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Altered Phase Behavior in Membranes of Aging Dry Pollen May Cause Imbibitional Leakage[1]

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Aging of dry pollen has been shown to coincide with increases of free fatty acids and lysophospholipids. These compounds reduce the integrity of hydrated liposomes made from isolated pollen phospholipids but do not lead to their total destruction. However, a massive, instantaneous leakage occurs upon imbibition of dry cattail pollen (*Typha latifolia*) that has aged to the point of complete loss of viability. To resolve the apparent discrepancy in stability between hydrated and dry membranes, the lyotropic phase behavior of two liposome systems containing lysophospholipid (12 mol%) was studied with differential scanning calorimetry and Fourier transform infrared spectroscopy. In both systems dehydration caused phase separation of the lipids. Fourier transform infrared data concerning phase behavior of isolated membranes from aging pollen and of membranes in situ did not show phase separations, probably because the assay technique was not sufficiently sensitive to detect them. However, aging of the pollen resulted in a permanent increase in the gel-to-liquid crystalline phase transition temperature (T_m) of isolated membranes and in a broadening of the transition in situ. We conclude that the increase in T_m of hydrated membranes may be more closely related to the leakage.

In an atmosphere of 40% RH (dry), *Typha latifolia* pollen has a maximum life span of approximately 120 d at 24°C (van Bilsen and Hoekstra, 1993). At the higher humidity of 75% (semidry), aging is considerably accelerated, and viability is lost within 20 d. In general, dry and semidry aging exhibit the same characteristics: (a) lipid deesterification, resulting in the accumulation of LPLs and FFAs; (b) decreased PL content; (c) insignificant lipid peroxidation; (d) increased leakage of endogenous K^+ upon reimbibition coincident with loss of viability (van Bilsen and Hoekstra, 1993; van Bilsen et al., 1994). Also in several other pollen species, loss of viability during storage has been shown to coincide with deesterification of membrane lipids (van Bilsen et al., 1994). The impact of the observed changes in lipid composition on bilayer permeability has been studied in detail in liposomes composed of pollen lipids (van Bilsen and Hoekstra, 1993). An increased leakage from the liposomes due to the products formed during aging was observed. However, this moderate reduction in liposomal integrity cannot explain the instantaneous, massive leakage that occurs upon imbibition of aged pollen (Hoekstra and Van Roekel, 1985).

Also in aging seeds membrane PLs deteriorate and FFAs (Senaratna et al., 1988) and LPLs (Nakayama et al., 1981) accumulate. This is accompanied by an increase in membrane microviscosity and leakage of cytoplasmic solutes, which is indicative of reduced membrane integrity (McKersie et al., 1988).

In general, an increased leakage of cellular solutes from pollen is caused by problems at the membrane level (Hoekstra and Van der Wal, 1988; Sack et al., 1988; Crowe et al., 1989b, 1989c; Hoekstra et al., 1989, 1992). Thus, a membrane phase transition from gel to liquid crystalline was suggested to be responsible for the leakage and death of initially viable, dry pollen during imbibition in liquid medium (Hoekstra and Van der Wal, 1988; Crowe et al., 1989c; Hoekstra et al., 1992). FTIR measurements of intact pollen have indicated that the membrane T_m rises during drying to exceed room temperature (Crowe et al., 1989b, 1989c; Hoekstra et al., 1992). This means that membrane PLs in dry pollen are at least partially in gel phase at room temperature but entirely so at low temperature. That the phase change from liquid crystalline-to-gel phase during drying does not lead to leakage and death has been attributed to the lack of free water for solute transport under those conditions (Hoekstra, 1992). The situation during imbibition is entirely different in that ample water is then available for solute leakage during the reverse transition back to liquid crystalline phase. Imbibitional damage can be prevented by treatments or conditions that return the PLs to the liquid crystalline state prior to imbibition, such as preheating at imbibition and pretreatment in humid air (Hoekstra, 1984; Hoekstra and Van der Wal, 1988; Crowe et al., 1989a). Even when carefully prehydrated in water vapor, aged pollen still shows decreased germination (van Bilsen and Hoekstra, 1993; van Bilsen et al., 1994). This suggests that membrane phase properties may have irreversibly changed during aging.

Pollen aged under dry or semidry conditions always con-

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; FFA, free fatty acid; FTIR, Fourier transform infrared spectroscopy; 16:0-LPC, 16:0-*sn*-1-palmitoyllysophosphatidylcholine; LPL, lysophospholipid; MLV, multilamellar vesicles; PC, phosphatidylcholine; PL, phospholipid; T_m, gel-to-liquid crystalline phase transition temperature.

tains LPL and FFA. In contrast to the situation in the liquid crystalline state, mixtures of LPC and various PCs are immiscible when the PC is in the gel state (Van Echteld et al., 1980). This may occur during production of the LPLs in aging dry pollen. Thus, the newly formed LPLs may induce a lateral phase separation in the dry membranes, which may cause irreversible damage during imbibition. The effect of the FFA palmitic acid on the behavior of DPPC liposomes during drying and rehydration has already been studied in detail (Crowe et al., 1989d). Apart from a phase separation in the dry liposomes, palmitate induces fusion between the liposomes during rehydration, which is responsible for leakage of solutes from them. Many authors have also reported lateral phase separations of membrane constituents in plant cells that had been frozen (Gordon-Kamm and Steponkus, 1984; Quinn, 1985; Bryant and Wolfe, 1992).

In this paper we report the behavior of LPL in dry and hydrated model membrane systems in the ratio in which they occur in aging pollen membranes. These systems were studied with DSC and FTIR and are compared with membrane behavior in situ as studied by FTIR. We present evidence that extensive phase separation occurs in model systems during drying, but the methods for assay are insufficiently sensitive to detect these separations in vivo or in isolated membranes. However, aging of the pollen resulted in permanent increase in the T_m of the isolated membranes.

MATERIALS AND METHODS

Plant Material and Treatments

Collecting and handling of *Typha latifolia* L. pollen were performed as previously described (Hoekstra et al., 1991). For aging purposes pollen was incubated at a constant temperature of 24°C in an atmosphere of 75% RH produced by a saturated NaCl solution (final moisture content 0.17 g of water g^{-1} of dry matter). Incubations were done in desiccators provided with a fan inside to stir the air. Control pollen was stored at −20°C. Germination and leakage of cellular K^+ were determined as described elsewhere (Hoekstra et al., 1992). Pollen was prehydrated in water vapor at 24°C for 1 h before germination and leakage experiments.

Membrane Isolation

Microsomal membranes from control and aged pollen were obtained as described previously (Hoekstra et al., 1991; van Bilsen and Hoekstra, 1993) with the following modifications. To the isolation medium were added 1 mM each EDTA, EGTA, and diethylenetriaminepentaacetic acid; 5 mM ascorbic acid; and 1 mM DTT. The isolated membrane fraction was either used directly or lyophilized and stored at −80°C until needed.

Liposome Studies

All purchased lipids were used without further purification. For the PC/LPC mixtures the lipids were dissolved in $CHCl_3$:methanol (1:1, v/v) to ensure a homogeneous distribution of the LPC. The solvents were evaporated in a stream of nitrogen, and after the sample was dried under vacuum for 1 h, buffer (10 mM Tes, 0.1 mM EDTA [pH 7.4]) was added. Vesicles for FTIR and DSC measurements were prepared by vortexing the lipids in buffer. Unilamellar vesicles were produced by extrusion through one 100-nm pore size polycarbonate filter (Nuclepore Corp., Pleasanton, CA) 35 times using the LiposoFast extruder (Avestin Inc., Ottawa, Canada) according to the method of MacDonald et al. (1991). A portion of the freshly prepared vesicles was lyophilized to determine the transition temperatures of the dry material. All dried samples were handled under dry nitrogen or dry air to prevent partial rehydration on exposure to ambient air.

IR Spectroscopy

IR spectra were recorded on a Perkin-Elmer 1750 Fourier transform IR spectrometer equipped with a deuterated triglycine sulfate detector or a Perkin-Elmer 1725 FTIR equipped with a liquid nitrogen-cooled mercury cadmium telluride detector and a Perkin-Elmer microscope. Data were acquired and analyzed with a Perkin-Elmer 7500 data station or an MS-DOS computer using Perkin-Elmer software. Each spectrum was the average of at least 10 scans at each temperature in the IR region 3500 to 900 cm^{-1}. The gel-to-liquid crystalline phase transition was monitored by observing the shift with temperature in the IR absorption bands attributed to the CH_2 symmetric stretch (at 2850 cm^{-1}) of the PL acyl chains. Precooled samples were slowly warmed (40°C h^{-1}), and spectra were sampled at intervals. Where necessary, samples were lyophilized prior to recording the IR spectra, removed from the lyophilizer under vacuum, and sandwiched between two BaF_2 or CaF_2 windows under dry nitrogen or air to avoid rehydration as much as possible (Hoekstra et al., 1991).

DSC

Phase transitions of approximately 4 mg of lipid samples were measured with a Hart series 7077 high-sensitivity differential scanning calorimeter, assisted by an IBM PC-XT data station and Hart Scientific (Provo, UT) software (Crowe et al., 1989d). Samples were scanned from −30 to 90°C or higher at a rate of 20°C h^{-1}. This calorimeter has three sample cells, and as many as three samples can be run simultaneously for direct comparison. Dry samples were loaded into the sample pans and sealed in a glove box flushed with dry nitrogen.

Chemicals

All organic solvents were purchased from Merck (Darmstadt, Germany), 16:0-LPC was obtained from Serva (Heidelberg, Germany), and egg PC was from Avanti Polar Lipids (Birmingham, AL) and Fluka (Buchs, Switzerland).

RESULTS

Imbibitional Leakage of Aged Pollen

Figure 1 shows that within the 1st min of imbibition at 24°C virtually all endogenous K^+ had leaked from aged pollen grains. In control pollen (germination capacity 91%) the leakage was slower and did not exceed 46% of the total

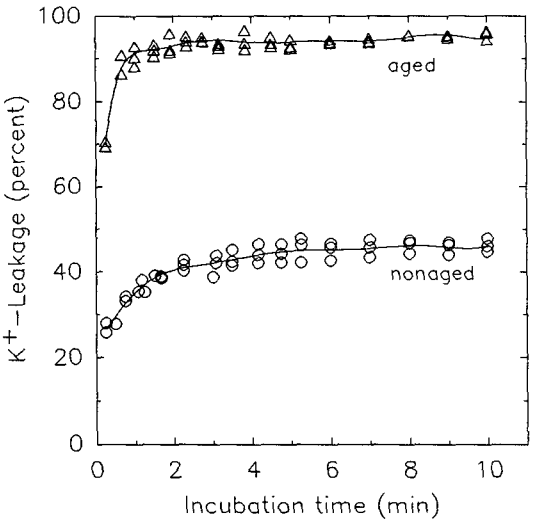

Figure 1. Leakage of endogenous K^+ from aged and nonaged *T. latifolia* pollen. Pollen was exposed for 1 h to humid air at 24°C prior to immersion in liquid medium. Aging was achieved by storage at 75% RH and 24°C for 12 d (0% germination). Leakage was monitored for 10 min after immersion of the pollen grains in germination medium without K^+.

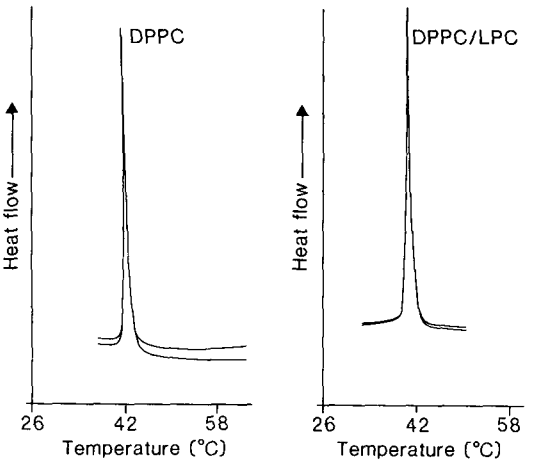

Figure 2. Two sequential DSC heating scans of hydrated MLV composed of pure DPPC and a DPPC/LPC mixture (12 mol% 16:0-*sn*-1-LPC). The samples were run simultaneously.

amount of extractable K^+. Care was taken not to cause injury during the change from the dry to the imbibitional state by carefully rehydrating the pollen in humid air for 1 h prior to imbibition. However, prehumidification did not reduce K^+ leakage from the aged pollen (data of leakage from nonprehumidified aged pollen not shown). This indicates that possible aging-related phase separations in the membranes are not properly reversed by this pretreatment.

Influence of LPL on Phase Behavior of Liposomes

To understand the massive leakage from the aged grains during rehydration, liposome studies were undertaken, with special reference to the effects of LPL on the melting behavior and miscibility of the lipids in the hydrated and dehydrated conditions. The gel-to-liquid crystalline phase transition of hydrated 16:0-*sn*-1-LPC, which is a naturally occurring LPC in aging cattail pollen (in addition to 18:2-*sn*-1-LPC and 18:2-*sn*-2-LPC [van Bilsen and Hoekstra, 1993]), was determined with DSC and FTIR (data not shown). With both methods, T_m for this phase change in the hydrated micelles occurred at 2°C, in accordance with previous results with DSC (Van Echteld et al., 1980, 1981). In the dry micelles, T_m was seen with DSC to rise to 94°C. An attempt to measure the same transition temperature with FTIR indicated that up to 75°C the transition had not yet occurred, but because of instrumental limitations, scanning at higher temperatures was not possible.

The phase transition of fully hydrated liposomes composed of DPPC (Fig. 2) or egg PC (Fig. 3A) was not affected by the addition of 12 mol% LPC, which is the amount of LPL occurring after a few days of aging (van Bilsen and Hoekstra, 1993). In general, larger amounts of LPC are required to generate a detectable phase separation in excess water (Van

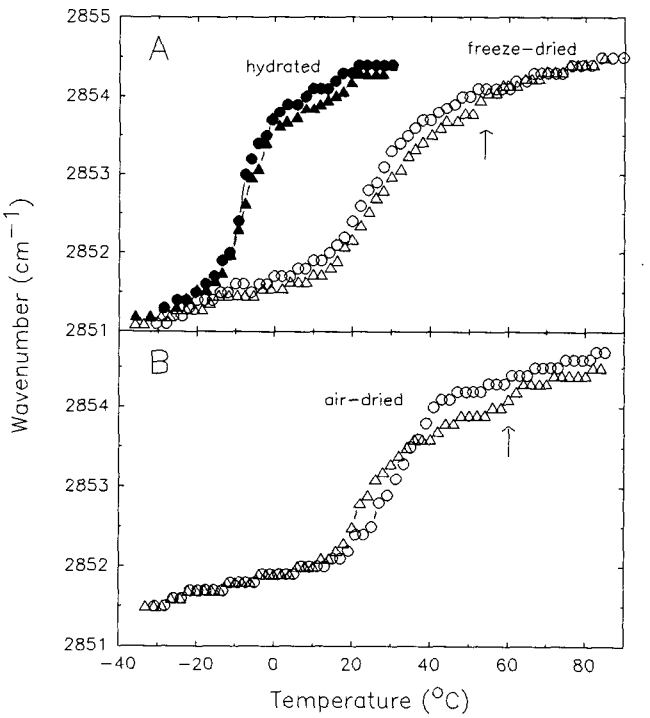

Figure 3. A, Frequency versus temperature plots (FTIR) of hydrated and freeze-dried vesicles composed of egg PC and an egg PC/LPC mixture (12 mol% 16:0-*sn*-1-LPC). Circles, Egg PC; triangles, egg PC/LPC. The data points represent vibrational frequencies of the symmetric CH_2 band. In the presence of LPC, a second transition is seen in the freeze-dried sample, indicated by the arrow. B, Same as A, except that the specimens were dried in a stream of dry air for 3 h (final water content less than 0.05 g of water g^{-1} dry weight as determined by ^3H_2O evaporation and described by Crowe et al., 1987).

Echteld et al., 1980, 1981). The phase behavior of hydrated egg PC was studied by FTIR and not by DSC, because the thermotropic phase transition of the lipid lies in the region of the ice endotherm.

Drying evokes large changes in phase behavior. Using DSC, we found increased T_ms for dried DPPC and egg PC liposomes compared to the hydrated controls (Figs. 4 and 5). The elevated T_ms were established at approximately 72 and 44°C for dry DPPC and dry egg PC, respectively. For DPPC this is less than reported by other authors (Kodama et al., 1985; Crowe and Crowe, 1988b). Based on published phase diagrams for DPPC (Kodama et al., 1985), the samples used here contained about 5% water.

During drying of mixtures of these PLs with LPC lateral phase separation occurred (Figs. 4 and 5). Dried liposomes composed of DPPC and LPC exhibited two phase transitions (Fig. 4), indicating the separate melting of two different lipid domains. When this phase-separated sample was heated for a short period above its highest transition, then rapidly cooled, and scanned again, its lower transition had vanished (Fig. 4, dotted line), which is indicative of remixing of the components. In dry egg PC with a T_m below that of dry 16:0-LPC, the presence of LPC also caused a dehydration-dependent phase separation (Fig. 5). In addition, FTIR spectra were run on freeze-dried and air-dried egg PC/LPC vesicles (Fig. 3). The wave number versus temperature plots of the dry egg PC/LPC mixture are complementary to the DSC scans. The freeze-dried samples showed a small apparent phase separation (Fig. 3A), whereas in the air-dried samples a clear phase separation was apparent (Fig. 3B). Furthermore, the cooperativity of melting of the egg PC-enriched fraction is diminished, in agreement with the DSC scan (cf. Fig. 3B and Fig. 5, right).

Phase Behavior of Isolated Pollen Membranes

To investigate whether phase separation occurs in dry membranes of aging cattail pollen, microsomal membranes were isolated after 0, 6, and 12 d at 75% RH and 24°C. Pollen viability declined steadily during the aging (91% germination on d 0, 31% on d 6, and 0% on d 12). It was previously shown that the membranes isolated from aged

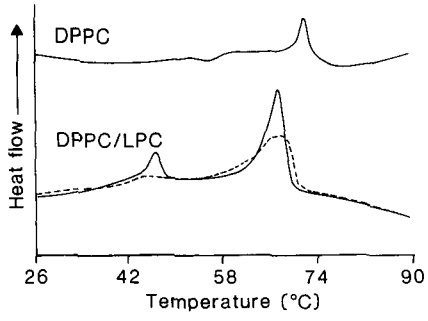

Figure 4. DSC scans of dried MLV composed of pure DPPC and a DPPC/LPC mixture (12 mol% 16:0-*sn*-1-LPC). The dotted curve represents the second scan of the DPPC/LPC mixture. The two samples were run simultaneously.

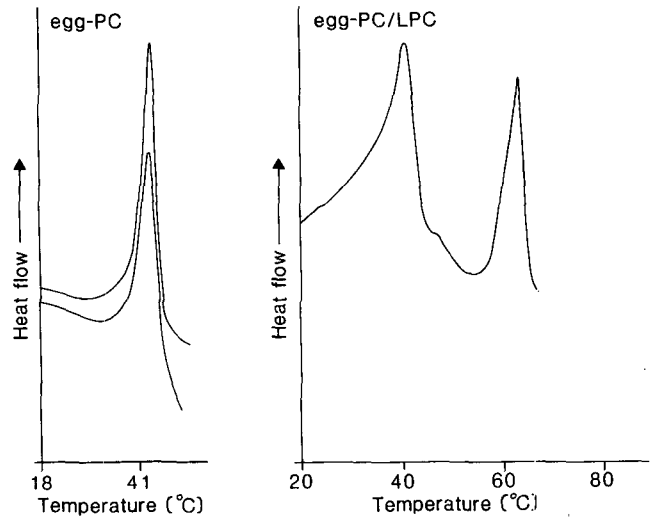

Figure 5. DSC scans of dried MLV composed of egg PC (two scans) and an egg PC/LPC mixture (12 mol% 16:0-*sn*-1-LPC). The two samples were run simultaneously. The egg PC alone showed no other melting event other than the one shown.

pollen contain LPL and that LPL accumulation was not an isolation artifact (van Bilsen and Hoekstra, 1993). Freeze-dried microsomal membranes isolated from viable (control) pollen showed a cooperative melting between 40 and 60°C when studied by FTIR (Fig. 6A). To our surprise no evidence of phase separation was seen in the dry membranes regardless of how long the pollen had been aged. However, upon rehydration of these freeze-dried membranes, clear differences emerged. Nonaged pollen membranes showed a phase transition centered at about 0°C, in agreement with previously published results (Hoekstra et al., 1991). Membranes from pollen aged for 12 d exhibited a phase transition of approximately 25°C, with that of 6-d-aged pollen being intermediate. This increased phase transition of membranes from the aged pollen was not due to the previous freeze-drying, since it also was seen in the hydrated membranes that had never been dried (Fig. 6B).

Phase Behavior in Membranes of Intact Pollen

The major disadvantage of using isolated membranes in these studies of membrane behavior in the dry state is that the pollen has to be rehydrated before the membranes can be isolated, thus potentially introducing artifacts. FTIR spectra of control and aged dry pollen were recorded to determine whether membrane phase separation could be seen in situ during aging. Before the measurements the moisture contents of the two pollen samples were adjusted to about 8%, representing the water content after exposure for 2 d to 50% RH over a saturated $Ca(NO_3)_2$ solution. In Figure 7 the wave number versus temperature plots for control and aged pollen are shown. Whereas the nonaged pollen has mostly a discrete wave number shift, giving a T_m of approximately 10°C, it is not possible to estimate T_m of pollen after 12 d of storage at 75% RH. The phase transition in the aged pollen is broader and more gradual than in the control pollen. Furthermore,

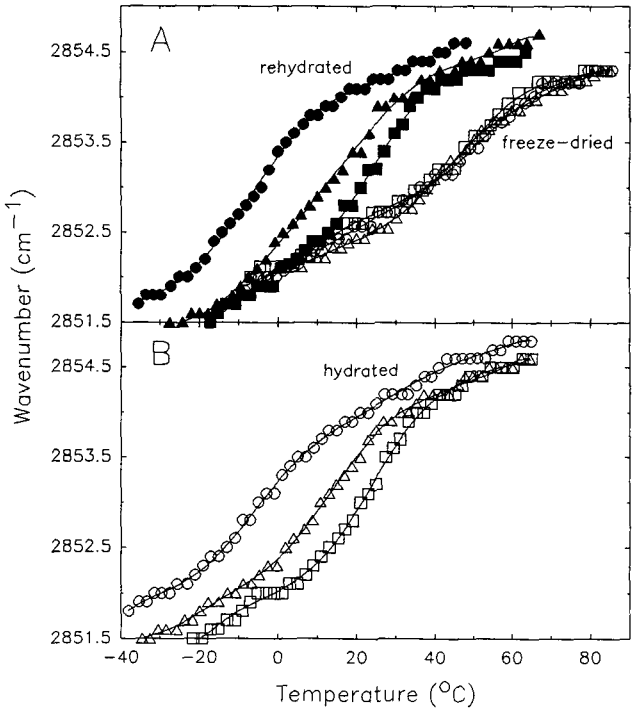

Figure 6. Frequency versus temperature plots (FTIR) of microsomal membranes isolated from nonaged (circles; 91% germination), 6-d-aged (triangles; 31% germination), and 12-d-aged (squares; 0% germination) cattail pollen (75% RH at 24°C). Curve fitting was by ninth-order polynomial. A, Freeze-dried and rehydrated membranes; B, hydrated membranes that have not previously been freeze-dried.

there is no evidence of phase separation. The much lower T_m in pollen compared to dry membranes is due to a difference in water content and the endogenous Suc in the pollen (Hoekstra et al., 1991).

DISCUSSION

With respect to dehydration, pure 16:0-LPC exhibits the same characteristics as DPPC and egg PC, i.e. T_m increases considerably (Crowe and Crowe, 1988a). In hydrated vesicles phase behavior does not change as a result of the presence of 12 mol% 16:0-LPC. In dry vesicles the effect of the lysolipid is of a totally different nature. Both the DSC scans and the FTIR data indicate that in the presence of 16:0-LPC the vesicles undergo a dehydration-dependent phase separation, irrespective of whether T_m of the PL is higher (DPPC) or lower (egg PC) than the T_m of the lysolipid. This is reflected by the two melting endotherms in the DSC scans and separate transitions seen in the vibrational frequency of the symmetric CH_2-stretching bands. About the exact nature of these domains we can only speculate. LPLs can form bilayers with interdigitated acyl chains, but the formation of this type of lysolipid domain involves large differences in bilayer thickness (Hauser et al., 1981). We suggest that for the egg PC/LPC mixture the first endotherm reflects the melting of egg PC domains, and the second endotherm reflects the melting of a mixture preferentially composed of 16:0-LPC. Because the temperature for the higher transition is much lower than that of pure, dry 16:0-LPC, it cannot be attributed to the melting of pure 16:0-LPC domains. In the case of the DPPC/LPC mixture the lower transition corresponds to the melting of domains preferentially consisting of LPC, possibly with traces of DPPC. The higher transition corresponds to the melting of DPPC domains. Apparently, LPC is not only immiscible in the gel phase of both tested PCs but also in the dry liquid crystalline phase the components are not homogeneously mixed.

Drying of hydrated nonaged cattail pollen causes the T_m of its membranes to exceed room temperature, which leads to gel phase domains in the dry state (Crowe et al., 1989c; Hoekstra et al., 1992). Considerable leakage occurs when this dry pollen is plunged in germination medium. Imbibitional leakage can be minimized by pretreatment in humid air, suggesting that possible phase separations can be reversed by this treatment (Hoekstra and Van der Wal, 1988; Crowe et al., 1989c). However, prehumidifying could not prevent the rapid and complete leakage of K^+ from the aged grains upon immersion in germination medium. This effect could conceivably be due to phase separations in the dry state, in view of the results with the model lipid system, but we were unable to obtain evidence for such phase separations in isolated membranes or intact pollen. At first we were puzzled as to why the phase separation is not seen in these biological membranes. However, considering the fact that only 12 mol% of the PLs is in the form of LPC, one cannot expect a large phase separation to be seen by FTIR; taking this into account, along with the fact that the LPL has only a single hydrocarbon chain, we calculate that the maximum frequency change due to the LPL cannot be more than 0.2 cm^{-1}. We cannot rule out the occurrence of phase-separated LPLs and their involvement in the imbibitional leakage; even a small amount of phase separation could lead to leakage.

It is more likely that the increased T_m of rehydrated mem-

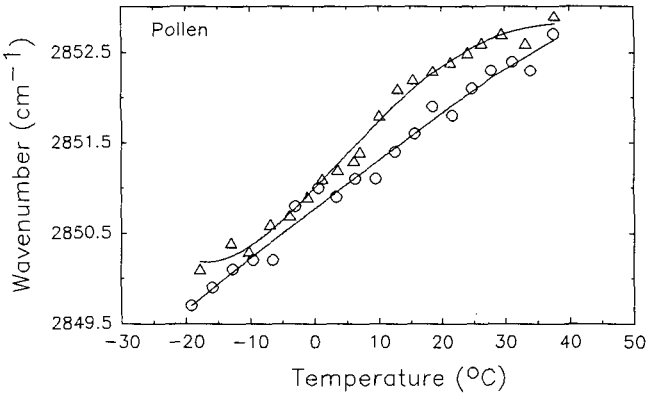

Figure 7. Frequency versus temperature plots (FTIR) of dry, nonaged, and aged cattail pollen. Aging was achieved by storage at 75% RH and 24°C for 11 d. The pollen samples were equilibrated at 50% RH for 2 d before the measurements, giving a water content of 0.095 g of water g^{-1} dry weight. The data points represent vibrational frequencies of the symmetric CH_2 band. Curve fitting was by fourth-order polynomial.

branes isolated from aged pollen is related to the imbibitional leakage. Pollen was allowed to imbibe at 24°C, which is near the T_m of the membranes isolated from aged pollen. Since the elevated T_m is apparently irreversible, existing before the membranes were freeze-dried, prehumidification in humid air of aged pollen is not expected to alleviate the leakage. Even in the presence of endogenous Suc in situ T_m of the membranes will be elevated.

Imbibitional damage of viable dry pollen can also be decreased by preheating during imbibition (e.g. at 35°C), allowing for the melting and remixing of membrane components before the pollen is allowed to imbibe (Hoekstra et al., 1992). For aged pollen, preheating to even higher temperatures, at which full melting and mixing occurs, might be a strategy to improve germination (cf. the dotted curve of the second scan in Fig. 4). However, because of the apparently increased T_m, the culture then has to be maintained at a higher temperature, too, until repair mechanisms restore T_m to its normal level. Previous attempts along these lines have been unsuccessful, possibly because the permanent elevation in T_m was not understood until the present investigation.

The chemical changes behind the increased T_m in membranes from aged pollen are not clear, but some comments can be made, nevertheless. FFAs in large amounts (5–10 mol%), particularly palmitic acid, can increase T_m in hydrated (McKersie et al., 1988, 1989) but not dry (Crowe et al., 1989d) model bilayers. Since the amounts of FFAs in aged pollen (75% RH, 24°C) can reach about 10 weight percent of the PL content (van Bilsen and Hoekstra, 1993), it would appear that FFA may play a role here. Furthermore, the presence of FFAs during drying of model liposomes is responsible for extensive leakage during rehydration, which is associated with fusion (Crowe et al., 1989d). This may explain the excessive leakage during rehydration of aged pollen. In contrast, readded FFAs from aged pollen cause only modest and relatively slow leakage from hydrated liposomes made from pollen PCs (van Bilsen and Hoekstra, 1993). Peroxidation of PLs cannot be the cause of the elevated T_m, since chemical analysis of aged pollen showed no preferential loss of unsaturated PLs (van Bilsen and Hoekstra, 1993). Chemical analysis did show an increase in phosphatidic acid and a considerable decrease in PLs, particularly in PC and phosphatidylethanolamine. Effects of such changes in PLs on the phase behavior of membranes have not yet been studied.

We conclude that LPLs incorporated in model membranes phase separate upon dehydration. It is not clear whether the LPLs formed during dry and semidry storage of pollen similarly phase separate and thus contribute to imbibitional leakage and death. We conclude instead that the increase in T_m in hydrated membranes may more closely be related to the leakage.

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