

An Assessment of Phase Transitions in Soybean Membranes

Received for publication January 27, 1982 and in revised form July 16, 1982

SHARMAN D. O'NEILL¹ AND A. CARL LEOPOLD

Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, New York 14853

ABSTRACT

Phase transitions were measured in vesicles of phospholipids, alone and in various combinations, and in pelleted mitochondrial membranes, using thermal (DSC) and optical methods. The objective was to consider their possible involvement in chilling injury of soybeans (*Glycine max* [L.] Merr. cv Wayne 1977). Saturated phospholipids showed clear transitions in the temperature range of 50°C to near 0°C. When mixtures of two phospholipids were examined, there was a marked lowering and broadening of the transition peaks, and a shift in the transition temperatures to intermediate temperatures. The unsaturated phospholipids that occur naturally in soybeans showed no detectable phase transitions in this temperature range, alone or in combinations. Examination of the polar lipids from soybean asolectin revealed no transitions in the biological temperature range; the additions of cations such as Ca²⁺ and La³⁺ did not evoke a detectable phase transition in them. Mitochondrial membrane pellets likewise showed no transitions. The application of these two direct methods of examination of membrane components without the addition of foreign agents did not support the suggested occurrence of a bulk phase transition which could be related to chilling injury in soybeans.

Analyses of the temperature dependence of metabolic processes in chilling-sensitive plants often show abrupt changes in the apparent activation energy between 10 and 20°C (10). The observed changes in activation energies have been attributed to a phase transition of the membrane lipids which could alter the conformational behavior of membrane-bound enzymes (17). This contention has been supported by studies that showed discontinuities of electron spin-label motion parameters and fluorescence polarization measurements, from which membrane fluidity changes are inferred.

The interpretation of ESR² and fluorescence polarization measurements must be viewed cautiously however, as the bulky probes often used in these measurements may themselves cause local molecular ordering. Even the use of relatively nonperturbing fluorescent phospholipid probes may not accurately reveal the properties of the bilayer, as has been pointed out by Sturtevant *et al.* (21), because fluorinated phospholipids may form nonideal mixtures with the unsubstituted bilayer resulting in measurement

of the properties of the mixture rather than the real lipid bilayer. The use of Arrhenius plots must also be carefully evaluated as many other temperature-dependent effects such as ion binding and protein ionization can influence enzyme activity in conjunction with fluidity effects (24).

Chilling injury is a major problem during the early germination of soybean seeds (3). We felt that the effects of thermal perturbation on the cellular membranes as related to chilling injury needed additional investigation. To this end we have examined soybean seed mitochondrial membranes and various combinations of their component phospholipids for phase transitions, using methods which did not involve the introduction of membrane probes: that is, with the Differential Scanning Calorimeter, and with a simple spectrophotometric method (14). Differential scanning calorimetry directly measures enthalpic changes, both endothermic and exothermic, associated with lipid phase transitions. The enthalpic change associated with a lateral phase separation where one lipid component has undergone a transition to a gel phase should be detectable by the sensitive thermal analysis technique if a large enough fraction of the membrane lipid is involved in the transition.

MATERIALS AND METHODS

Plant Material. The plant material used throughout this investigation consisted of soybean seeds (*Glycine max* [L.] Merr. cv Wayne 1977) obtained from Maumee Valley seeds (Woodburn, IN). Germination assays showed high viability with approximately 90% germination. Whole seeds were used for total and polar lipid extracts. Axes were removed from seeds which had been held in saturated atmospheres 1 to 6 h for both mitochondrial isolation and extraction of axis total lipids. Hypocotyls were taken from 6-d-old etiolated seedlings for the isolation of hypocotyl mitochondria and mitochondrial lipids.

Isolation of Mitochondrial Membranes. Soybean mitochondria were isolated from 1 to 6 h imbibed seed axes and 6-d-old etiolated hypocotyls. The cells were disrupted by homogenization in 0.3 M sucrose, 5 mM EDTA-Na₂²⁺, 5 mM KH₂PO₄, 10 mM Hepes (pH 7.0) buffer in the presence of 0.1% defatted BSA at 0 to 4°C. Following filtration and centrifugation, the mitochondrial pellet was resuspended in buffer and layered on a 0.6-M sucrose pad. After final centrifugation at 14,000 rpm for 10 min, a hard pellet was obtained and subsequently used for either lipid extraction, absorbance measurements, or calorimetry. Proteins in the mitochondrial membranes (3-5 mg/DSC sample) were determined according to the method of Bradford (1), using a commercially available color reagent (Bio-Rad).

Lipid Preparation and Analysis. Total seed lipids were extracted from 1 kg of finely ground dry whole seeds with chloroform:methanol (2:1, v/v) under argon as previously described (16). The solvent extract was filtered and subsequently partitioned against 0.2 volumes of 1% (w/v) NaCl and once against 0.5 volumes of methanol 1% (w/v) NaCl (1:1, v/v). The washed total lipid extract was subsequently evaporated to near dryness *in vacuo* and resuspended in chloroform:methanol (2:1, v/v). Seed polar

¹ Present address: Section of Plant Biology, Cornell University, Ithaca, NY 14853.

² Abbreviations: ESR, electron spin resonance; DMPC, dimyristoyl phosphatidylcholine; DPG, diphosphatidylglycerol; DPPA, dipalmitoyl phosphatidic acid; DPPC, dipalmitoyl phosphatidylcholine; DSC, differential scanning calorimetry or calorimeter; DSPC, distearoyl phosphatidylcholine; MLV, multilamellar vesicles; OD, optical density; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; T_c, midpoint of the gel to liquid crystalline phase transition.

lipids (phosphatides) were separated from the neutral lipids by two procedures for comparative purposes. In the first procedure, phosphatides were precipitated for 1 h from 200 to 300 μ l total lipid extract by adding 5 ml cold acetone plus 100 μ l 10% (w/v) $MgCl_2 \cdot 6H_2O$ in methanol. The precipitated phosphatides were centrifuged, washed, and resuspended in chloroform as described by Kates (5), and finally, stored under argon in the dark at $-20^\circ C$ until future use. In the alternate procedure, phosphatides were separated by adsorption chromatography using an acid-washed floril column (5). The two partitioning procedures gave essentially the same results so the acetone precipitation method was used in the reported experiments except where noted.

Fatty acids were prepared for analysis by esterification in methanol:benzene: sulfuric acid (100:5:5, v/v/v) for 2 h at $80^\circ C$. The fatty acid methyl esters were partitioned into *n*-hexane and analyzed using a Hewlett-Packard 5730 A gas chromatograph equipped with a flame ionization detector and coupled to a Hewlett-Packard 3380 S integrator. Separations of the methyl esters were done on a 180-cm glass column (4 mm i.d.) packed with 3% (w/w) Silar-5 CP on 100/120 mesh Gas-chrom Q (Applied Science Labs) at $200^\circ C$ with N_2 as the carrier gas. Heneicosanoic acid (C21:0) was used as the internal standard (Sigma). Fatty acid methyl esters were identified by comparison to known standards (Sigma).

Liposome Preparation. Multilamellar vesicles were prepared from soybean membrane lipids extracted from seed axis mitochondria, whole axes, and whole seeds. Asolectin (Associate Concentrates, Inc.), a crude mixture of soybean phosphatides, was made into vesicles following purification as described by Kagawa and Racker (4). Vesicles composed of individual phospholipids or mixed lipids were prepared from purified soybean phosphatidylcholine (Soy PC), soybean phosphatidylinositol (Soy PI), soybean phosphatidylethanolamine (Soy PE), soybean phosphatidylglycerol (Soy DPG), and DPPA obtained from Sigma. The soybean phospholipids were approximately 99% pure showing a single spot on thin layer chromatograms. Aqueous dispersions were also prepared from highly purified DMPC, DPPC, and DSPC obtained from Calbiochem and were used without further purification.

Liposomes (MLV) were prepared from basic stock solutions of phospholipids, usually stored under argon in the dark at $-20^\circ C$ or used fresh. Dissolved lipids were dried in small test tubes under a stream of argon, dissolved in a small volume of ether to remove residual chloroform, and dried again under argon (22). Lipid vesicles used for differential scanning calorimetry were further dried at $50^\circ C$ under argon in a high vacuum desiccator for 1 h to ensure complete removal of solvent and then equilibrated to $24^\circ C$ under argon. Multilamellar liposomes were prepared by Vortex mixing the lipids in deionized H_2O , buffer, or the appropriate salt-buffer solution. Deionized H_2O was used in preparing zwitterionic phospholipid (PC and PE) liposomes. Acidic negatively charged phospholipid (PI, PG, PA) liposomes were prepared in 10 mM Hepes (pH 7.0) plus or minus 2 mM EDTA. The lipid mixture was then heated several degrees above the T_c temperature of the highest melting phospholipid and given Vortex mixing intermittently for 2 min. Liposomes obtained in this manner were used immediately for temperature scans. The final concentrations of the MLV suspensions were 0.01% to 0.1% (w/v) for spectrophotometric measurements and 50% (w/v) for calorimetric measurements. Single lipid component dispersions of DMPC, DPPC, and DSPC (Calbiochem) were used for temperature calibration. Binary mixtures of these same lipids were used to construct phase diagrams. A simplified model system was constructed from ternary mixtures of purified soybean PC, PE, and PI (Sigma) based on relative proportions of phospholipid classes reported for soybean membrane lipids (15, 23).

DSC. DSC was performed on a Perkin-Elmer DSC-2. Samples were hermetically sealed in stainless steel large volume capsules

(75 μ l capacity). All weights were determined on a Cahn 26 automatic electrobalance. Heating and cooling rates of 2.5 or $5^\circ C/min$ were used. Scans were performed close to full scale sensitivity (0.1–0.5 mcal/s). Synthetic lipids were often scanned at 1.0 mcal/s because of the large transition enthalpy. The instrument was temperature calibrated in the biological range of interest (0 – $100^\circ C$) with crystalline diphenylamine and tristearin standards, and with 25 and 50 μ l MLV dispersions of DMPC (Calbiochem) equivalent to 0.5 and 1.0 mg lipid, respectively. Temperature calibration of the DSC-2 at higher temperature was checked with reference to a pure indium standard. Large volume capsules were used for temperature calibration to assess thermal lag, which was found to be less than $1.5^\circ C$ at a scan rate of $5^\circ C/min$.

Base line calibration from 0 to $100^\circ C$ was carried out sequentially from range 1.0 to 0.1 mcal/s for each scan rate of interest. During base line calibration the sample and reference pans contained 50 μ l H_2O to optimize thermal balance between the sample and reference holders and to minimize specific heat differences resulting from the use of large volume capsules and aqueous biological samples. Both synthetic and biological samples were scanned against the reference capsule containing 50 μ l water. Phase transitions were determined relative to the base line calibration, which was repeated whenever the base line became unstable. Samples were scanned from 2 to 5 times in the temperature range of interest. Three separate preparations of the mitochondrial membranes samples were examined.

Optical Absorbance Measurements. Absorbance changes associated with phase transitions of MLV suspensions were continuously recorded by measuring OD changes at 400 or 600 nm during temperature scanning with a Gilford Recording Spectrophotometer by a modification of the procedure of Petersen *et al.* (14). Temperature was monitored in the cuvette by an internally attached thermocouple. The samples were temperature scanned from close to 0 to $95^\circ C$ at a scan rate of 0.5 or $1.0^\circ C/min$.

RESULTS

Synthetic Saturated Phosphatidylcholines. We first compared the phase transitions observable for a series of phospholipid samples of known composition. Using the DSC and the optical scanning method, it was found that close agreement is obtained as to the phase transition temperatures for various phosphatidylcholines, the T_c decreasing in a linear manner as chain lengths of the fatty acids were lowered from 18 to 14 carbons. Agreement between the two methods was within $0.6^\circ C$ in each instance.

Results from heating scans of vesicles of DMPC and DSPC using the two methods are superimposed in Figure 1. A large and sharp endothermic transition is observed at $23^\circ C$ for DMPC and at $54^\circ C$ for DSPC, visible not only in the DSC scan but also in the OD scan as a region of abruptly altered light scattering. Further, in at least some instances, the smaller pretransition that occurs at slightly lower temperatures than the main transition is detectable in the OD as well as the DSC scan (6, 7).

The binary mixture of DMPC and DSPC did not appear to segregate into discrete populations in the gel phase, indicating a high miscibility of the solid and liquid phases (8, 11). Mixtures of DMPC and DSPC (Fig. 1, B and C) showed a lower enthalpy of the transition, a broadening of the transition peak, and a shifting of the T_c to an intermediate temperature. Similar alterations of transitions were obtained with mixtures of DMPC and DPPC.

Soybean Lipids. The transitions of fully saturated pure phospholipids generally occur at temperatures in the biological range, between 0 and $40^\circ C$. However, the fatty acids of soybean phospholipids are highly unsaturated, as shown in Table I. The dominance of unsaturated fatty acids, especially in soybean PC and PE, would be expected to lower the T_c values. Scans of soybean phospholipids in purified asolectin reveal no transitions in the range of 3 to $80^\circ C$ (Fig. 2A). This lack of a visible transition was

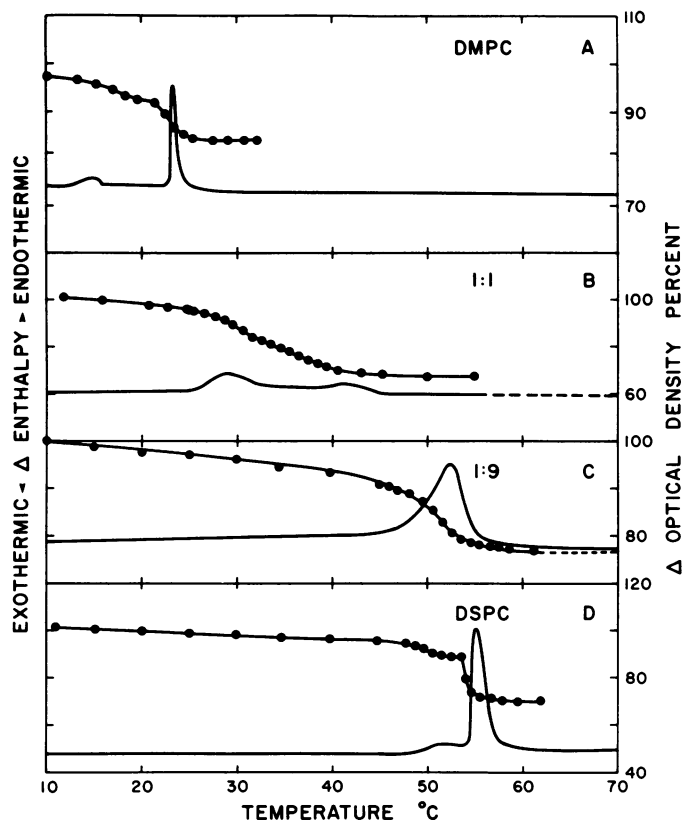


FIG. 1. Effects of mixing two phosphatidylcholines (DMPC and DSPC) on transition temperatures. DSC scans shown as lines, OD scans as dots. A, DMPC alone; B, DMPC/DSPC 1:1; C, DMPC/DSPC 1:9; and D, DSPC alone. DSC scans on range 0.5 mcal/s heating at 2.5°C/min; OD scans heating at 1°C/min.

Table 1. Fatty Acid Composition of Soybean Mixed Phospholipids and Purified Individual Soybean Phospholipids as Percentage of Total Fatty Acids

Data points represent the mean of three to five determinations for extracted mixed phospholipids.

Sample	Fatty Acids				
	16:0	18:0	18:1	18:2	18:3
	* % composition				
Seed polar lipids	19.87	4.94	7.92	59.10	8.18
Axis polar lipids	21.87	4.80	3.84	56.43	13.06
Mitochondria polar lipids	32.62	6.22	3.14	50.03	8.00
Asolectin polar lipids	20.00	7.42	9.89	51.92	10.78
Soy PC	14.39	4.81	9.37	64.64	7.12
Soy PE	24.76	2.99	6.90	58.80	6.85
Soy PI	37.88	10.74	6.83	40.96	3.59

confirmed at various scanning rates (2.5–10°C/min) and sensitivity levels (0.2–0.5 mcal/s), and was unaltered by the inclusion of Ca^{2+} or La^{3+} (5 and 1 mM, respectively). Likewise, the OD method could detect no transition in this temperature range. We suggest then that the mixture of phospholipids from soybean asolectin would undergo transitions only at temperatures below the biological range.

The major phospholipid in soybean seed membranes is PC, constituting approximately 46 mol % of total phospholipid (16, 23). The phase transitions of soybean PC and PE have been reported to be between approximately -25° and -11°C , respectively (6), well below the range of our temperature scanning

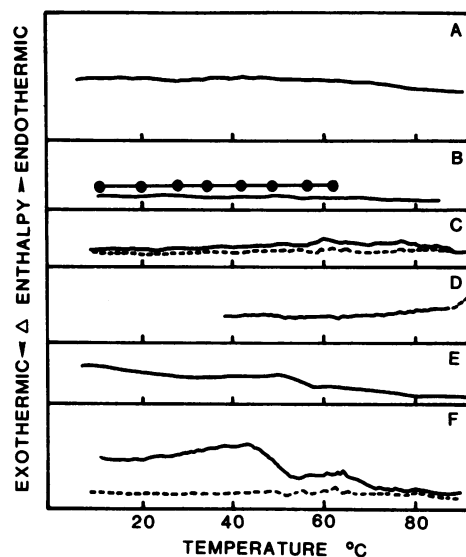


FIG. 2. Temperature scans of some soybean polar lipids. A, MLV dispersions of polar lipids from asolectin; B, scans by DSC and OD of soybean phosphatidylinositol; C, scans of soy PI plus 1 mM La^{3+} in a heating cycle; D, same as C in a cooling cycle; E, mixture of soy PI and DSPC 1:1; and F, same as E but with 2 mM La^{3+} . Broken lines are base lines. Settings at 0.5 mcal/s at 5°C/min for A and B; 0.2 mcal/s for C, D, and F; and 2.5°C/min for E.

capacity and below the biologically relevant range. Spectrophotometric scanning of soy PC or PE alone and in the presence of 1 mM La^{3+} did not reveal a phase transition above 0°C (data not shown). It is evident, then, that the major phospholipid of soybeans does not show a transition in the chilling temperature range even in the unmixed state. The presence of a strong cation such as La^{3+} did not evoke a transition in the biological temperature range (cf. 13).

Acidic Phospholipids. The phase behavior of the acidic phospholipids are less well known, especially with regard to cation interaction. Soy PA, soy PI, soy PG, and soy DPG are of interest because these acidic phospholipids carry a net negative charge, are present in small quantities in soybean seeds, and might be influenced by strong cations such as Ca^{2+} or La^{3+} . PI is the most abundant charged species ranging from 12 to 17.4 mol % of the total soybean phospholipid. Lesser amounts of PG (1.5–3.6 mol %), DPG (1.5–3.4 mol %), and PA (0–1.5 mol %) have also been reported (15, 23). The major acidic phospholipid in animal membranes, phosphatidylserine, is not detected in soybean. The best candidates for cation-induced phase separation in soybeans, and for a possible involvement in chilling injury, are PI and PG, each of which is known to have a Ca^{2+} binding capacity in sarcoplasmic reticulum phospholipids (13). A phase transition of soy PI in the presence of Ca^{2+} or La^{3+} has not been described.

Using buffered MLV dispersions of pure soy PI (1.0–2.0 mg), we found no phase transition above 5°C by DSC analysis nor above 3°C by spectrophotometric analysis (Fig. 2B). Likewise, no transition occurred in the presence of either 1 mM Ca^{2+} (not shown) or 1 mM La^{3+} (Fig. 2C). No exothermic transition during cooling to approximately 36°C was found (Fig. 2D) verifying that small peaks during heating were base line disturbances and not true endotherms.

If the transition temperature of soy PI was indeed below 0°C and below our range for a direct measurement, soy PI would be expected to lower the T_c of a phospholipid such as the fully saturated DSPC. A mixture of PI and DSPC (1:1) showed a lowering from the value of 54°C for DSPC alone to a point near 45°C (Fig. 2E). A mixture of 1:9 had a lesser lowering effect (data not shown). The effect of 2 mM La^{3+} on the 1:1 soy PI/DSPC

mixture is presented in Figure 2F. Two endothermic peaks appear with T_c values above and below that of the single endothermic peak seen in Figure 2E. This suggests that La^{3+} can induce a localized separation of the soy PI from the DSPC, resulting in lipid domains either enriched or depleted of DSPC, and consequently small transitions at higher or lower temperatures, respectively.

Soy PI had a similar effect on the transition of DPPA in lowering the onset and broadening the phase transition (data not shown). The effect of 2 mM La^{3+} on this mixture is not as straightforward though, as both components are negatively charged and would bind La^{3+} .

The observed effects of soy PI in lowering the T_c of DSPC and DPPA, the apparent lack of a transition in soy PI above 3 to 5°C, and the preponderance of unsaturated fatty acids in soy PI are three reasons to believe that the transition of soy PI in the presence or absence of cations is below 0°C, and thus may not contribute to a phase transition in the biological chilling range of 0 to 15°C. This was again confirmed by a simplified model soybean system constructed of soy PC, soy PE, and soy PI in the relative proportions normally found in the total seed polar lipids. No phase transition could be detected in DSC scans of this model system above the base line noise at a range close to full scale sensitivity. The addition of Ca^{2+} or La^{3+} did not focus a phase separation detectable above 3°C by the DSC method (data not shown).

Soy PG was investigated as another charged lipid which may be induced to undergo a phase transition in the presence of cations. Soy PG incorporated into the mixture of soybean PC, PE, and PI showed no detectable phase transition, nor was a transition observable in the presence of 2 mM La^{3+} . When soy PG was combined with DSPC in a ratio of 1:9 there was a lowering of the T_c of DSPC from 54°C to approximately 46°C. The inclusion of La^{3+} shifted the transition of the mixture to a slightly higher temperature (data not shown).

Phosphatidic acid was next considered as a possible acidic contributor to phase transitions in soybean lipids. Although present in only minor amounts (15, 16), PA may accumulate under perturbed conditions, particularly through the hydrolytic activity of phospholipase D (19). PA has the highest melting point of the phospholipids commonly found in plant and animal membranes. DPPA is seen to have a transition at 62°C (Fig. 3D). The difference in transition temperature for DPPC and DPPA in Figure 3, A and D, respectively, can be attributed solely to the differences in head groups as both have identical fatty acyl chains. The presence of PA in soybean lipids even with unsaturated acyl chains might introduce a phase transition at temperatures in the biological range. But it can be seen (Fig. 3, B and C) that a mixture of DPPC and DPPA yields two poorly defined thermotropic transitions of reduced enthalpy and over broad temperature bands. These are detectable whether one is scanning in the warming or the cooling direction. The effects of mixes containing DPPA indicate that such acidic phospholipids are miscible with nonacidic components and in such mixtures they do not retain their higher transition temperatures.

With the addition of small amounts of DPPA to asolectin vesicles (1:9 w/w), a long broad endotherm was observed that was not seen in a similar scan of asolectin alone. During slow cooling, a broad exotherm was detected verifying that the steady decline in the DSC scan during heating was attributable to a reversible lipid phase transition (data not shown). No sharp transition was observed, however. When larger amounts of PA were mixed with asolectin vesicles (1:1), a broad endotherm was observed with a peak enthalpy close to that of pure DPPA, indicating that domains of DPPA were formed at this high concentration.

Mitochondrial Lipids. Since mitochondrial membranes have been implicated in chilling injury (9), DSC scans were made of soybean mitochondria and of the lipids extracted from mitochon-

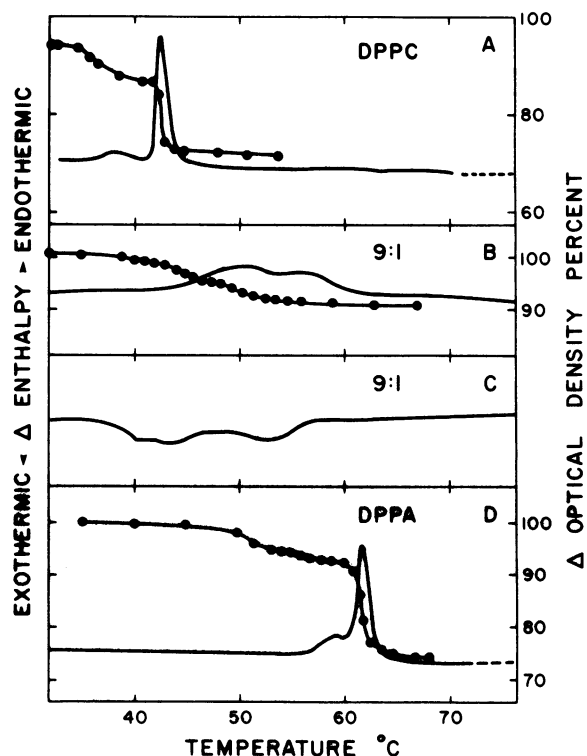


FIG. 3. Effects of mixing DPPA, an acidic phospholipid, on transition behavior of DPPC. A, DPPC alone; B, DPPC/DPPA 9:1 in a heating cycle; C, same as B in a cooling cycle; and D, DPPA alone. DSC scans on range 0.5 mcal/s, 2.5°C/min.

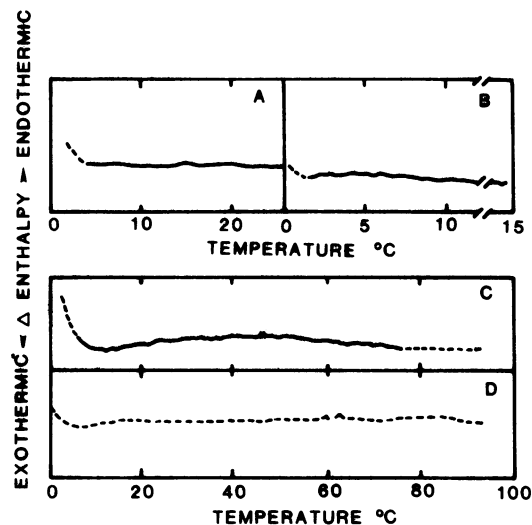


FIG. 4. DSC scans of soybean mitochondrial membranes and extracted membrane phospholipids. A, Soybean mitochondrial membranes, scanned at range 0.2 mcal/s, 2.5°C/min; B, same as A but scanned at 1.25°C/min; C, scans of phospholipids extracted from soybean mitochondria scanned at 0.2 mcal/s, 5°C/min; and D, base line on range 0.2 mcal/s at 5°C/min.

dria. At scan rates from 1.25 to 5°C/min, no transitions were detected (Fig 4, A and B). Scan rates above 5°C/min had been previously reported to result in anomalous peaks in biological samples (18). Using slow scanning conditions with full scale sensitivity, some small endothermic events above 50°C were observed which were attributable to protein denaturation since they were not seen in the cooling scans or upon re-scanning of the membrane samples. The addition of La^{3+} to the mitochondrial sample had no apparent effect. Scans of extracted mitochondrial

polar lipids yielded no detectable transitions (Fig. 4C). Care was taken not to interpret base line curvature or offset (Fig. 4D) as an endothermic phase transition.

DISCUSSION

The theory that chilling injury of plants may be related to phase changes in the membrane lipids rests on the observations that the apparent activation energies of metabolic activities are markedly increased in the chilling temperature range of susceptible species (10). Published attempts to find more direct evidences of phase transitions in membranes have almost always utilized ESR probes or fluorescence polarization probes introduced into the membranes. Attempts to find phase transitions in membranes without the addition of chemical probes have either revealed only broad transitions over a 60°C temperature span (2) or a small transition signal occurring in the bulk lipids (12).

Two methods for direct examination of membranes or phospholipid components of membranes can be used: differential scanning calorimetry and changes in optical diffraction properties. Each of these has sensitivity limits but they have the specific advantage of not requiring the addition of exogenous compounds to membranes which can change the characteristics being studied (20, 21).

Our experiments with DSC and optical scanning indicate that no bulk phase transition is detectable in the membranes of the chilling-sensitive soybean seed in the temperature range where chilling occurs. Likewise, the unsaturated phospholipids obtainable from soybeans yielded no detectable transitions in the chilling injury range. The addition of divalent and trivalent cations, Ca^{2+} and La^{3+} , caused apparent small thermal changes in simple mixtures of charged species of phospholipids, but not in the more complex mixtures of natural soybean membrane components. The scans of mixtures of soybean phospholipids show a profound depression and spreading of phase transitions, with even binary mixtures reducing the phase change to a weak and spread out signal.

We were able to observe phase changes in vesicles when DPPA was present in as little as 10% of the total phospholipid, from which we infer that a phase separation of this magnitude due to PA in the membrane samples should have been detectable by our measurements if it were occurring. Our results cannot exclude the possibility of a lateral phase separation or phase transition occurring in less than 10% of the membrane lipid. Except for the possible contribution of saturated phosphatidic acid which may be present in small amounts (less than 1.5 mol%, 16), it seems unlikely that a temperature-induced phase transition occurs in soybean seeds in the chilling temperature range.

Collectively, the data presented here generate five arguments that do not support the idea that low temperatures cause chilling damage by inducing a bulk phase transition in the lipids of membranes.

(a) Soybean phospholipids and soybean mitochondrial membranes show no detectable transition in the temperature range at which chilling damage occurs. The preponderance of unsaturated fatty acids precludes T_c values in the biologically relevant temperature range.

(b) Arrhenius plots of metabolic activities, which have formed the basis for the phase transition theory, indicate a relatively sharp alteration in apparent activation energies at a specific threshold temperature; sharp phase transitions are obtained only in samples of unmixed phospholipids, and are not detectable in mixes. Of course the natural membrane represents a complex mixture of phospholipids.

(c) Although the DSC has the advantage of not altering the membrane components, it does have limits of resolution; but even

if small local aggregations of soybean phospholipids were to occur in membranes, the phase transitions would have to occur in the chilling temperature range to be causal of injury, and none of the soybean phospholipids is known to have a T_c in the chilling temperature range.

(d) Although it is possible that membrane proteins might cause local phase separations, again it would be essential that the localized phospholipids have a characteristic T_c in the chilling temperature range, and no soybean phospholipid is known to do so.

(e) Although it is known that strong cations such as Ca^{2+} can elevate the T_c values for some phospholipids, no such elevation of the T_c for soybean phospholipids or isolated mitochondrial membranes was detectable.

Acknowledgments—The authors would like to express their sincere appreciation to Drs. D. Barbano, J. W. Sherbon, and A. Wood for the generous use of equipment used in this investigation. A special thanks is extended to Dr. G. W. Feigenson for many helpful suggestions and critical advice.

LITERATURE CITED

- BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254
- DALZIEL AW, RW BRIEDENBACH 1979 Differential thermal analysis of tomato mitochondrial lipids. *In* JM Lyons, D Graham, JK Raison, eds, *Low Temperature Stress in Crop Plants*. Academic Press, New York, pp 319-325
- HOBBS PR, RL OBENDORF 1972 Interaction of initial seed moisture and imbibitional temperature on germination and productivity of soybean. *Crop Sci* 12: 664-667
- KAGAWA Y, E RACKER 1971 Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J Biol Chem* 246: 5477-5487
- KATES M 1972 Techniques of Lipidology. *In* TS Work, E Work, eds, *Isolation, Analysis, and Identification of Lipids*. Elsevier/North Holland, Amsterdam pp 347-353; 397-405
- LADBROOKE BD, D CHAPMAN 1969 Thermal analysis of lipids, proteins and biological membranes. A review and summary of some recent findings. *Chem Phys Lipids* 3: 304-367
- LEE AG 1977 Lipid phase transitions and phase diagrams. I. Lipid phase transitions. *Biochim Biophys Acta* 472: 237-281
- LEE AG 1977 Lipid phase transitions and phase diagrams. II. Mixtures involving lipids. *Biochim Biophys Acta* 472: 285-344
- LEOPOLD AC, ME MUSGRAVE 1979 Respiratory changes with chilling injury of soybeans. *Plant Physiol* 64: 702-705
- LYONS JM, JK RAISON 1970 Oxidative activity of mitochondria isolated from plant tissue sensitive and resistant to chilling injury. *Plant Physiol* 45: 386-389
- MABREY S, JM STURTEVANT 1976 Investigation of phase transitions of lipids and lipid mixtures by high sensitivity differential scanning calorimetry. *Proc Natl Acad Sci USA* 73: 3862-3866
- MCMURCHIE EJ 1979 Temperature sensitivity of ion-stimulated ATPases associated with some plant membranes. *In* JM Lyons, D Graham, JK Raison, eds, *Low Temperature Stress in Crop Plants*. Academic Press Inc, New York, pp 163-176
- PHILIPSON KD, DM BERS, AY NISHIMOTO 1980 The role of phospholipids in the Ca^{2+} binding of isolated cardiac sarcolemma. *J Mol Cell Cardiol* 12: 1159-1173
- PETERSEN NO, PA KROON, M KAINOSHO, SI CHAN 1975 Thermal phase transitions in deuterated lecithin bilayers. *Chem Phys Lipids* 14: 343-349
- PRIESTLEY DA, AC LEOPOLD 1980 The relevance of seed membrane lipids to imbibitional chilling effects. *Physiol Plant* 49: 198-204
- PRIESTLEY DA, AC LEOPOLD 1979 Absence of lipid oxidation during accelerated aging of soybean seeds. *Plant Physiol* 63: 726-729
- RAISON JK 1973 Temperature-induced phase changes in membrane lipids and their influence on metabolic regulation. *Symp Soc Exp Biol* 27: 485-512
- SCHIEDLER PJ, JM STEIM 1975 Differential scanning calorimetry of biological membranes: instrumentation, Report 2. *In* ED Korn, ed, *Methods in Membrane Biology*, Vol. 4, pp 77-95
- SCHERER GFE, DJ MORRÉ 1978 Action and inhibition of plant phospholipases. *Plant Physiol* 62: 933-937
- SHIMSHICK EJ, HM McCONNELL 1973 Lateral phase separation in phospholipid membranes. *Biochemistry* 12: 2351-2360
- STURTEVANT JM, C HO, A REIMANN 1979 Thermotropic behavior of some fluorodimethylphosphatidylcholines. *PNAS* 76: 2239-2243
- SZOKA F, D PAPAHAJIOPOULOS 1980 Comparative properties and methods of preparation of lipid vesicles (liposomes). *Annu Rev Biophys Bioenerg* 9: 467-508
- WILSON RF, RW RINNE 1974 Phospholipids in the developing soybean seed. *Plant Physiol* 54: 744-747
- WOLFE J 1978 Chilling injury in plants—the role of membrane lipid fluidity. *Plant, Cell Environ* 1: 241-247