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# MINI-REVIEW

# Phase Transitions and Permeability Changes in Dry Membranes During Rehydration

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## Abstract

Dry phospholipid bilayers are known to undergo transient changes in permeability during rehydration. In this review, we present evidence from which we suggest that this permeability change is due to a gel to liquid-crystaline phase transition accompanying rehydration. If the transition is avoided, as in lipids that remain in gel phase whether dry or rehydrated, the problem of leakage during rehydration is obviated, at least in part. Further, the evidence that the transition temperature for dry bilayers can be depressed by certain sugars is discussed. Finally, we show that these principles can be extended to intact cells. Using pollen grains as a model, we have measured the transition temperature for membrane phospholipids and show that the transition is correlated with physiological measurements including permeability changes and subsequent germination. From the $T_m$ values taken from pollen grains at different water contents, we have constructed a phase diagram for the intact pollen that has high predictive value for physiological properties.


**Key Words**: Phase transitions; phospholipids; water; desiccation; dehydration; anhydrobiosis; imbibition.

## Introduction

Studies on natural membranes and pure phospholipids have demonstrated over the past two decades that dramatic alterations in the organization of membrane lipids are induced as a result of thermotropic and dehydration-induced phase transitions (reviewed in J. Crowe *et al.*, 1987, 1988a; L. Crowe and Crowe, 1986, 1988; Quinn, 1985). Among these alterations are phase

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separations of membrane constituents (L. Crowe *et al.*, 1985, 1986), leading to such phenomena as aggregation of membrane proteins (J. Crowe *et al.*, 1983) and formation of nonbilayer phases (L. Crowe and Crowe, 1982); fusion between adjacent bilayers (L. Crowe *et al.* 1986; Womersley *et al.*, 1986); and transient permeability changes, resulting in leakage of the contents of vesicles or cells to the surrounding medium (reviewed in L. Crowe and Crowe, 1986; J. Crowe *et al.*, 1988b).

The events described above are usually catastrophic for a living cell. Nevertheless, numerous organisms are capable of surviving conditions under which their membranes would be expected to undergo phase transitions that usually result in death. For example, seeds and pollen of plants, spores of bacteria and fungi, yeast cells, and even some species of animals are capable of surviving almost complete dehydration, a living state known as anhydrobiosis (Keilin, 1959; J. Crowe and Clegg, 1978). Under these conditions, we would expect, from what we know about the lyotropic effects of water on the physical properties of phospholipids, that membrane phospholipids would undergo a liquid-crystalline to gel phase transition during dehydration (Chapman *et al.*, 1967; Kodama *et al.*, 1982, 1985a, b), leading to the kinds of damage mentioned above. This minireview deals with the mechanisms by which these organisms escape this damage. Since we have written several more general reviews on this subject in the past several years (J. Crowe *et al.*, 1987, 1988a–c; L. Crowe and Crowe, 1986, 1988), we will focus on one aspect of the problem: permeability changes in bilayers as a function of their hydration state.

## Permeability of Bilayers During Thermotropic Phase Transitions

As phospholipid bilayers pass through the thermotropic transition from gel ($L_\beta$) to liquid–crystalline ($L_\alpha$) phase, they undergo transient changes in permeability (e.g., Hammouhdah *et al.*, 1981). We illustrate this point through a specific example. Small (100-nm diameter) unilamellar vesicles composed of dimyristoylphosphatidylcholine (DMPC) were prepared with carboxyfluoroscein trapped in the aqueous interior, and leakage of the fluorescent probe was then monitored as the vesicles passed through the main calorimetric phase transition, which, in the case of DMPC, is centered on 23°C. The results (Fig. 1) show that leakage of the probe commences at $\sim 20$°C and is complete by 27°C. Thus, the midpoint of the leakage curve occurs at 23.5°C, occurring simultaneously with the calorimetric transition.

It is also possible to measure phospholipid phase transitions with infrared spectroscopy, based on the temperature dependence of the frequency and bandwidth of the $CH_2$ vibrations in the hydrocarbon chains (reviewed in

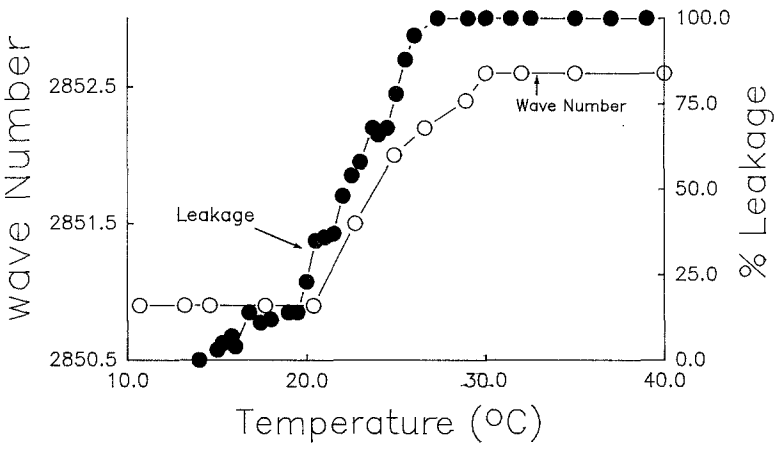


**Fig. 1.** Leakage of carboxyfluorescein from unilamellar vesicles of DMPC as they are heated through their transition temperature. Also shown are data for vibrational frequency (wave number) of the $CH_2$ symmetric stretch in the hydrocarbons as a function of temperature.

Cameron and Dluhy, 1987). This method for measuring lipid phase transitions is particularly useful with complex mixtures of lipids (Blazyk and Rana, 1987; Cameron and Dluhy, 1987; Chapman *et al.*, 1980) and even intact cells (Cameron *et al.*, 1983; Loeffelholz *et al.*, 1987), as we will discuss later. When the hydrocarbon chains melt, there is an abrupt increase in vibrational frequency and bandwidth of the $CH_2$ bands in the 3000 to 2800-$cm^{-1}$ region. From the midpoint in the frequency (or bandwidth)–temperature diagram, $T_m$ can be evaluated. Such a diagram for DMPC (Fig. 1) shows that there is good agreement between this measurement of the phase transition and leakage.

It is still not entirely clear why permeability of bilayers increases as they pass through the phase transition, but the most predominant idea found in the current literature is that packing defects exist at boundaries between liquid-crystalline and gel phase domains. Leakage would occur, according to this notion, through the defects (reviewed in Cameron and Dluhy, 1987). Such defects would exist even in phospholipid vesicles composed of single species of phospholipid (e.g., the DMPC vesicles discussed here); since the phase transition is not completely cooperative, occurring over a temperature range, liquid-crystalline and gel phase domains must coexist during the transition. With complex mixtures of phospholipids, this effect may be exaggerated by the following sequence of events. There is good evidence that lateral phase separations of at least some phospholipid classes may occur in the plane of the bilayer during cooling (Quinn, 1985) or drying (L. Crowe and Crowe, 1986), mainly due to the immiscibility of some gel phase phospholipid

classes. When such a phase-separated mixture composed of phospholipids with different transition temperatures is rewarmed, liquid-crystalline and gel phase domains will unquestionably be found in the bilayer simultaneously until the domains are melted.

## Dry Bilayers Pass Through $T_m$ as They Are Rehydrated

Phospholipids are hydrated to some extent, with, in the case of phosphatidylcholines, 10–12 water molecules of hydrogen bonded around the polar head groups (Chapman *et al.*, 1967; Kodama *et al.*, 1982, 1985a, b; reviewed in L. Crowe and Crowe, 1988). When that water is removed by dehydrating the bilayer, the lateral spacing of the head groups decreases, leading to increased van der Waals' interactions between the hydrocarbon chains (reviewed in L. Crowe and Crowe, 1988). As a result, the transition temperature for the phospholipid increases, often by large amounts. Hydration-dependent phase diagrams have been prepared for only a few phospholipids, so it is not possible to generalize about the magnitude of the increase in $T_m$ as a result of dehydration. In the case of DPPC, $T_m$ increases from 41°C to nearly 120°C when it is dehydrated (Kodama *et al.*, 1982), while, with POPC, $T_m$ increases from $-3$°C to 57°C (L. Crowe *et al.*, 1986). Based on the limited evidence available, it appears that dehydration can lead to increases in $T_m$ of 60–70°C for phosphatidylcholine. This transition is fully reversible so that, as the dry bilayer is rehydrated, $T_m$ falls. Consequently, phosphatidylcholines like POPC that would be in liquid-crystalline phase at room temperature ($T_m = -3$°C) would be in gel phase when dry ($T_m = 57$°C), and return to liquid-crystalline phase during rehydration. Vesicles of POPC would not be expected to lose their contents during dehydration since the liquid-crystalline to gel transition would occur after bulk water into which the contents could be leaked was removed. However, during rehydration, when the vesicles undergo the gel to liquid-crystalline transition, they would be expected to leak into the surrounding medium. It follows that the gel to liquid-crystalline phase transition is likely to be a primary vector for leakage across dry bilayers as they are rehydrated.

The only other phospholipids that have been studied in any detail in this regard are several phosphatidylethanolamines. The situation is more complex with these lipids since they readily enter the nonbilayer hexagonal-II phase at temperatures above $T_m$. In such lipids, the gel to liquid-crystalline transition temperature rises during dehydration, and the lamellar to hexagonal transition temperature falls, as a result of which the two transitions merge into a single transition at an intermediate temperature (Seddon *et al.*, 1983). Accordingly, dehydrated phosphatidylethanolamines

enter hexagonal-II phase far more readily than do hydrated ones. Since this phase is clearly incompatible with maintaining low permeability of a bilayer, its formation would undoubtedly lead to increased permeability. However, this phase has never been detected in membranes of organisms that normally survive dehydration (reviewed in L. Crowe and Crowe, 1986; J. Crowe *et al.*, 1988b), so we will not discuss it further in the present context.

## How Anhydrobiotic Organisms Escape Leakage: Effects of Sugars on $T_m$

Since, unlike the DMPC vesicles shown in Fig. 1, anhydrobiotic organisms usually do not leak extensively during rehydration, they must possess mechanisms to escape the gel to liquid-crystalline phase transition that would be expected to occur in their membranes at this time. The first clues concerning these mechanisms were the findings that many anhydrobiotic organisms contain large quantities of disaccharides, the most common of which is trehalose. This molecule may be present in quantities exceeding 20% of the dry weight of such organisms as baker's yeast, fungal spores, anhydrobiotic micrometazoa (e.g., certain nematodes), and the desert resurrection plant (see Crowe *et al.*, 1984, for references). Astonishingly, comparatively little is known about the biochemistry of the embryos found in what are probably the most important anhydrobiotic organisms with respect to human welfare —seeds—but the limited evidence available suggests that the analogue of trehalose in these organisms is sucrose (Leopold and Vertucci, 1986).

Trehalose has the ability to preserve dry bilayers (J. Crowe *et al.*, 1982; L. Crowe *et al.*, 1985, 1986; Madden *et al.*, 1985) and labile soluble proteins (Carpenter and Crowe, 1988). For example, when unilamellar vesicles composed of POPC and phosphatidylserine (9 : 1) were dried in the presence of a sufficient amount of trehalose, upon rehydration the vesicles were seen to retain 100% of their original contents (Fig. 2). Sucrose has somewhat similar effects, but is not quite as effective as trehalose in stabilizing vesicles composed of this particular mixture of lipids (L. Crowe *et al.*, 1984, 1986).

We have investigated the mechanisms by which trehalose stabilizes dry bilayers, with the results summarized in Fig. 2. One source of damage during the dehydration is fusion between vesicles, which usually leads to leakage (reviewed in Duzgunes and Bentz, 1988). We quantified the fusion that occurs as a result of dehydration, using resonance energy transfer between fluorescent probes to assay fusion (L. Crowe *et al.*, 1986; Womersley *et al.*, 1986). The results (Fig. 2) show that a relatively small amount of trehalose is sufficient to inhibit fusion or aggregation of the vesicles (called "probe intermixing"; see Duzgunes and Bentz, 1988, for discussion), while much more trehalose is needed to stabilize them against leakage. It follows that

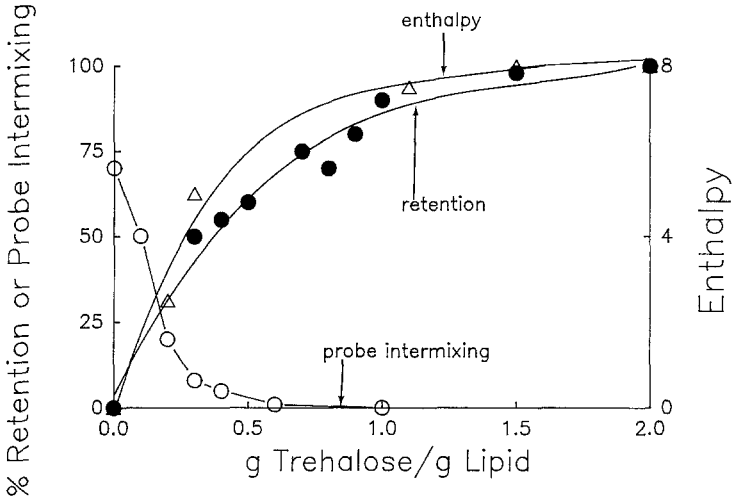


**Fig. 2.** Retention of trapped solute by unilamellar vesicles composed of POPC and PS (9 : 1) as a function of the amount of trehalose with which the vesicles had been dried previously. Also shown are data for effects of trehalose on fusion (probe intermixing) and enthalpy of the low-temperature transition. Data from L. Crowe *et al.* (1986).

inhibition of fusion is not in itself sufficient to preserve the vesicles. Trehalose also effectively depresses $T_m$ for the dry vesicles. With increasing amounts of trehalose present, the enthalpy of the low-temperature transition for the dry lipids used here (which is considerably below room temperature) increases steadily (L. Crowe *et al.*, 1986). The increase in enthalpy coincides with the increased retention of trapped solute by the dry vesicles. The vesicles with $T_m$ fully depressed are in liquid-crystalline phase even when they are dry and, consequently, upon rehydration they do not pass through the gel to liquid-crystalline transition, and they do not leak.

Since we proposed this model for stabilization of dry bilayers (J. Crowe *et al.*, 1984; L. Crowe *et al.*, 1986), several laboratories have investigated the mechanism of interaction between sugars and phospholipids (e.g., Gaber *et al.*, 1986; Lee *et al.*, 1986; Strauss and Hauser, 1986). The results indicate that trehalose and some other sugars depress $T_m$ by direct interaction with the polar head groups of phospholipids, probably involving hydrogen bonding between $-$OH groups on the sugar and the phosphate of the polar head group (reviewed in J. Crowe *et al.*, 1987, 1988a, b).

## Stabilization of Lipid Vesicles with High $T_m$

If the model proposed above is correct, it has the following corollary. DPPC has a hydrated $T_m$ of 41°C, which rises when the lipid is dehydrated.

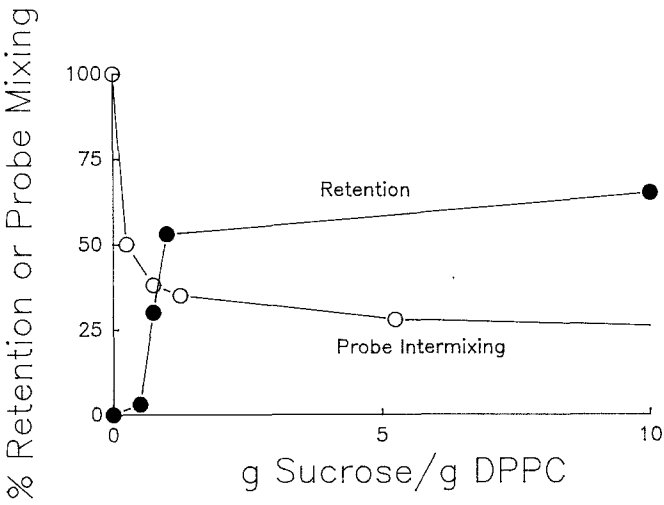


**Fig. 3.** Effects of sucrose on retention of trapped solute by and fusion between unilamellar vesicles of DPPC.

Thus, this lipid will never pass through its phase transition when it is dried at room temperature. Under these circumstances, it follows that inhibition of fusion alone may be sufficient to stabilize dry vesicles composed of DPPC. That this is the case is illustrated in Fig. 3, which shows that retention of trapped carboxyfluorescein by dry unilamellar vesicles of DPPC reaches maximal value at the same concentration of sucrose as that required to inhibit fusion maximally. This point is made more clearly in Fig. 4. Under

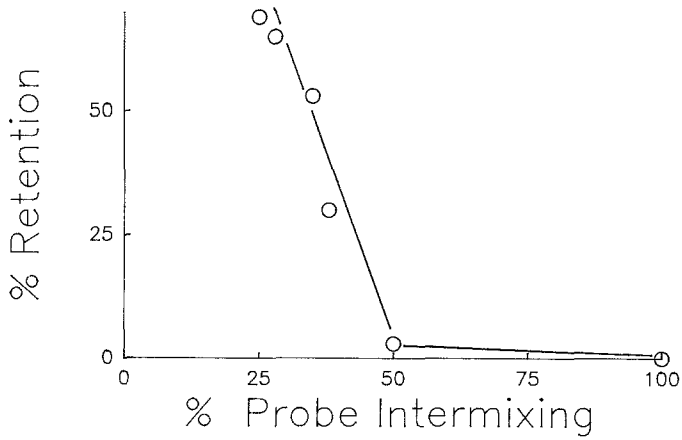


**Fig. 4.** Relationship between probe intermixing and retention of trapped solute by vesicles of DPPC. Under the conditions of these measurements, 50% probe intermixing represents an average of one fusion event per vesicle.

the conditions in which these experiments were conducted, 50% probe intermixing represents, on average, one fusion event per vesicle. When we plotted retention of trapped solute at intermixing, we found that when probe intermixing was > 50% the vesicles leaked all of their contents, but when probe intermixing was depressed to > 50% retention of trapped solute rose linearly. Thus, it appears that fusion is the major damaging event in these vesicles. We hasten to point out, however, that retention of trapped solute by vesicles of pure DPPC is never very high (cf. Figs. 2 and 3), suggesting that other factors play a role in leakage as well.

## Effects of Sugars on $T_m$ in Dry DPPC

The discussion above does not imply that sugars do not have an effect on $T_m$ in a saturated lipid like DPPC. Indeed, the effects of trehalose on $T_m$ were discovered from studies on its interaction with DPPC (J. Crowe *et al.*, 1984). In our first studies, we used homogeneous mixtures of DPPC and trehalose prepared from organic solvents as a model and showed that trehalose depresses $T_m$ below that for the fully hydrated lipid. Subsequent studies on other phospholipids have been on mixtures dried from aqueous solution (L. Crowe *et al.*, 1986). Under these conditions, DPPC behaves quite differently than in the previousy published studies. Tsetkova *et al.* (1988) have recently shown that DPPC must be dried from above the hydrated $T_m$ if the trehalose is to depress $T_m$ of the dry preparations maximally. They interpreted this effect to suggest that the trehalose must have access to the head groups if it is to have an effect on $T_m$ in the dry state. They also reported that the minimal $T_m$ observed was 45–49°C, in contrast with results that we had previously reported showing a transition well below that of hydrated DPPC (Crowe *et al.*, 1984). The "dry" preparations used by Tsetkova *et al.* (1988) were actually DPPC dihydrate, as they state in the paper. In our hands, $T_m$ for DPPC vesicles rises from 41°C to 112°C when it is dehydrated without trehalose, suggesting that we are working with much drier preparations. When it is lyophilized with trehalose, on the other hand, on the first scan it is seen to have a $T_m$ of ~ 70°C—an apparent depression of $T_m$ by 40°C. On the second scan, however, $T_m$ declines to a stable value of 24°C. We interpret these results as follows. When DPPC is dried with trehalose from below its hydrated $T_m$, the polar head groups are tightly packed, thus limiting access of the sugar to the head groups, as Tsetkova *et al.* (1988) suggest. On the first scan, $T_m$ of this dry preparation shows a depressed, but metastable transition. Once the DPPC–trehalose mixture has melted, the trehalose would be expected to have full access to the head groups, thus forming a structure with a stable $T_m$ depressed by a total of > 80°C. Thus, we suggest

that the interesting difference between our results and those reported by Tsetkova *et al.* (1988) may be due to the differences in hydration state of the samples.

## Measurement of Phase Transitions in Intact Cells

We can now ask this question: Do the phase transitions discussed above have any bearing whatsoever on what goes on in an intact cell of an anhydrobiotic organism during dehydration and rehydration? To address this question, it was necessary to devise means for detecting the phase transitions in the intact cells. The usual way of measuring the transitions has been to isolate phospholipids from the cells to be studied and to record the transitions, usually with calorimetry. Since there is some evidence that isolation of membranes from cells (let alone separating out the phospholipids) significantly alters $T_m$ (Cameron *et al.*, 1983), it is desirable to do the measurements on intact cells. That is particularly true in the present context, where we suspect that the sugars in the anhydrobiotic organisms may affect $T_m$. A superior way of measuring these phase transitions is with Fourier-transform infrared spectroscopy, which provides a fast, accurate measurement (reviewed in Gendreau, 1987). We have simply adapted the methods used by others for measurements of $T_m$ in phospholipids (reviewed in Cameron and Dluhy, 1987) and intact bacterial cells (Cameron *et al.*, 1983; Loeffelholz *et al.*, 1987) for use in this context. We believe these are the first such measurements with eukaryotic cells.

The first measurements of $T_m$ in intact cells have been made on pollen grains of cattail (*Typha latifolia*). These plant propagules have made an excellent model system with which to study phase transitions; infrared spectra can be obtained easily from the intact organisms, and portions of the spectra can be assigned to phospholipids. Representative spectra in the hydrocarbon-chain-stretching region (3000–2800 $cm^{-1}$; Fig. 5) illustrate all of the details seen in spectra of pure phospholipids, including bands assigned to stretching vibrations of the $CH_2$ and $CH_3$ groups in hydrocarbons. Further, shifts in frequency and bandwidth can be seen with temperature as the hydrocarbons undergo their phase transition from gel to liquid-crystalline phase (Fig. 5). A detailed description of the procedure used to obtain these spectra and to assign them to phospholipids is presented elsewhere (J. Crowe *et al.*, 1988c). Briefly, care must be taken to minimize light scattering by distributing the experimental material uniformly as possible on the windows of the sample holder. In assigning the phase transitions evident in the data, we biochemically analyzed the contents of the pollen. The results showed that these organisms contain three major classes of hydrocarbons: (a)

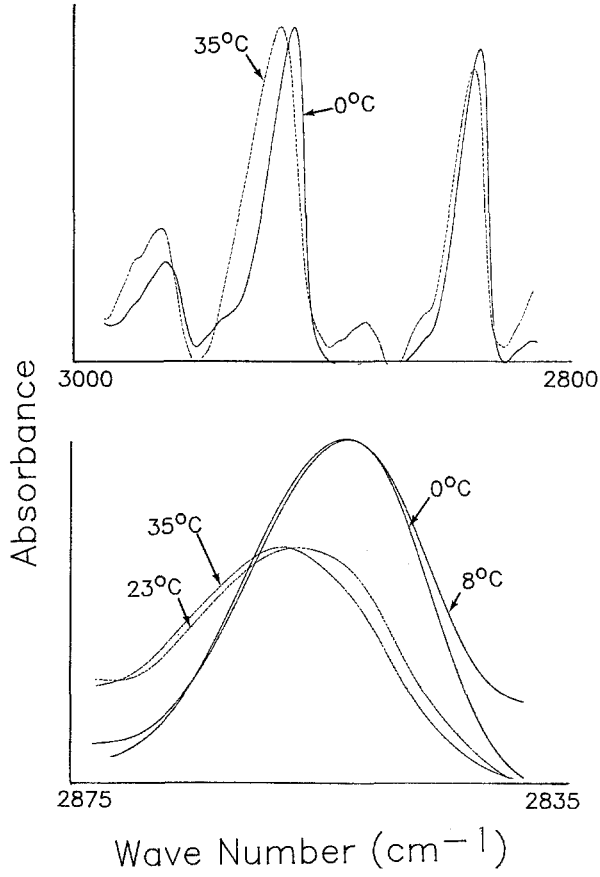


**Fig. 5.** Representative infrared spectra of dry pollen grains, showing the hydrocarbon-stretching region. (Top) All four major bands assigned to the hydrocarbons, at two temperatures. (Bottom) Clear frequency shifts and band broadening in the $CH_2$ symmetric stretch as the temperature is changed.

An extracellular hydrocarbon, which can be removed by washing the pollen in organic solvents, thus eliminating this fraction from consideration. This treatment does not alter viability of the pollen. (b) Neutral lipids, which we found to have a major transition centered on $\sim 15°C$. This transition does not change with water content of the pollen. (c) Phospholipids, which have $T_m$'s varying from $-6°C$ in the fully hydrated pollen to 32°C in the dry pollen. Accordingly, the hydration sensitivity of $T_m$ in the phospholipids can be used to distinguish their transition from that of the interfering neutral lipids. A representative plot of the change in frequency with temperature for the $CH_2$ symmetric stretch is shown in Fig. 6. From the midpoint of the

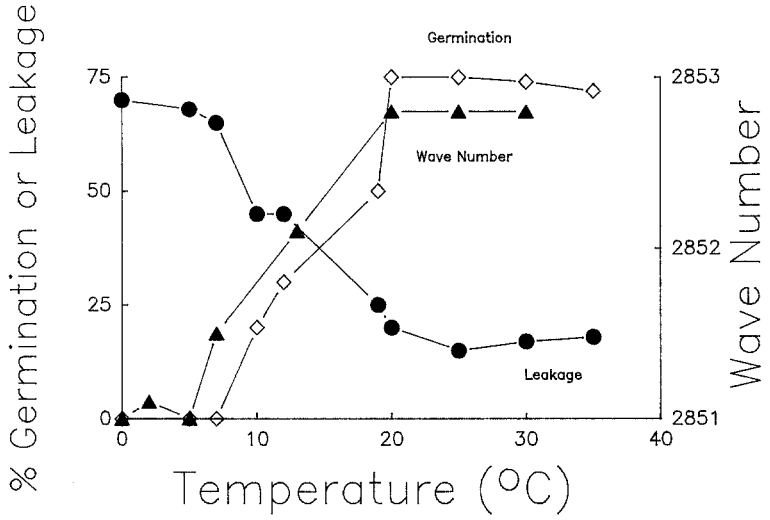


**Fig. 6.** Frequency of the $CH_2$ symmetric stretch in pollen grains containing ~0.05 g water/g dry weight as a function of temperature. From the midpoint of the transition, a value for $T_m$ can be extracted. Also shown are data for leakage from pollen grains hydrated at the indicated temperatures. Subsequently, the pollen grains were transferred to room temperature and germination was measured. Infrared data from Crowe *et al.* (1988d). Leakage and germination data from Hoekstra (1984).

frequency change, an average value for $T_m$ can be extracted. Similar measurements have been made on a variety of cells, including newly emerged hypocotyls from seeds of several plant species and sperm of several animal species (J. Crowe *et al.*, 1988c), indicating that such measurements are possible with a wide variety of cell types.

## The Relationship of $T_m$ to Physiological Measurements

When dry cells are placed in water, particularly at low temperatures, they often show a transient leakage of intracellular solutes. In the case of dry pollen grains, Hoekstra (1984) showed that this leakage is alleviated by heating above a species-specific temperature before the pollen grains are placed in water. He also showed that germination of the pollen was enhanced if they were heated before they were placed in water. Leakage declined in concert with the increase in germination. His data for one species, *Typha latifolia*, are reproduced in Fig. 6. The temperature at which germination reaches half-maximal value ($G_m$) can be extracted from these data. It is clear that the increase in germination coincides also with the increase in vibrational

frequency for the $CH_2$ bands in the pollen; in other words, $T_m$ is approximately equal to $G_m$. Based on these findings, we suggest that, at the low temperatures, gel phase domains exist in the plasma membranes of cells in these pollen grains. As a result, when they are rehydrated, these gel phase domains undergo a transition to liquid-crystalline phase, and the pollen grains leak. Heating them above the transition temperature before they are rehydrated obviates the hydration-dependent phase transition, and they do not leak.

## A Phase Diagram for Pollen Grains

From measurements of $T_m$ extracted from frequency–temperature plots taken for pollen at different water contents, it was possible to construct the phase diagram shown in Fig. 7. The data show that $T_m$ falls sharply when the pollen grains are hydrated, reaching a minimal value at a water content of $\sim 0.25$ g water/g dry weight. We have also measured $G_m$ for pollen grains treated similarly. Such measurements have been made for pollen at only a few water contents, but the limited data available agree remarkably well with the phase diagram constructed from the $T_m$ measurements (Fig. 7).

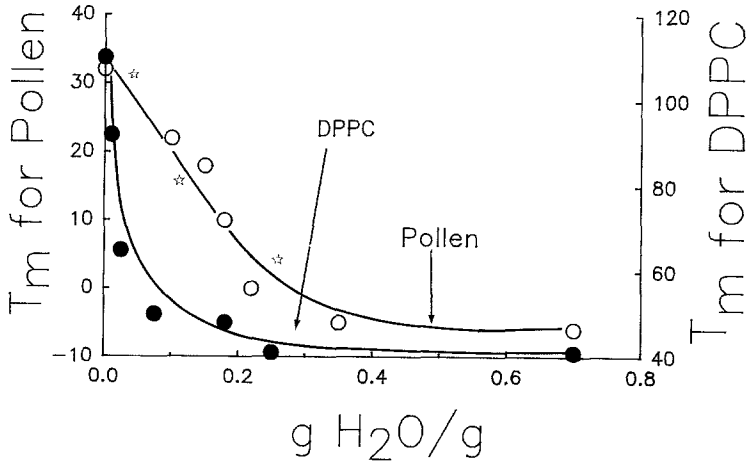


**Fig. 7.** A hydration-dependent phase diagram for pollen grains, extracted from measurements of $T_m$ (open circles) on pollen at the indicated water contents. Also shown are data for $G_m$ (stars) extracted from temperature–germination curves similar to the one shown in Fig. 6. The closed circles represent a phase diagram for DPPC. Data for pollen from J. Crowe *et al.* (1988d). Data for DPPC from Chapman *et al.* (1967) and Kodama et al. *(1985a)*.

## Comparison of the Pollen Phase Diagram with that for a Phospholipid

For comparison with the phase diagram for the pollen, a similar phase diagram for DPPC is also shown in Fig. 7. The shapes of the two phase diagrams are only roughly similar, but, with progressive dehydration, $T_m$ for the pollen grains rises by a small amount (from $-6$°C to 32°C) relative to that seen in DPPC (from 41°C to 110°C). In both cases, however, the limiting value for $T_m$ is reached at a water content of $\sim 0.25$ g water/g dry weight. Although the phase diagrams differ in detail, the probable reasons for which will be discussed below, we suggest that this comparison supports the proposition that we are measuring the hydration-dependent phase transition in membrane phospholipids in the pollen. The phase diagram for the pollen shown here is the first such phase diagram for an intact cell.

## Why is $T_m$ for Dry Pollen So Low?

We can now ask why $T_m$ for the pollen grains does not rise as much as that for DPPC. The answer to this question is not yet settled and constitutes an active area of research in our laboratories currently. However, we have sufficient data available already to suggest a probable solution. The isolated phospholipids taken from the pollen have a $T_m$ in excess water near $-6$°C, in agreement with results obtained from the intact pollen, but the dry phospholipids have a $T_m$ between 60 and 70°C— $\sim 30$°C higher than that seen in the intact pollen, for which $T_m$ is 32°C (Fig. 7). This result clearly indicates that $T_m$ is depressed in the dry pollen (but not in the hydrated ones) by an as yet unknown mechanism. While it is possible that other membrane constituents contribute to depressing $T_m$ in the dry pollen, we suggest that an important contributor to this phenomenon is the presence of sugars in these organisms. The pollen grains that we are studying contain $\sim 25\%$ of their dry weight in the form of sucrose (F. A. Hoekstra, unpublished data). We propose that this molecule is responsible for depressing $T_m$ in the dry pollen.

## Summary and Conclusions

The available evidence suggests that leakage of intracellular solutes across the plasma membrane may occur simultaneously with a gel to liquid-crystalline phase transition. The temperature at which this transition occurs in dry bilayers is depressed by certain sugars, including sucrose and trehalose. It has been possible to measure such phase transitions in intact cells and to

relate the transition to their permeability and subsequent viability. It has even been possible to construct a hydration-dependent phase diagram for the cells. We believe that this procedure will be of immedite importance in assessing the conditions under which economically important dry cells such as yeasts, seeds, and pollen will survive rehydration optimally.

## Acknowledgment

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