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Membrane phase transitions are responsible for imbibitional damage in dry pollen

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ABSTRACT **We have found that the most probable cause of the leakage seen when dry cells or organisms such as seeds, pollen, or yeast cells are plunged into water is a gel to liquid crystalline phase transition in membrane phospholipids accompanying rehydration. By using Fourier transform infrared spectroscopy we have recorded infrared spectra of CH_2 stretching vibrations in dry and partially hydrated intact pollen grains of *Typha latifolia*. The vibrational frequency changes abruptly as phospholipids pass through the gel to liquid crystalline phase transition. Below the apparent transition, viable pollen shows low germination and high leakage when placed in water, but above the transition germination increases and leakage decreases. The apparent transition temperature falls with increasing water content, much as in pure phospholipids. By using this phenomenon, it was possible to construct a hydration-dependent phase diagram for the intact pollen. This phase diagram has immediate applications since it has high predictive value for the viability of the pollen when it is placed in water.**

When dry seeds (1, 2), pollen (3), yeast cells (4), and anhydrobiotic microscopic animals (5) are rapidly rehydrated they leak their soluble cellular contents into the surrounding medium, as a result of which they are often killed. This imbibitional leakage has long been known to be a major source of damage to the dry organisms, particularly if the imbibition is accomplished at low temperatures (6). Economic losses result if, for example, dry soybean seeds are imbibed in the field during cold weather. Consequently, numerous workers have investigated the physical basis for the leakage. Simon (1, 7) suggested that it is due to formation of nonbilayer phases, particularly the inverse hexagonal (H_{II}) phase in the plasma membrane of the dry cells. According to this hypothesis, the presence of H_{II} phase lipids would be incompatible with maintenance of a low-permeability bilayer. When the cell is rehydrated, the H_{II} lipids would then enter lamellar phase, and leakage would be abated. This appealing idea was quickly embraced by the scientific community, and many workers began to search for H_{II} phase lipids in plasma membranes of dry cells. However, there is good evidence that H_{II} lipids are not found in membranes of dry but viable organisms (8–10), and we have sought an alternative explanation for the leakage.

When pure phospholipids are dehydrated their transition temperature rises, in some lipids by 70°C or more (11, 12). Consequently, a fully hydrated phospholipid that is in liquid crystalline phase at room temperature might very well undergo a transition to gel phase when it is dried at the same temperature, followed by a transition back to liquid crystalline phase when it is rehydrated (see ref. 13 for recent discussion). It is also well known that leakage occurs when hydrated phospholipid vesicles are heated through their thermotropic gel to liquid crystalline phase transition (14) or when dry gel phase vesicles are rehydrated and pass through the gel to liquid crystalline transition (15). Thus, it seems possible *a priori* that the leakage from dry organisms during rehydration might be due to a gel to liquid crystalline lipid phase transition. According to this idea, membrane phospholipids in the dry organism would be in gel phase. When the dry organism is placed in water, the gel phase lipids would undergo a transition to liquid crystalline phase, during which leakage would occur. We now show that imbibitional leakage from dry intact pollen grains is almost certainly due to such transitions.

MATERIALS AND METHODS

Sample Preparation. Pollen of cattail (*Typha latifolia*) was chosen as a model system to use in these studies primarily because the pollen is available in large quantities. The pollen was collected from the field and stored at −20°C until use. The only preparation given to the pollen samples before the experiments were conducted was to remove a hydrocarbon layer on the outside of the cell wall and to adjust the water content. In a recently published study (16), a crystalline phase hydrocarbon in the same pollen studied here was detected by x-ray diffraction. This crystalline phase was attributed to a hydrocarbon on the surface of the pollen, the presence of which not surprisingly makes it impossible to obtain infrared spectra of cellular portions of the intact pollen, as described below. Thus, this extracellular hydrocarbon was removed by washing the dry pollen in hexane briefly, after which treatment the pollen remain fully viable. Water contents of the samples were adjusted by equilibrating them over known relative humidities. At equilibrium the samples were transferred to the sample chamber in a cold room at 4°C to minimize changes in water content during the transfer. To obtain dry samples, the pollen grains were evacuated on a VirTis lyophilizer for at least 24 hr at 0.01 mmHg, after which they were removed from the lyophilizer under vacuum and transferred to a glove box flushed with dry nitrogen. The samples were removed from the vacuum flask in the glove box and loaded in the sample chamber.

Infrared Spectroscopy. Infrared spectroscopy was done with a Perkin–Elmer 1750 Fourier transform infrared spectrometer assisted by a Perkin–Elmer 7500 data station. Temperature control was by means of a Peltier device, and sample temperature was recorded with a thermocouple against the window. The samples were loaded in a monolayer between two BaF_2 windows for acquisition of the spectra. Ten spectra were recorded at each temperature and stored without further data processing. Frequencies of the CH_2 bands were computed on the data station to the nearest 0.1 wavenumber by the center of gravity algorithm of Cameron *et al.* (17).

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Abbreviations: T_m, midpoint of phase transition; G_m, midpoint of germination curve.

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Measurement of Germination and Leakage. Germination of the pollen and leakage of NAD^+ were measured as described (3).

RESULTS AND DISCUSSION

Use of Infrared Spectroscopy to Measure Phase Transitions. We have found that Fourier transform infrared spectroscopy is particularly useful for measuring phospholipid phase transitions in intact, dry pollen. Spectra were obtained between 3000 and 2800 wavenumbers, the region where CH_2 vibrations in hydrocarbon chains are strongest. With increasing temperature the vibrational frequency (or wavenumber) of these bands increases abruptly in pure phospholipids (18) and in intact cells (19) as they pass through the gel to liquid crystalline transition temperature. Surprisingly, it was possible to detect CH_2 bands clearly in the spectra from intact pollen (Fig. 1). When the pollen was heated in the spectrometer (14, 15) and scans were taken at different temperatures, a clear increase in vibrational frequency of the CH_2 groups was seen (Fig. 1). This elevation in frequency as a function of temperature can be recorded to produce the results shown in Fig. 2, and the transition temperature can be estimated from the midpoint of this frequency change (T_m in Fig. 2).

Infrared spectroscopy is far more useful for measuring phase transitions in a complex mixture of lipids, proteins, etc., such as that found in a biological membrane or in the pollen studied here than is the more widely used differential scanning calorimetry for reasons that we will document in detail elsewhere (20). Briefly, when differential scanning calorimetry scans are done on a native biological membrane small melting endotherms are seen during warming that probably represent melting of small domains of gel phase lipid. These small endotherms are often of such low enthalpy that it is difficult to distinguish them from noise in the baseline. When the same sample is studied with infrared spectroscopy, by contrast, successive melting of small gel phase domains results in a smooth, progressive increase in the average vibrational frequency. The result is the kind of curve seen in Fig. 2. The apparent T_m can be extracted from the midpoint of the curve by probit analysis, as described elsewhere (20).

Hydration-Dependent Phase Transitions in Intact Pollen. Spectra similar to those shown in Fig. 1 were taken from

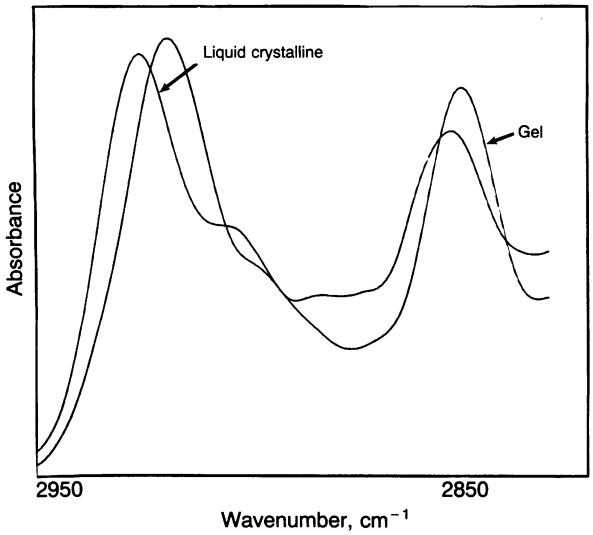


FIG. 1. Representative infrared spectra for intact pollen grains of *T. latifolia* containing about 0.7 g of water per g of dry weight. As temperature is increased the vibrational frequency (wavenumber) for the CH_2 bands increases (14, 15).

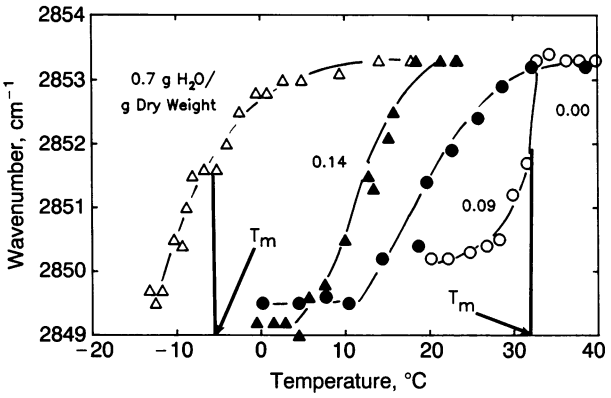


FIG. 2. Vibrational frequencies for CH_2 symmetric stretch as a function of temperature in samples of pollen grains with different water contents. Water contents (expressed as g of water per g of dry weight) are shown in the numbers by each curve. The midpoint of the transition (T_m) is indicated for two of the curves (15).

pollen at different water contents, and the resulting vibrational frequencies were measured, yielding the curves in Fig. 2. According to these data, the apparent T_m changes with water content. The four representative plots shown in Fig. 2 suggest that T_m rises from about −6°C in the hydrated poller to about 32°C when the pollen is dried. In pollen with intermediate water contents, intermediate T_m values were obtained (Fig. 2).

Assignment of Phase Transitions to Phospholipids. The following studies were conducted to establish that the data seen in Figs. 1 and 2 represent phase transitions in membrane phospholipids. The procedures used in these studies and the detailed results are presented elsewhere (20). Briefly, the major hydrocarbons in these pollen grains are triglycerides, phospholipids, and a hydrocarbon on the surface that can be removed by washing the pollen grains briefly in hexane. This treatment does not alter viability, so all studies reported here were conducted with pollen from which this extracellular hydrocarbon had been removed. To distinguish between transitions in the remaining triglycerides and phospholipids, we isolated both of these lipid fractions and measured their T_m values with differential scanning calorimetry and Fourier transform infrared spectroscopy. The triglycerides undergo melting at 10–15°C. This transition does not change with water content, indicating that the hydration-dependent transitions seen in Fig. 2 cannot be due to melting of the triglycerides. By contrast, the fully hydrated phospholipids have a T_m of −6°C, whereas that of the dry phospholipids is in excess of 30°C. We conclude that the hydration-dependent transitions recorded in Fig. 2 can be assigned to the phospholipids.

Physiological Significance of the Phase Transition. Our hypothesis that membrane phase transitions are responsible for imbibitional damage in dry pollen suggests that the transition temperature should closely predict rates of leakage from the pollen during rehydration and the subsequent germination success. Physiological data on leakage from and germination of the pollen as a function of imbibitional temperature (17) indeed agree well with the measurement of T_m. Pollen grains with various water contents (produced by equilibrating the pollen to known relative humidities) were imbibed in germination medium at several temperatures. Leakage of NAD^+ was recorded immediately, and germination was measured subsequently. Some representative results, shown in Fig. 3, demonstrate that germination increases with temperature coincidentally with the increase in vibrational frequency of the CH_2 bands. Furthermore, leakage declines over the same temperature range as germination and vibrational frequency increase (Fig. 3). It is possible to extract from the germination/temperature plots the midpoint

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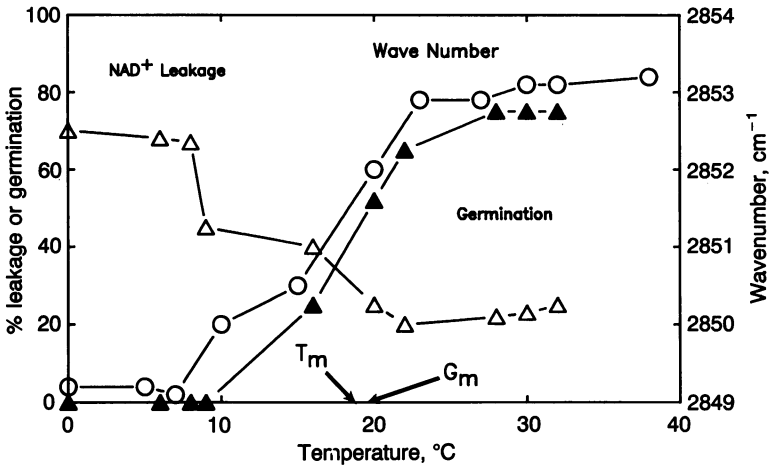


FIG. 3. Vibrational frequencies for CH_2 symmetric stretch as a function of temperature in a pollen sample containing about 0.1 g of water per g of dry weight. Also shown are data for germination of and leakage of NAD^+ from pollen adjusted to about 0.1 g of water per g of dry weight by exposing them to moist air before they were imbibed in germination medium at the indicated temperatures. Germination and leakage data are expressed as % of maximal values recorded. The temperature at which germination reaches half-maximal value (G_m) is also indicated.

of the germination curve, a parameter we have called G_m in Fig. 3. It is apparent from the data shown in Fig. 3 that G_m is approximately equal to T_m.

Phase Diagram for Intact Pollen. Data similar to those shown in Fig. 3 were produced for pollen with a variety of water contents, and T_m and G_m were extracted from plots of germination or vibrational frequency of the CH_2 bands. From these data we have constructed the hydration-dependent phase diagram for the intact pollen shown in Fig. 4. The phase diagram shows unquestionably that T_m rises steeply when water content is decreased below about 0.25 g of H_2O per g of dry weight, a figure that agrees well with values for hydration dependence for T_m in pure phospholipids (8). Above the line, the phospholipids will be in liquid crystalline phase; below the line, they will be in gel phase. Also shown in Fig. 4 are the values for G_m, which agree with those for T_m; as T_m falls with increasing water content, G_m falls coincidentally. Above this line, the pollen grains do not leak when they are placed in water, and they show maximal germination. Below the line, they leak extensively and show minimal germination.

Mechanism of Imbibitional Leakage: A Hypothesis. Based on the data presented in Figs. 1–4, we suggest the following hypothesis concerning the cause of imbibitional leakage, summarized in Fig. 5. When pollen, and probably other organisms are dehydrated, T_m for membrane phospholipids

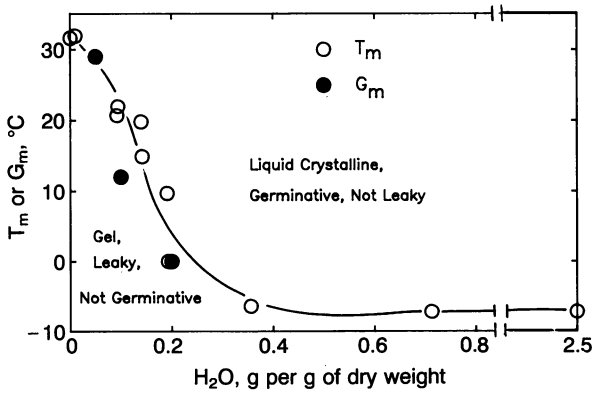


FIG. 4. Hydration-dependent phase diagram for pollen obtained by extracting values for T_m from the data shown in Fig. 2 and similar data not shown. For comparison, G_m values extracted from germination data shown in Fig. 3 and similar data for pollen imbibed at other water contents are also shown.

increases. As a result, when the cells are rehydrated, the phospholipids undergo a transition from gel to liquid crystalline phase. Consequently the cells leak their contents to the medium, and they are killed. By contrast, if the dry cells are heated to above the gel to liquid crystalline phase transition temperature before they are placed in water, they do not undergo a phase transition during rehydration and they do not leak (Fig. 5). Similarly, if the cells are partially rehydrated by exposing them to water vapor before they are placed in bulk water, T_m may be reduced so that the partially hydrated phospholipids are in liquid crystalline phase. In either case, during rehydration phospholipids in membranes of the dry cells pass from liquid crystalline phase to liquid crystalline phase when they are rehydrated, and they do not leak (Fig. 5). If one is to predict the conditions under which this situation can be obtained, it is essential to have available a detailed phase diagram of the kind shown in Fig. 4.

Utility of the Phase Diagram. It is now feasible to produce phase diagrams similar to that shown in Fig. 4 for all cells that are capable of surviving dehydration, including plant propagules of commercial importance. By using such phase diagrams it will be possible to predict the combination of temperature and water content at the time the cells are placed in bulk water that will result in minimal leakage and maximal germination. This procedure should be particularly useful since a full phase diagram can be constructed in a matter of a few hours with Fourier transform infrared spectroscopy, whereas the comparable germination studies under the variety of conditions that would be required may take weeks or even months to complete. We conclude that the results presented here not only explain an old puzzle—imbibitional damage in dry organisms—but also may have considerable practical importance.

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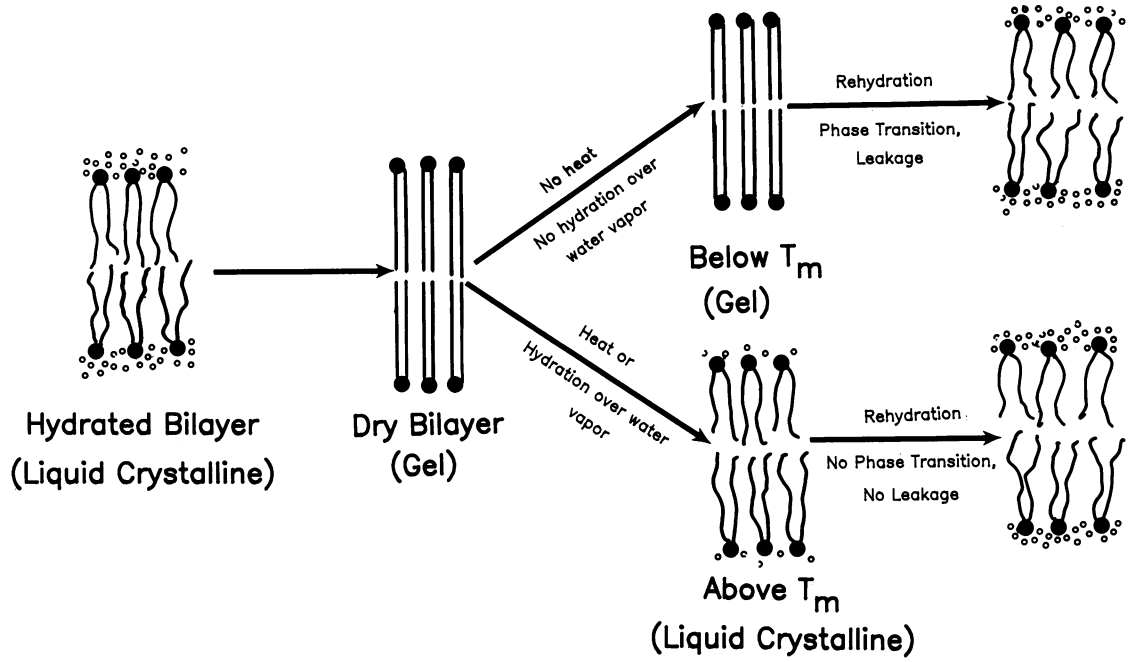


FIG. 5. Diagrammatic representation of the proposed mechanism for imbibitional leakage. As a hydrated bilayer (water molecules are represented by small, open circles) in liquid crystalline phase is dehydrated, it may enter gel phase, depending on the temperature. If this dry bilayer is not heated to above its transition temperature (T_m) before it is returned to water (upper pathway), it will undergo a phase transition during rehydration during which it would be expected to leak. On the other hand, if it is heated to above T_m or partially hydrated by exposure to water vapor (lower pathway), the bilayer can pass through the phase transition in the absence of bulk water. Under these conditions it will not undergo a phase transition when it is placed in water and it will not leak.

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