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Phospholipid Motional Characteristics in a Dry Biological System[1]

A ^{31}P-NUCLEAR MAGNETIC RESONANCE STUDY OF HYDRATING *TYPHA LATIFOLIA* POLLEN

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ABSTRACT

Analysis of the proton-decoupled ^{31}P-nuclear magnetic resonance (NMR) spectrum of fully hydrated *Typha latifolia* pollen revealed the presence of two main peaks: A broad asymmetrical component of a 'bilayer' lineshape and a much narrower symmetrical component originating from phosphorus compounds undergoing rapid isotropic motion. From (a) ^{31}P-NMR experiments on the hydrated total pollen phospholipids, (b) saturation transfer ^{31}P-NMR experiments, and (c) the fraction of lipid phosphate in the pollen, it can be concluded that the great majority of the endogenous phospholipids are arranged in extended bilayers in which the lipid phosphates undergo fast ($\tau_c < 10^{-6}$ second) long axis rotation. This bilayer arrangement of phospholipids was observed in the pollen down to hydration levels of at least 10.9% moisture content. At the lowest level of pollen hydration examined (5.2%) the ^{31}P-NMR spectrum had a solid state lineshape demonstrating that all the phosphorus-containing compounds (including the phospholipids) were virtually immobile.

The seeds and pollen of many higher plants are capable of withstanding severe desiccation without loss of viability. The extent to which cellular and metabolic integration is maintained in such dry systems is very imperfectly understood. Some studies have suggested that metabolism may take place in 'dry' seeds and pollen, albeit at a very low rate (2, 26). Undoubtedly, however, our knowledge of the biochemical restrictions acting in such anhydrobiotic systems is severely limited (3, 24). One particularly important aspect of cellular organization in the dry condition lies in the response of membranes to dehydration. Primarily on the basis of leakage kinetics from imbibing seeds, Simon (22, 23) suggested that dry cellular membranes were probably incapable of functioning as selective permeability barriers. Extrapolating from the x-ray diffraction data of Luzzati and others (12) working on animal systems, he suggested that membrane lipids generally might undergo a mesomorphic phase change from a bilayer (lamellar) to a hexagonal (H_{II})[3] arrangement upon dehydration. In the hexagonal (H_{II}) conformation, groups of phospholipids aggregate to form porous or tubular structures, in which water-filled areas are contained within a lining derived from the hydrophilic head groups of the lipids (see explanatory illustrations in [4]). Simon's interesting hypothesis has received only scant empirical support (*e.g.* 25). Indeed, some recent reports have voiced mild scepticism as to the widespread occurrence of such membrane-related changes in dehydrated seeds (14, 21) whereas others have doubted the heuristic power of Simon's hypothesis when translated to the tissue level (16). Admittedly, however, none of these criticisms has completely disproved Simon's idea and the physical state of membranes in dry and partially hydrated systems has remained enigmatic.

Nuclear magnetic resonance offers several paths of approach to the understanding of problems in seed and pollen physiology. Proton-NMR has been successfully used for a number of years to analyze the status of water in dry systems. Thus, in an earlier study we used ^{1}H-NMR to demonstrate the first appearance of free water in soybean cotyledons at about 9% to 11% moisture content (21). ^{13}C-NMR has also been applied with considerable success to problems of seed composition. Procedures are now available which permit nondestructive estimation of oil, protein, and carbohydrate content in seeds (18, 19). Even quantitation of the individual fatty acids in a single living seed is currently possible using ^{13}C-NMR (20). Recent studies using ^{31}P-NMR have proved of great value in understanding the problems of lipid polymorphism in fully hydrated model and biological membranes (4). ^{31}P-NMR has proved especially useful in discriminating between hexagonal and bilayer phases and there is a growing body of evidence suggesting that non-bilayer lipid structure may be considerably more prevalent in membranes than once thought (5). We have examined several dry systems using ^{31}P-NMR and have found that *Typha latifolia* pollen is particularly suitable for analysis by this technique. We report here on the phospholipid motional characteristics of *Typha* pollen at various stages of hydration and present evidence showing that the membrane lipids are arranged in a bilayer structure at hydration levels at least as low as 11%.

MATERIALS AND METHODS

Pollen. *T. latifolia* L. pollen was collected during the month of June 1981 from field populations growing near Nijmegen. The harvested pollen was allowed to air-dry for at least 3 d, at which stage its moisture content was approximately 8% to 9% (all values of moisture content are expressed on a wet weight basis). The dry pollen was subsequently stored at −25°C, a process that did not impair germinability.

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[3] Abbreviations: H, magnetic field strength; H_{II}, hexagonal lipid phase of type II as defined by Gulik-Krzywicki *et al.* (8); NMR, nuclear magnetic resonance; r.f., radio frequency; τ_c, correlation time.

Modification of Moisture Content. In most cases, the moisture content of the pollen was manipulated by 'air-imbibition' in a water-saturated atmosphere at 2°C to 4°C. Batches of pollen weighing approximately 15 g were exposed on Petri plates for periods of up to 24 h. Fully imbibed pollen was produced by shaking it for at least 5 min at room temperature in a medium containing: 6.84% (w/v) sucrose, 0.03% (w/v) $Ca(NO_3)_2 \cdot 4H_2O$, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.01% (w/v) KNO_3, 0.01% (w/v) H_3BO_3, and 0.1 M Mes acid, adjusted to pH 6.0 with KOH. The imbibed pollen was collected on a Sartorius membrane filter (8 μm pore size). Prolonging the incubation period to 3.5 h had no additional effect on the NMR spectrum. Production of pollen with an especially low water content (approximately 5%) was accomplished by drying over P_2O_5 for 18 h at room temperature. Moisture content was determined by drying at 110°C for 4 h.

Lipid Preparation and Phosphorus Analysis. For the preparation of the relatively large amounts of material needed for NMR analysis of isolated phospholipids, 125 g of pollen were extracted using the boiling isopropanol procedure described by Kates (10). The solvent extracts were concentrated *in vacuo*, redissolved in chloroform:methanol (2:1, v/v) and partioned against 0.2 volumes of 0.9% (w/v) NaCl. The solvent phase was again concentrated to an oil and a crude phospholipid precipitate was produced by cold solvent fractionation using acetone (10). The precipitate was washed twice with acetone. This crude phospholipid fraction was used for the NMR study. (Additional purification of the phospholipids on a column of acid-treated Florisil [10] had no effect on the NMR spectrum.) For the NMR analysis, about 50 mg phospholipids were dried from chloroform and dispersed by vortexing in 1 ml of 100 mM NaCl, 4 mM EDTA, 10 mM Tris-HCl (pH 7.0) in ^2H_2O. The critical determination of phospholipid content in pollen demanded a more efficient extraction procedure than the large-scale method detailed above. Dry pollen (approximately 750 mg) was dispersed in water and rapidly frozen into a pellet using liquid N_2. This pellet was disrupted at high pressure in a X-Press disintegrator (AB Biox, Stockholm). Ice remaining in association with the pollen was removed by freeze-drying. Examination under the microscope confirmed that nearly all the pollen grains were ruptured by this treatment. The subsequent scheme of solvent extraction followed a pattern similar to that already outlined above. Lipid phosphorus was determined on the total lipid extract following the salt wash. Wet digestions of lipid and whole pollen samples were accomplished using a ternary acid mixture, which included $HClO_4$ (full details are given in [17]). The phosphorus present was determined using a phosphomolybdate colorimetric reaction. The reagent formulation of Lindeman (11) was employed.

NMR. ^{31}P-NMR experiments were performed on a Bruker WP 200 wide-bore spectrometer operating at 81.0 MHz. The pollen sample used to fill the cavity had a volume of approximately 8 ml. Spectra were recorded at 30°C from 1,000 transients using a 50 kHz sweep width, 4 K data points, a 27 μs 90° r.f. pulse and a 1 s interpulse time using high power (50 w input power during the 41 ms acquisition time) proton-noise decoupling. No ^{2}H lock was used. Spectra of the hydrated isolated lipids were recorded similarly in 10 mm tubes using the ^{2}H-lock. Saturation transfer experiments were performed using the DANTE pulse technique (6, 15). The pulse sequence is: $\{D_0 - (P_1 - D_1)_{N_p} - P_1 - D_2 - P_w - D_e - \text{Acquisition}\}_{N_s}$. D_0 and D_2 are variable delays, P_1 and D_1 are the pulse width and delay between the saturation pulses. This sequence is repeated N_p times. P_w is the 90° r.f. pulse used for data acquisition and D_e is the 10 μs delay between P_w and the data acquisition. With this short delay it is even possible to detect the rigid lattice ^{31}P-NMR spectra of anhydrous phospholipids (7). N_s is the number of times the whole sequence is repeated. In our experiments: $D_0 = 0$; $P_1 = 0.4$ μs; $D_1 = 100$ μs; $N_p = 10{,}000$; $D_2 = 10$ μs; $P_w = 27$ μs; $N_s = 500$.

RESULTS AND DISCUSSION

^{31}P-NMR provides a rapid, noninvasive method for investigating the state of membranes in isolated cellular fractions and in living tissue. Inasmuch as no artificial probes are introduced into the system, the possibility of artefactual results is essentially eliminated. Further, the measurements can be made under physiologically realistic conditions and the sample remains unchanged at the end of the experiment. A detailed review of the technique has been given by Cullis and de Kruijff (4). A more condensed account has also appeared (5).

When hydrated phospholipid samples are subjected to ^{31}P-NMR analysis under conditions of proton decoupling (a process which removes the dipolar interactions between protons and phosphorus nuclei) a variety of spectral lineshapes may be encountered. These spectra are sensitive indicators of the physical phase adopted by the lipids. The major types of spectral lineshape have been catalogued and illustrated by Cullis and de Kruijff (4). Thus, phospholipids arranged in bilayers in large vesicles (radius > 100 nm) give rise to a highly characteristic spectrum that consists of a low field shoulder and a high field peak separated by a chemical shift of about 40 ppm. This spectrum is determined by the residual chemical shift anisotropy of the lipid phosphate group, which is only partially averaged by fast ($\tau_c < 10^{-6}$ s) axial rotation of that group. Phospholipids arranged in a hexagonal (H_{II}) phase have a much narrower lineshape and an asymmetry which is the reverse of the bilayer spectrum. The differences between bilayer and hexagonal phase spectra arise because in the former system the lipids are essentially restricted in movement to the plane of the membrane. In the case of the hexagonal phase, the two-dimensional constraints of the bilayer disappear, and rapid motion in a third dimension (*i.e.* diffusion around the aqueous channels) becomes an effective averaging mechanism of the residual chemical shift anisotropy. Phospholipids organized in structures in which the molecules can undergo rapid isotropic motion, such as in (inverted) micelles or small (<100 nm diameter) vesicles, give rise to narrow symmetrical ^{31}P-NMR spectra (4).

An aqueous dispersion of isolated pollen phospholipids has a ^{31}P-NMR spectrum typical of phospholipids organized in extended bilayers (Fig. 1, lower). There is a high field peak at about 15 ppm and a low field shoulder extending to about −30 ppm. The ^{31}P-NMR spectrum of fully hydrated pollen shows some similarities

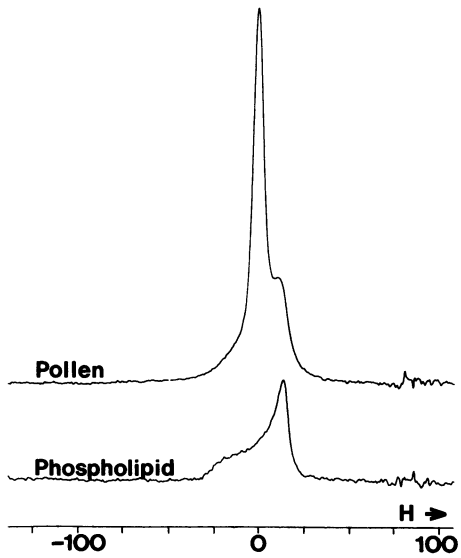

FIG. 1. ^{31}P-NMR spectra of fully hydrated *T. latifolia* pollen (above) and extracted phospholipid (below). The horizontal scale (chemical shift) is in ppm. The spurious signals in the 75 to 100 ppm range in this figure and in Figure 2 are instrumental artefacts.

(Fig. 1, upper). Again, there is a high field peak at about 15 ppm and signs of a low field shoulder. However, a large central peak (at 0 ppm) has apparently been superimposed on the basic bilayer spectrum. The position and shape of the central peak is diagnostic of phosphorus-containing compounds undergoing rapid isotropic motion ($\tau_c < 10^{-5}$s). Our initial interpretation of the pollen spectrum of Figure 1 was that it consisted of a basic bilayer spectrum due to phospholipids on top of which a signal from smaller soluble phosphorus-containing compounds (such as sugar phosphates, nucleotides, inorganic phosphate, etc.) had been superimposed. Further evidence supporting this interpretation will be given below. Examination of a hydration series of pollen samples showed dramatic changes in the ^{31}P-NMR spectrum with increasing hydration. In the driest pollen (5.2% moisture content, Fig. 2) a very broad lineshape was observed. This spectrum is similar to a solid-state 'powder' spectrum, in which all phosphates are immobilized and randomly orientated (1). Under these conditions, there is essentially no molecular motion. At 8.5% moisture content, narrowing of the spectrum is evident with the appearance of a central isotropic signal. Increasing the moisture content further to 8.8% leads to an enhancement of these processes but at a value of 10.9% a significant new feature appears in the spectrum. The high field shoulder diagnostic of phospholipids in the bilayer phase is readily apparent. At this hydration, therefore, the phospholipid phosphates must be rotating rapidly about the long axis of the lipid, since this motion generates the characteristic spectrum. Increasing the hydration state of the pollen above 10.9% sharpens the spectrum somewhat as further motional restrictions are removed, until finally the fully hydrated spectrum is attained (Fig. 2, upper). At no stage is there any suggestion that any significant proportion of the cellular phospholipid is arranged in a hexagonal phase. However, it should be noted that below a hydration level of 10.9% ^{31}P-NMR cannot provide structural information on the phospholipid arrangement as the molecular motions which give rise to the typical lineshapes are no longer present. It remains conceivable, but it is in no way suggested by the data, that a hexagonal phase could occur at a hydration level below 10.9%.

Our initial assumption was that the hydrated pollen ^{31}P-NMR spectrum consisted of two principal components: The phospholipids and the other phosphorus-containing compounds. In principle, the relative amount of a particular ^{31}P-NMR signal corresponds to the relative amount of the particular phosphate giving rise to the signal. This is only true when certain conditions are fulfilled, *i.e.* the NMR spectrometer should be capable of detecting all signals and no saturation of resonances should occur. In our experiments both conditions were satisfied. The spectrometer used and the experimental conditions are such that even the rigid lattice (*e.g.* no motion) ^{31}P-NMR spectra of anhydrous phospholipids (width $\simeq$ 200 ppm) can be detected (7). Furthermore, by increasing the interpulse time by a factor of ten, no change in the spectrum was observed (in the case of a pollen sample with 16.5% moisture content), thereby demonstrating the absence of saturation effects and the stability of the pollen preparation. By superimposing the lineshape of the phospholipid spectrum onto the hydrated pollen spectrum (Fig. 1) the relative amount of 'bilayer' lineshape can be estimated to be approximately 35%. The proportion of lipid phosphorus to total phosphorus in the pollen was also determined by direct chemical analysis. The lipid phosphorus content was 62 μmol/g dry weight. The total amount of phosphorus in the pollen was 238 μmol/g. Thus at least 26% of the phosphorus in the pollen was lipid phosphorus. Although the value for lipid phosphorus content is somewhat higher than that previously reported by others (9), it is probably still a slight underestimation inasmuch as the extraction procedure may not have been completely exhaustive. From these data it can be concluded that lipid phosphorus accounts for a significant proportion of the total phosphorus in the tissue and that the great majority of the phospholipid in hydrated pollen is organized into extended bilayers.

Saturation transfer ^{31}P-NMR is a particularly useful technique for artificially enhancing the signal due to the phospholipids in extended bilayers relative to that of the signal of the water-soluble phosphates (for an extensive treatment of this method, the reader should refer to [6]). Selective saturation with a pulse train is applied to the high field side of the 'bilayer' type of spectrum (Fig. 3A). At this chemical shift position, the ^{31}P-NMR spectrum has its maximum and saturation will be most efficient. This results in saturation and loss of signal at that position. However, as the chemical shift position of a phospholipid molecule in an extended bilayer is dependent on its relative orientation towards the magnetic field and inasmuch as the time it takes for a lipid molecule

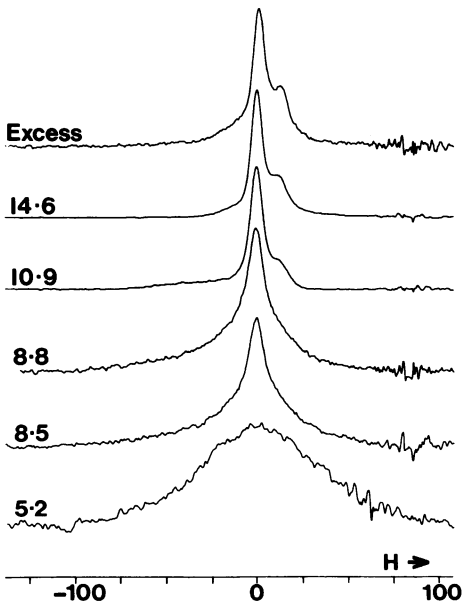

FIG. 2. ^{31}P-NMR spectra of *T. latifolia* pollen at various degrees of hydration. Each spectrum is labeled with the appropriate moisture content (wet weight %) of the pollen. The horizontal scale is in ppm.

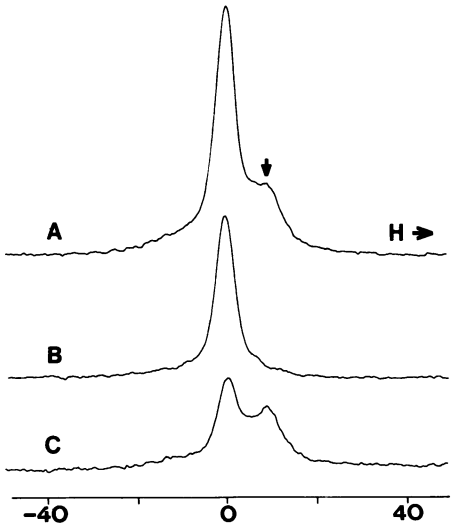

FIG. 3. ^{31}P-NMR spectra of *T. latifolia* pollen at 16.5% moisture content. (A), The 'normal' pollen spectrum without conditions of saturation transfer. (B), The pollen spectrum under conditions of saturation transfer. (C), The enhanced phospholipid spectrum from whole pollen (*i.e.* the difference spectrum of spectrum A minus spectrum B). The frequency of the saturating pulse chain is marked with a vertical arrow. The horizontal scale is in ppm. The ^{31}P-NMR spectrum of pollen at this moisture content was stable for the duration of the measurements.

to diffuse around a curved bilayer surface is short with respect to the duration of the saturation, the majority of the bilayer signal will be saturated and thus eliminated from the spectrum. This is shown for *Typha* pollen in Figure 3B. In the difference spectrum (Fig. 3C) the bilayer component is considerably enhanced. It should be noted that in the difference spectrum considerable intensity can be observed at the low field side of the bilayer spectrum, demonstrating the saturation of the phospholipid resonance at this position. The saturation of some of the isotropic signal is due to the limited selectivity of the saturation pulse train (6). These experiments thus confirm our spectral assignment of the broad resonance in the spectrum of the hydrated pollen and in addition demonstrate that the correlation time for isotropic motion caused by lateral diffusion of the phospholipids around curved bilayer surfaces is much shorter than the duration of the saturation (*i.e.* 1 s). Assuming spherical vesicles this would correspond to lateral diffusion rates in the same order of magnitude as has been found for liquid-crystalline bilayers (6).

Although the potential value of applying ^{31}P-NMR to studies of plant membranes *in vivo* is great, there are definite limitations. First, the information we have obtained does not discriminate between membranes of different cellular compartments. Fortunately, in the case of partially hydrated pollen this limitation is scarcely significant because virtually all the phospholipid must be in a bilayer state. The sharp downfield peak normally attributable to a hexagonal arrangement is absent from the spectra and the amount of lipid which could be in this particular nonbilayer conformation must therefore be vanishingly small. Second, problems may be encountered if less favorable tissues are employed. We have attempted to apply the technique to soybeans (*Glycine max* cv Wayne) for example, but in this material the large quantities of storage phosphate present (13) obscure the phospholipid signal. Only rather generalized conclusions could be drawn. Thus, in soybean cotyledons at 8.1% moisture content, a powder spectrum was observed which was very similar to the 5.2% spectrum in pollen (Fig. 2, lower). At 8.7% moisture content, a small amount of isotropic motion could be identified. This component thereafter increased rapidly with hydration, although some motional restriction was evident even at 15.1%. No characteristic phospholipid lineshapes were observed, which could be due to the dominating isotropic signal.

The data we have presented here suggest that ^{31}P-NMR can be used to monitor the status of membranes in *Typha* pollen at various levels of hydration. It is evident that there is considerable motion in the phospholipid fraction at moisture contents as low as 11% and that the membranes of the pollen form an extended bilayer. Certainly at 16.5% moisture (and possibly lower) our data suggest that the lipids in the membranes have motional properties similar to those of liquid-crystalline phospholipid bilayers. Obviously this tells us nothing directly about the state of other membrane components, such as membrane-bound enzymes, although appropriate structural configuration of the phospholipids and some degree of molecular motion are fundamental requirements for normal membrane-associated functions. From the point of view of membrane organization, at least, our data suggest that the basic structural prerequisites for cellular metabolism in pollen might be potentiated even at quite low levels of hydration.

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