed plants can be elicited by the inclusion of increased free fatty acids in the bilayer

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