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Studies on Lipid Changes Associated with Frost Hardiness in Cortex in Woody Plants*

By

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Abstract

Seasonal changes in lipid components were studied with special reference to the variation of frost hardiness of poplar. Changes in the total phospholipids in the cortical tissues were associated with hardiness changes. Major phospholipids in the cortex were found to be phosphatidylcholine and phosphatidylethanolamine, and the amounts of these phospholipids in the cortex were found to be inversely related to the environmental temperature. It was also observed that an increase in hardiness is accompanied by a concomitant increase in phosphatidylcholine and phosphatidylethanolamine. Thus, compositional changes in phospholipids appear to play an important part in increasing the hardiness of the cortical cells. Opposite responces in the content of phospholipids and triglycerides, and of monogalactosyl diglyceride and digalactosyl diglyceride, to low temperatures were also observed. These facts indicate that environmental temperature significantly controls lipid metabolism and the development of hardiness.

A striking degradation of phosphatidylcholine into phosphatidic acid was observed in the cortical tissues of less hardy poplar, when the tissues were frozen below a lethal temperature. No change in phospholipids was, however, detected in winter cortical tissues, which survived even immersion in liquid nitrogen after prefreezing at −50°C. Thus, these facts suggest that some deleterious changes associated with freezing injury occure in cellular membranes.

On the basis of these results, it is likely that the seat of the principal chemical changes involved in both resistance and injury of cells is in the membranes and in the substances comprising them.

Contents

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I. Introduction

Most of plants growing in cold climates have the ability to survive freezing below −30°C. Some woody plants such as willows, poplars, white birchs etc. are extremely resistant to freezing in winter, while they will sustain freezing injury at temperatures just below −5°C in summer. Thus, the freezing tolerance (frost hardiness) of these plants varies dramatically during the year.

In attempts to elucidate the mechanism of hardiness changes in plants, some 10 to 20 years ago, research was mainly focussed on the parallelism between accumulation of sugars, amino acids and other metabolites and the increase in hardiness. A vast amount of research in this area has been reviewed by Levitt[1] and Alden et al.[2] Of these substances, the accumulation of sugars during natural hardening from autumn to winter (cold acclimation) was particularly studied by many workers[3–10]. Also, a high degree of protective action of sugar and related compounds against freezing injury of cells[5] and membrane systems[11] has been well established. However, some reports revealed that hardiness could sometimes be developed without any increase in sugars[8]. On the other hand, sometimes an increase in sugar content failed to induce a rise in hardiness[1].

Siminovitch and Briggs some 20 years ago observed a relation between hardiness of the bark of the black locust tree and soluble protein content of the tissues[12]. Recent studies have confirmed and extended this in black locust tree[13,14] and demonstrated it in alfalfa[15,16] and other plants[17]. The rise in soluble protein content was demonstrated to be accompanied by a rise in RNA[13–15]. Weiser and his collaborators have also found, during cold acclimation, changes in total protein content[17], specific protein content[18], organic and inorganic phosphorus[17], total ribonucleic acid[18,19], and transfer and ribosomal ribonucleic acids[19]. More recently, research has been focussed on the changes in proteins[15, 19,20], specific enzymes[20,21] and nucleic acids[13,19].

Levitt[1] and his collaborators have found a number of correlations (both possitive and negative) between sulfhydryl (SH) and disulfide contents of tissues and frost hardiness. Levitt[1,22] postulated that, during hardening, membrane proteins enriched in SH may be replaced by SH depleted membrane proteins thus reducing the chance of injurious intermolecular SS bonding between membrane proteins during freezing.

These are the major components of plant tissues for which correlations may exist. However, none of them seem to be perfect. Thus, despite these correlations, the question remains unsolved. Probably, the natural hardening process involves a sequence of processes which are mutually dependent.

There is some evidence to show that membranes may be the primary site of freezing injury in plant cells. In his electrical low frequency resistance studies of alfalfa, Greenham[23] obtained evidence to show that the intactness of plasmalemma was lost during freezing as a result of injury. Using electrophoretic measurements to study the patterns of freezing in various plant materials, Olien[24] and Sukumaran et al.[25] obtained essentially the same results. These facts suggest that some deleterious changes occur in cellular membranes of plants frozen below the critical temperatures. Thus, again it may be considered that hardening process in plants probably involves some profound changes in membrane components. In connection with this consideration, some changes in phospholipids, glycolipids[14,26,27,28] and lipid unsaturation[29,30] have been reported in association with hardiness change. Little attention has, however, been paid to qualitative changes in the individual phospholipids and neutral lipids and their temperature dependencies in hardy woody species.

This study was designed to elucidate the intimate relation of lipid changes and temperature-induced hardiness in poplar. For this, seasonal changes in lipid components of cortical tissues, intracellular localization of lipids, and degradation of phospholipids associated with freezing injury were mainly studied.

II. Materials and Methods

Materials

As experimental materials, current year twigs from 6-year poplar (*Populus euramericana* cv. gelrica) growing on the campus of our Institute were chiefly used. In late August the terminal growth ceased in this poplar. In mid October the leaf color began to change to yellow at the basal parts of the stems and the leaves fell off by the end of the month. In early May the poplar began to put forth young leaves.

In the experiments under artificial environmental conditions, potted cuttings

of poplar, *Populus euramericana* cv. I–475, were used. On October 11, two weeks before defoliation, all leaves were removed prior to the experiments to exclude the effect of temperature on the leaves.

Methods of treatment at different temperatures

These potted poplars were maintained at 24 (day)–19 (night) °C and 15 (day-night)°C in a temperature controlled green house under natural light conditions for 2 months. The treatment at 0°C was made in a hardening chamber with illumination of about 10,000 lux for 8 hr a day. Some potted poplars remained outdoors as controls.

Methods for evaluating frost hardiness

Cut stem or twig pieces of poplar were used to evaluate cold hardiness. These pieces, enclosed in polyethylene bags, were frozen at –5°C. They were then cooled in 5°C steps at hourly intervals to successively lower temperatures down to –30°C. They were then further cooled to –70°C (cooling rate from –30 to –70° C: 0.8°C/min). After standing at the selected temperatures for 16 hr, the frozen pieces were rewarmed in air at 0°C. When the ability of poplars to survive immersion in liquid nitrogen (–196°C) was to be evaluated, pieces were prefrozen for 16 hr at –20 or –30°C before immersion in liquid nitrogen. In hardy species, which are not killed by normal freezing temperatures, the temperature of the prefreezing treatment is related to the relative hardiness[31]; e.g. hardier tissues required less prefreezing at higher temperatures. After immersion in liquid nitrogen, samples were transferred to air at 0°C.

To evaluate the viability of poplars after freezing, thawed pieces were placed in polyethylene bags with water vapor at room temperature for 20 days. Thereafter, freezing injury was evaluated visually or microscopically. Browning was generally used as a criterion for rating injury of the tissues. In some cases, injury was determined from the extent of release of compounds reactive with $FeCl_3$. For this test 1 g of the frozen thawed cortical tissues was leached in 5 ml of distilled water at 27°C for 4 hr. Then 0.1 ml of 10% $FeCl_3$ solution was added to the leaching solution. A dark greenish color developed, with intensity dependent on the degree of injury. Absorbance was read at 620 nm, against the leaching solution from unfrozen samples. Along with this method, release of amino acids [32] and electrical conductivity[25] were also determined with the leaching solutions. The results obtained by these methods were found to be comparable with those obtained by the visual browning test and a plasmolysis test in which plasmolysis and deplasmolysis were repeated twice with a 2-fold isotonic balanced salt solution and water.

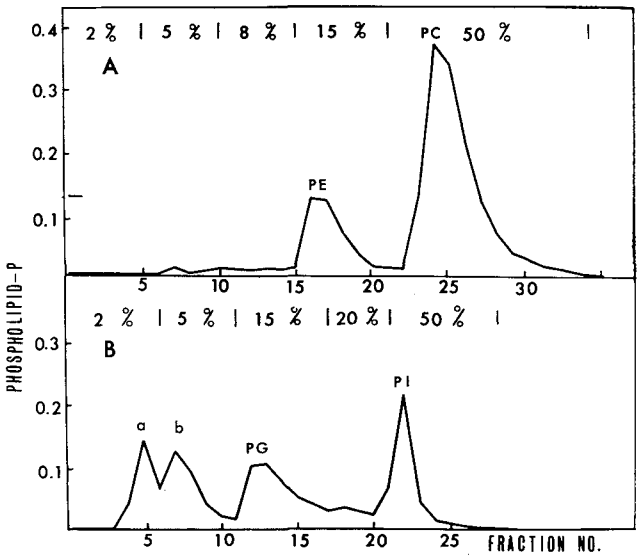

Fig. 1. Typical silica gel elution profiles of neutral (A) and acidic phospholipids (B) from DEAE cellulose column chromatography. Relative phospholipid content is expressed as absorbance at 700 nm. Phospholipids were eluted with chloroform-methanol mixtures through a stepwise increase in methanol content as indicated in the upper portions of the Figures. PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PG: Phosphatidylglycerol, PI: Phosphatidylinositol, a and b: Unidentified phospholipids

Extractions and purifications of phospholipids for quantitative analysis

From current year poplar twigs, 1.5 g of cortex or xylem was sampled at various stages of growth. The small pieces were then ground with isopropanol in a motor-driven mortar. All these procedures were performed at 4°C. After grinding, 25 ml of chloroform-methanol (2:1, v/v) were added and lipids were extracted for 30 min in centrifuge tubes at room temperature. Extraction was repeated twice more with the same solvent, then the combined lipid extracts were subjected to Folch's procedure[33] to remove nonlipid contaminants. The purified lipid samples were separated into neutral and acidic phospholipid fractions on a DEAE cellulose column (Whatman DE 32 in acetate form, 1×7 cm) according to Allen et al.[34] The neutral phospholipid fraction was eluted by 60 ml of chloroform-methanol (2:1, v/v) followed by 30 ml of methanol. The acidic phospholipid fraction was then eluted by chloroform-methanol-water (2:3:1, v/v/v) containing 0.05 M ammonium acetate. Both phospholipid fractions were further separated into several individual phospholipid components by silicic acid column chromatography (Fig. 1). In some cases, lipid samples were subjected

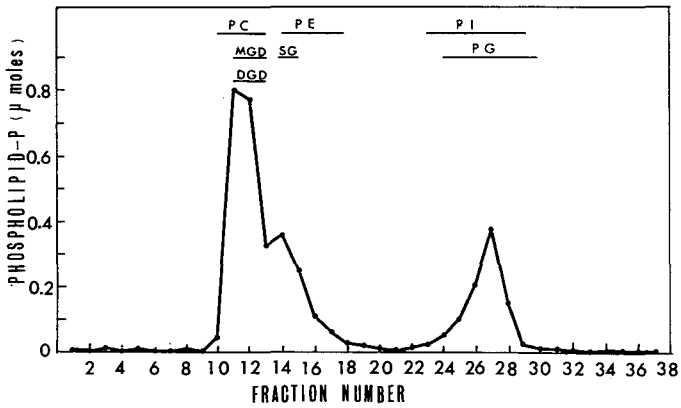

Fig. 2. Typical Sephadex LH-20 elution profile of phospholipids from poplar twig cortex. A lipid sample, prepared from 1 g of fresh tissues collected on November 11 in the field, was eluted with chloroform-methanol (2:1, v/v), and 4 ml of each fraction was collected. 0.15 ml of the aliquot was assayed for lipid phosphorus. DGD: Digalactosyl diglyceride, MGD: Monogalactosyl diglyceride, SG: Steryl glycoside The other abbreviations are the same as described in Fig. 1

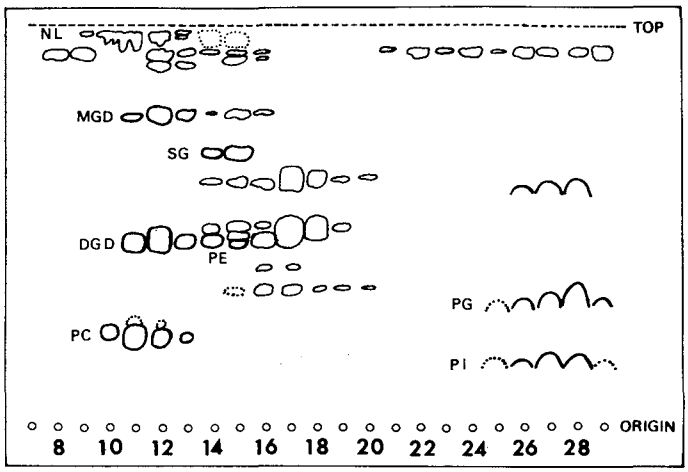

Fig. 3. Thin-layer chromatogram of lipid fractions from Sephadex LH–20 column. Numbers at the bottom corresponded to fraction numbers in Fig. 2. Plate, Silica gel H; Solvent, chloroform-methanol-water (65:25:4, v/v/v); Color reagent, 50% H_2SO_4 NL, Neutral lipid. Abbreviations are the same as described in Fig. 2

to Sephadex LH–20 column chromatography (1.8×50 cm, eluted with chloroform-methanol, 2:1, v/v) which separated phospholipids into two fractions as indicated in Figs. 2, 3. The first peak included phosphatidylcholine and phosphatidylethanolamine and the second peak included phosphatidylinositol, phosphatidylglycerol

and unidentified acidic phospholipids. Again, both phospholipid fractions were further separated into several individual phospholipid components by silicic acid column chromatography as indicated in Fig. 1.

Identification of lipids

Lipids were identified and their purity was tested by thin-layer chromatography on Silica gel H. Solvent used were chloroform-methanol-water (65:25:4, v/v/v), chloroform-methanol-acetic acid (65:25:8, v/v/v) and petroleum ether-ethyl ether-acetic acid (80:35:1, v/v/v). Positions of separated lipids were tested against authentic lipid samples. To identify each spot on the plates, the following spray reagents were used: 50% H_2SO_4 heated to 105°C for general lipid components; ammonium molybdate-perchloric acid reagent for phospholipids; ninhydrin reagent for amino group containing phospholipids; Dragendorf reagent for choline containing phospholipids; and anthrone reagent for glycolipids.

Quantitative analysis of lipids

Quantitative analysis of phospholipids was performed according to the method of Marinetti[35] with a slight modification. Small amounts of lipid samples (containing 0.05–0.3 μmoles of phosphorus) in test tubes were dried in a hot water bath and heated at 180°C for 45 min with 0.5 ml of 70% perchloric acid. After cooling, 3 ml of water, 0.5 ml of 2.5% ammonium molybdate and 0.2 ml of Fiske-Subbarow reagent were added to the test tubes. After heating them in a boiling water bath for 7 min, the absorbance was read at 700 nm.

The content of neutral glycerides, which were separated by preparative thin-layer chromatography on Silica gel H with a solvent of petroleum ether-ethyl ether-acetic acid (80:35:1, v/v/v), was determined according to the method of Hanahan and Olley[36].

For the purpose of separating galactolipids, an aliquot of lipid samples was loaded on a Silica gel H plate and developed with chloroform-mathanol-water (65:25:4, v/v/v). The separated galactolipids were then hydrolysed with 1 N HCl in methanol at 105°C for 2 hr. The content of galactose was quantitatively analysed using anthrone reagent[37].

Analysis of fatty acids

Each phospholipid fraction which was separated on silicic acid column chromatography was further purified on preparative thin-layer chromatography on Silica gel H with chloroform-methanol-water (65:25:4, v/v/v). The purified phospholipids were transesterified in 5% HCl-methanol by heating at 105°C for 2 hr. The fatty acid methylesters were further purified by thin-layer

chromatography and analysed on a Hitachi gas chromatograph, with a hydrogen ionization detector. A Hitachi Golley column BDS–45 was used. The column temperature was changed proportionally at the rate of 5°C per min from 160 to 200°C. The fatty acid methylesters were identified with authentic samples of fatty acid methylesters.

Cell fractionation procedures

Ten g of cortical tissues of poplar was minced with a razor blade and homogenized by grinding in a motor-driven mortar in 45 ml of medium consisting of 1.0 M sorbitol, 10 mM EDTA and 0.2 M tris-HCl buffer (pH 8.0), with 3 g of sea sand and 3 g of Polyclar AT for 5 min. The brei was strained through 3 layers of gauze. The crude cell-free homogenate was centrifuged successively at 200 $\times$g for 10 min, 1,000 $\times$g for 10 min, 12,000 $\times$g for 20 min and 78,000 $\times$g for 60 min. All pellets thus obtained were suspended in 0.01 M tris-HCl buffer (pH 7.8). An aliquot of the suspension was subjected to quantitative analysis of protein, and the remainder was subjected to quantitative analysis of lipid compounds. Lipids were extracted from the pellet suspension with isopropanol and subsequently with chloroform-methanol (2:1, v/v). Protein determination was carried out by amino acid analysis after hydrolysis with 6 N HCl for 20 hr at 105°C and bovine serum albumin was used as the standard[38].

Incorporation of glycerol-1-^{14}C

A piece of poplar twig (1.5 g weight, 0.7–0.8 cm diameter and 4–5 cm long) was excised immediately before use. Both ends of the sample were cut evenly with a sterilized blade. The sample was held vertically with the original lower cut end upward, and 2 μC_i of glycerol-1-^{14}C (purchased from Daiichi Pure Chemicals Co.; specific activity, 10 mC_i/m mole) was transfused to the cut end[39]. The transfused twig sample was covered with Parafilm and kept for various lengths of time at 20°C. At the end of incubation, the samples were chilled on ice and separated into bark and xylem fractions. This was followed by grinding with 1 g of sea sand in the presence of 5 ml of 0.1 N perchloric acid at 4°C. The brei was centrifuged at 5,000 rpm for 10 min and the precipitate was neutralized with $KHCO_3$ and washed with 10 ml of distilled water. Lipids were extracted from the washed residues with chloroform-methanol (2:1, v/v). The crude lipid extracts were purified by passing through a Sephadex G 25 column (1$\times$20 cm)[40]. The aqueous fractions were neutralized with $KHCO_3$ to pH 7.5 and fractionated into neutral fraction and basic fraction (amino acid fraction) on a Dowex-50 column (1$\times$2 cm). The basic fraction was eluted with 2 N ammonium hydroxide. Sugar phosphates were precipitated as barium salts from the netural fraction. The radioactivity in

each fraction was determined with a gas flow counter.

Procedures for phospholipid degradation studies

The cortical tissues from current year twigs of poplar were cut into small pieces (0.5×3 cm) and then frozen in a test tube (1.3×18 cm) at −5°C. The frozen tissues were cooled in 5°C steps at hourly intervals to successively lower temperatures down to −30°C. They were further cooled down to −50°C and then immersed in liquid nitrogen. The frozen tissues were thawed in air at 0°C. After standing at 0°C for 1 hr, they were placed at room temperature for more than 3 days. Freezing injury was then evaluated as described above.

Grinding of the frozen samples was performed in a cold room at −10°C. To minimize the degradation of phospholipids by phospholipases during grinding, isopropanol at −10°C was added. Isopropanol is known to eliminate lipolytic activity during grinding[41]. After grinding, 25 ml of chloroform-methanol (2:1, v/v) cooled at −10°C was added and then lipids were extracted for 1 hr at room temperature. Extraction was repeated twice more with the same solvent and the combined lipid extracts were subjected to Folch's procedure[33] to remove nonlipid contaminants. Aliquots of the purified lipid samples dissolved in a known amount of chloroform-methanol (2:1, v/v) were loaded on Silica gel H plates (5× 20 cm) and were developed with chloroform-methanol-acetic acid (65:25:8, v/v/v) at 25°C for 50 min. After exposing the plates in iodine vapor, the area corresponding to each standard phospholipid was scraped into test tubes and directly heated with 0.5 ml of 70% perchloric acid for 30 min at 180°C. The lipid phosphorus was determined according to the method described before. Nonloaded areas on the same plates were treated in the same way and used for blanks. Before reading the absorbance, each test tube was centrifuged at 3,000 rpm for 4 min to remove the silica gel powder.

Two μC_i of acetate-1-^{14}C (purchased from Daiichi Pure Chemicals Co.; specific activity, 45 mC_i/m mole) was transfused on the cut surface of a small twig piece sampled in mid-July (0.5 cm diameter, 3 cm long) and were then incubated at 20°C for 24 hr. The twig pieces frozen at −5°C for 2 hr were cooled to −10°C, resulting in serious injury. After standing at −10°C for 6 hr, lipids were extracted from the frozen sample. The crude lipid extracts from control and frozen samples were purified by passing through a Sephadex G 25 column[40]. The distribution of the radioactivities in the lipid samples was determined by thin-layer chromatography. Chloroform-methanol-acetic acid (65:25:8, v/v/v) and petroleum ether-ethyl ether-acetic acid (80:35:1, v/v/v) were used as solvents for the separation of polar and nonpolar lipids, respectively.

Ten g of cortical tissue collected on early November were minced with a razor

blade and homogenized with a Polytron PT 35 (Kinematica GmbH, Luzern) in 50 ml of 0.2 M tris-HCl buffer, pH 8.0, 1.0 M sorbitol, 1.5 g Polyclar AT and with or without 10 mM EDTA, at 60% of full speed for 1 min at 0°C. The brei was strained through 3 layers of gauze. The crude homogenate was centrifuged at 200 $\times$g for 10 min to remove cell debris and the supernatant was further centrifuged at 78,000 $\times$g for 60 min. The pellet prepared in the medium with EDTA present was suspended in 0.01 M tris-HCl buffer (pH 7.8) and then centrifuged again at 78,000 $\times$g for 60 min.

The reaction mixture consisted of 0.5 ml of pellet suspension, 0.5 ml of a buffer solution with or without 50 μmoles of Ca^{++}, Mg^{++} and various amount of EDTA. The final volume was 1.2 ml. The reaction was started by addition of a buffer solution; glycine-HCl buffer (pH 2.4–3.0), acetate buffer (pH 3.5–5.8) and tris-HCl buffer (pH 6.0–8.1). After incubation at 27°C, the reaction was stopped by adding 5 ml of isopropanol and then lipids were repeatedly extracted with chloroform-methanol mixture. In this extraction, polarity was decreased by means of a decreasing content of methanol. The crude lipid extracts were subjected to Folch's procedure[33] to remove nonlipid contaminants. An aliquot of purified lipid sample was loaded on a thin-layer plate of Silica gel H and run with chloroform-methanol-acetic acid (65:25:8, v/v/v). The separated phospholipids were analysed as described before.

Measurement of phospholipase D activity in poplar cortical tissues

Seasonal changes in phospholipase D activity were determined on poplar cortical tissues. Fresh tissues were homogenized by grinding in 1.0 M sorbitol, 0.1 M tris-HCl buffer (pH 7.8) with 1.0 g of sea sand and 1.0 g of Polyclar AT. The brei was strained through 3 layers of gauze and centrifuged at 200 $\times$g for 10 min to remove cell debris. The supernatant was used for the enzymatic sources. The reaction mixture consisted of 35 μl of 1.0 M $CaCl_3$, 13 μmoles of purified egg lecithin, 1.0 ml of the enzyme solution, 3 ml of 0.2 M acetate buffer solution (pH 4.8) and 1.0 ml of ethyl ether. Incubation was carried out at 27°C. The reaction was stopped by adding 1.0 ml of 1 N $HClO_4$. After washing the aqueous layer with ethyl ether, protein was removed by centrifugation. An aliquot of the clear aqueous solution was used for quantitative analysis of the liberated choline according to Kates and Sastry.[42]

III. Results

Changes in lipid components associated with frost hardiness

As presented in Fig. 4, twigs collected on September 1 were seriously injured at temperatures slightly lower than –3°C. In late September, the cortical tissues

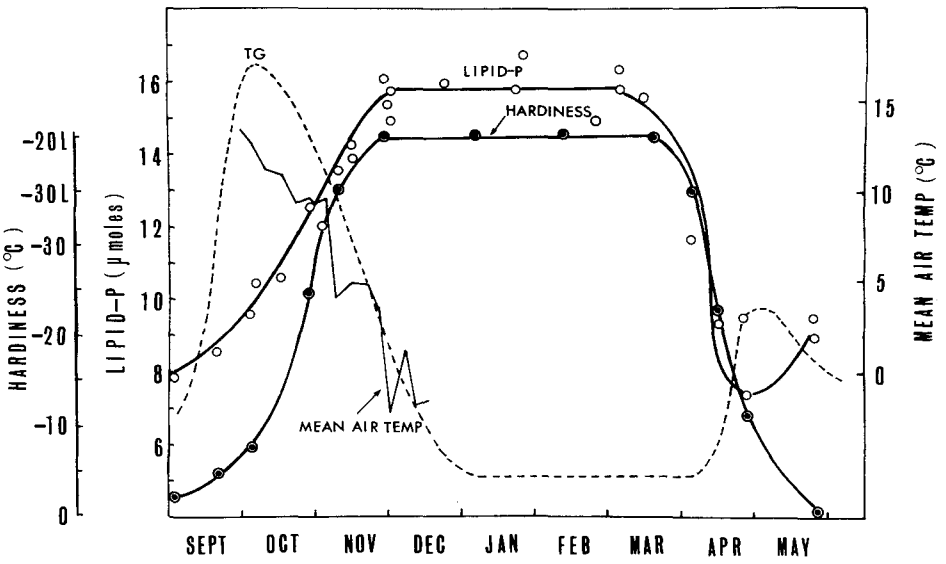

Fig. 4. Seasonal changes in frost hardiness and total amount of phospholipids in poplar cortex. Frost hardiness was expressed as the minimum temperature at which the cortex survived freezing without injury. –20 L and –30 L on hardiness scale: Tissues were slowly prefrozen to –20 or –30°C prior to immersion in liquid nitrogen (–196°C) TG: Triglyceride from cortex

became hardy to –5°C. Then, hardiness increased abruptly from mid-October to mid-November as the environmental temperatures decreased, reaching a maximal level in late November. These twigs survived immersion in liquid nitrogen after prefreezing to –20°C or below. Hardiness remained unchanged throughout winter, and then decreased rapidly until late May, reaching a summer minimum level.

The total amount of phospholipids in cortical tissues increased remarkably from early September to late November (Fig. 4). The high level of the total amount of phospholipids was maintained until late March and then decreased rapidly by the end of April. These results indicate that change in hardiness is accompanied by a change in total amount of phospholipids.

To determine the compositional changes in the phospholipids associated with hardiness, the lipids extracted from twig cortex were further separated into several components, and they were analysed quantitatively. As presented in Fig. 5, the most pronounced changes among phospholipids were observed in phosphatidylcholine and phosphatidylethanolamine. Phosphatidylinositol, phosphatidylgycerol and two unidentified acidic phospholipids appear to be minor phospholipids in poplar twig cortex and showed only slight seasonal changes. Thus, the major part of the increase in total phospholipids can be attributed to the increases in

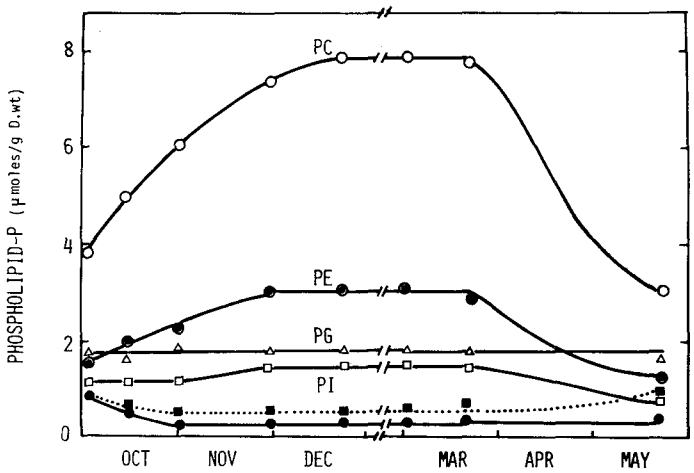

Fig. 5. Seasonal changes in individual phospholipids from poplar cortex. Phospholipids were separated first on a DEAE cellulose column into neutral and acidic phospholipid fractions and then each fraction was further separated into individual phospholipids on a silicic acid column. ▪·····▪, •·····• correspond to unidentified acidic phospholipids (a and b in Fig. 1). Abbreviations are the same as described in Fig. 1

Table 1. Fatty acid composition of phospholipids in cortical tissues from different growth periods*

Fatty acids**	Retention time(min)	PC		PE		PG		PI	
		Oct 1	Nov 30	Oct 1	Nov 30	Oct 1	Nov 30	Oct 1	Nov 30
14:0 (Myristate)	9.5	0	0	0	0	0	0	0	0
Unknown	12.4	0	0	20.5	19.1	0	0	0	0
16:0 (Palmitate)	13.4	20.1	20.8	16.7	13.6	38.6	36.4	46.8	49.8
16:1 (Palimitoleate)	14.8	0	0	0	0	0	3.3	0	0
18:0 (Stearate)	18.6	2.5	0.7	0.4	0.6	1.5	1.7	1.7	0.9
18:1 (Oleate)	19.8	6.8	0.4	2.7	1.3	7.7	1.9	3.0	0.6
18:2 (Linoleate)	22.2	64.3	67.4	50.7	58.5	46.1	48.1	42.9	37.4
18:3 (Linolenate)	25.8	6.2	10.7	9.1	6.9	6.1	8.7	5.6	11.3

* Percent by weight of each fatty acid calculated from area of each peak

** Numbers following fatty acids indicate number of carbons and number of double bonds respectively

Abbreviations are the same as described in Fig. 1

phosphatidylcholine and phosphatidylethanolamine.

The fatty acid compositions of the phospholipids of poplar cortex were determined (Table 1). Fatty acid composition was observed to differ for each phospholipid to some degree. Phosphatidylcholine was found to show a relatively high

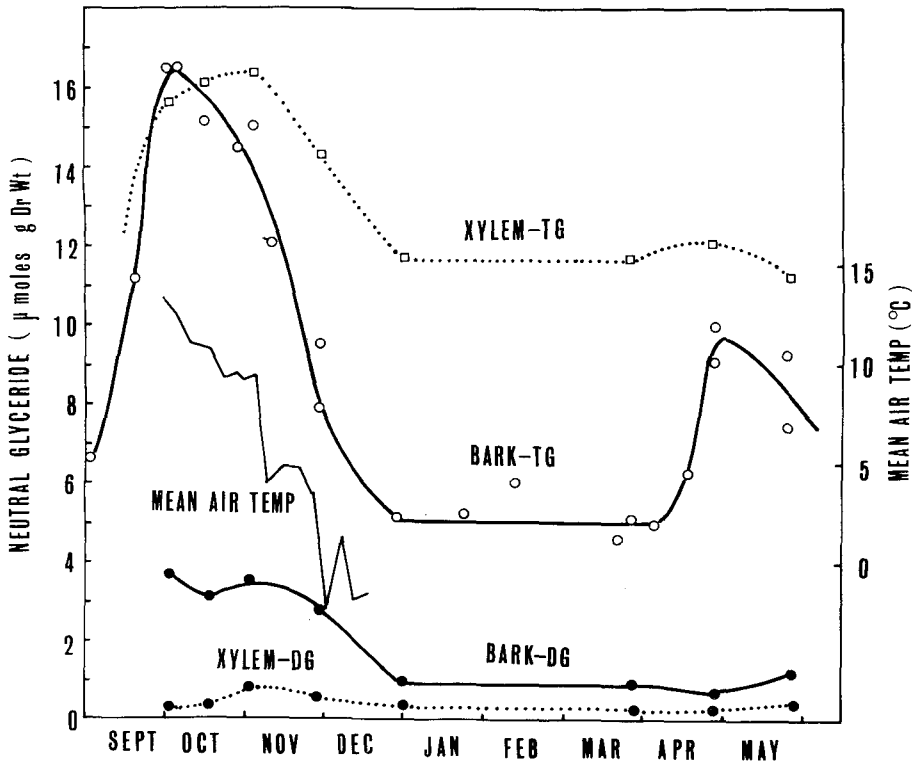

Fig. 6. Seasonal changes in neutral glycerides in poplar twigs. TG: Triglyceride DG: Diglyceride

percentage of linoleate and a low percentages of palmitate. Phosphatidylglycerol and phosphatidylinositol, on the other hand, showed a relatively high percentage of palmitate. Phosphatidylethanolamine was characteristic in being esterified with an unknown fatty acid. From early October to late November, the fatty acid compositions of phospholipids changed to some degree, although the patterns differed in individual phospholipids. In every phospholipid, oleate decreased and linoleate or linolenate increased. Thus, it appeared that during the natural hardening process the unsaturation of fatty acids proceeded in each phospholipid to some degree.

Seasonal changes in the amount of neutral glycerides in both twig cortex and xylem are summarized in Fig. 6 along with autumnal changes in mean air temperature. The amount of triglycerides in cortical tissues increased dramatically from early September to mid-October, and then decreased rapidly until late December. Triglyceride content remained at the low level until early April. There followed a rapid moderate increase immediately before bud opening. A similar pattern

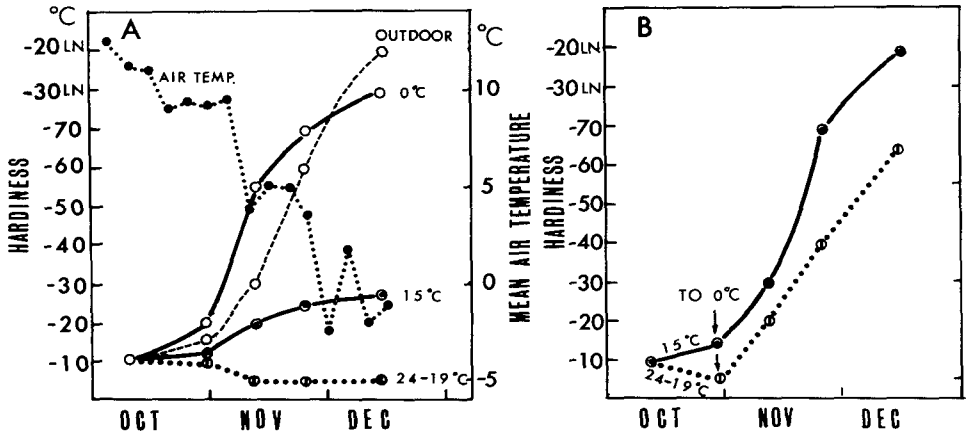

Fig. 7. Changes in hardiness of poplar cortex held at different temperature regimes of 24(day)-19(night)°C, 15(day-night)°C under natural day-length, respectively, and at 0°C (day-night) under illumination of about 10,000 lux for 8 hr. Arrows in Fig. 7–B indicated the time at which poplars from 24–19°C or 15°C were transferred to a hardening chamber at 0°C. -20L, -30L: Twigs were immersed in liquid nitrogen following prefreezing at -20 or -30°C, respectively

was also observed in xylem tissues, but triglyceride content in xylem tissues was maintained at a much higher level than that of cortex in mid-winter. As seen in Fig. 6, a decrease in triglyceride content from October to November was closely parallel to the decrease in mean air temperature. Seasonal changes in phospholipid and triglyceride contents suggest the likelihood of interconversion between these lipids, which appears to be inversely dependent on the environmental temperatures. On the other hand, the content of diglyceride in cortical tissues was maintained at a relatively high level from October to late November, and then decreased to a winter minimal level, which remained unchanged until late April. The same trend was observed in xylem diglyceride.

To clarify further that the hardiness of poplar cortical tissues is closely associated with phospholipid changes, some experiments were performed with potted poplars which were held at different temperature regimes for 2 months. Hardiness changes in cortical tissues from poplars held at different temperature regimes are summarized in Fig. 7 along with mean air temperature. In cortical tissues from the poplars held at 15°C, the hardiness gradually increased from -10 to -25°C over a period of 60 days. A dramatic increase in hardiness was observed in the poplars held at 0°C and in those kept outdoors. In mid-December they even survived immersion in liquid nitrogen following prefreezing to -30°C or -20°C. The mean air temperature outdoors remained below 5°C after mid-November (Fig. 7). How-

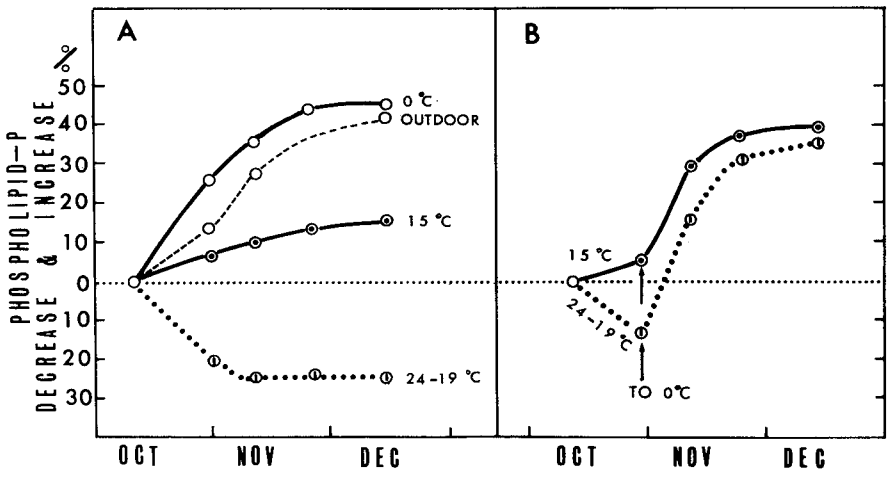

Fig. 8. Changes in phospholipid content in poplar cortex held at different temperature regimes. Phospholipid content is the percentage of increase or decrease in the original values. Arrows in Fig. 8–B indicate the time at which poplar from 24–19°C or 15°C was transferred to a hardening chamber at 0°C

ever, with cortical tissues held at 24–19°C, the hardiness gradually decreased from –10 to –5°C, then remained at the same level. As shown in Fig. 8-A, with cortical tissues held at 24–19°C, the phospholipids gradually decreased during the treatment and then remained at constant level, showing a pattern similar to the observed change in hardiness. In cortical tissues from poplars held at 15°C, a gradual increase in phospholipids was observed for 2 months. A remarkable increase in phospholipids was observed in poplars at 0°C and those kept outdoors. Poplars held at 24–19°C and 15°C for 20 days were transferred to a hardening chamber at 0°C. As shown in Fig. 8-B, in poplars held at 24–19°C, as well as 15°C, transfer to 0°C produced a rapid increase in phospholipid content with a concomitant increase in hardiness. Total phospolipids extracted from the cortical tissues of poplars were separated into individual phospholipids. The major part of the increase in total amount of phospholipids at low temperatures can be attributed to the increase in phosphatidylcholine and phosphatidylethanolamine (Fig. 9). However, phosphatidylglycerol, phosphatidylinositol and two unidentified acidic phospholipids appear to be minor phospholipids, showing a slight change in amount with temperature changes. Thus, the trends of the changes in individual phospholipids under low temperature conditions were substantially coincident with the results obtained previously in autumnal poplars in the natural environment.

As shown in Fig. 10, a rapid decrease in triglycerides in the cortical tissues was observed when poplars were transferred from outdoor to 0°C in mid-October and

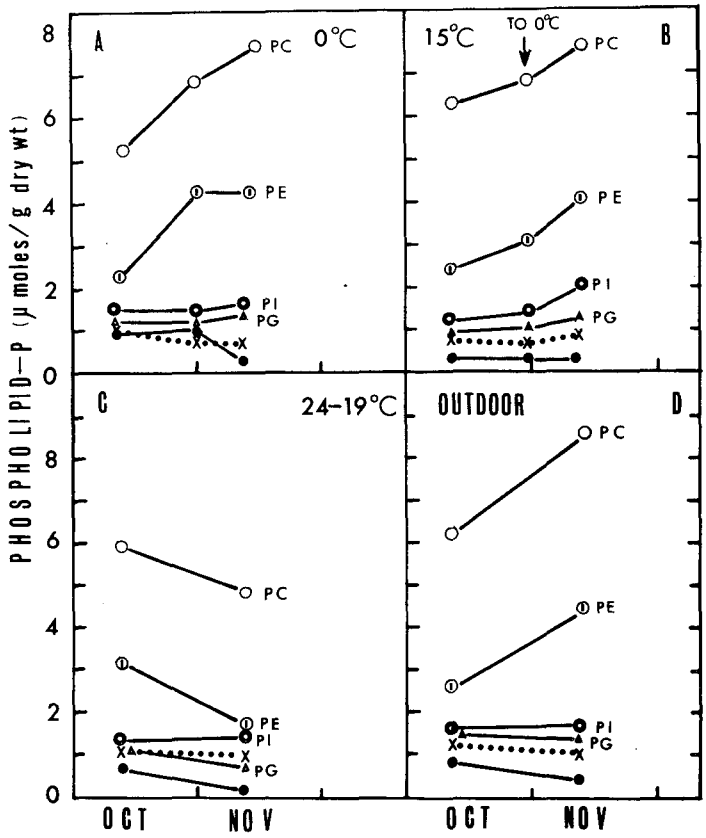

Fig. 9. Changes in individual phospholipid contents in poplar cortex held at varying temperatures. The arrow in Fig. 9–B indicates the time at which poplars from 15°C were transferred to a hardening chamber at 0°C. ×····×, ○····○: Unidentified acidic phospholipids. Abbreviations are the same as described in Fig. 1

from 24–19°C and 15°C in early November. The same interconversion between phospholipids and triglycerides under low temperature were observed with poplar twigs collected in different seasons, except for poplars collected in September (Table 2). In early September, an exposure to low temperature resulted in little or no increase in hardiness. At any rate, it seems likely that the environmental temperature may significantly control lipid metabolism and the development of hardiness.

Incorporation of glycerol-1-^{14}C into lipids and other fractions at different seasons

To get some information on the seasonal changes in lipid metabolism in poplar, the incorporation of glycerol-1-^{14}C into lipids and into several other fractions were

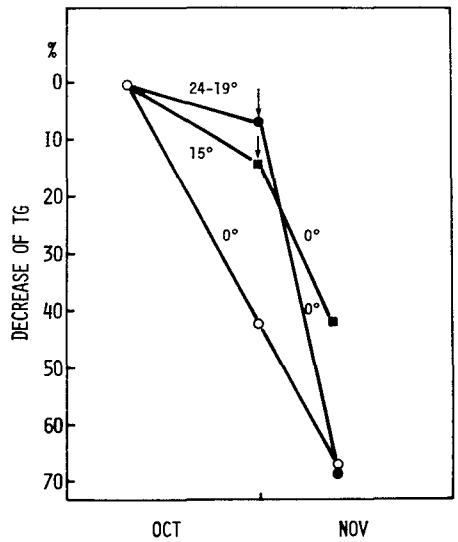

Fig. 10. Decrease in triglyceride in poplar cortex transferred to 0°C. Arrows are the same as described in Fig. 8. Triglyceride content is represented as the percentage of decrease from the original values

Table 2. Changes in the contents of phospholipid and triglyceride in cortical tissues of poplar during an artificial hardening

Expt date		Hardiness (°C)	Phospholipid μmoles/g dr.wt.	Triglyceride μmoles/g dr.wt.
Sept. 1	N	-3	8.0	6.6
	H (0°C)	-5	8.1	4.0
Sept. 18	N	-5	8.5	11.2
	H (0°C)	-12	10.2	11.6
Oct. 5	N	-7	10.5	16.4
	H (0°C)	-20	12.2	13.6
	H (-3°C)	-25	12.8	15.2
April 25	N	-12	7.5	9.1
	H (0°C)	-25	9.3	7.3
	H (-3°C)	-25	9.6	7.1
	H (-5°C)	-25	9.7	8.0

N: Before hardening, H: After hardening at the indicated temperatures for 2 weeks

examined. Table 3 shows the result of incorporation experiments with glycerol-1-^{14}C in different seasons. Seasonal changes in the incorporation of activity into lipids were closely observed in both cortex and xylem. From August to mid-

Table 3. Incorporation of glycerol-1-^{14}C into various fractions of poplars at different growth stages

Expt. date	Incubation time(hr)	Lipids		Radioactivity in Amino acids		Sug-P		Sugars		Insol.residues	
		C	X	C	X	C	X	C	X	C	X
						cpm x 10^{-3}					
Aug. 24	3.5	119.5	115.8	13.0	26.7	44.2	63.2	44.0	322.9	46.0	81.3
Sept. 16	3.5	206.5	157.5	29.2	40.0	25.4	13.5	238.6	639.1	6.4	8.4
Oct. 2	3.5	74.4	81.8	28.8	53.4	10.1	12.2	360.0	961.6	4.5	5.6
Oct. 28	3.5	13.6	17.9	4.4	5.2	3.5	3.4	467.3	957.8	0.8	1.3
Jan. 9	3.5	3.4	9.0							0.2	0.6
April 8	3.5	40.0	12.3								
April 20	4.0	275.0	72.1								
May 19	4.0	337.5	16.3								
June 20*	3.5	287.8	107.1	91.4	77.5	109.8	97.4	273.0	345.0	77.0	28.3
June 25*	3.5	231.0	136.8	135.4	113.3	60.0	143.2	239.0	334.0	57.5	37.1
July 14*	3.5	177.5	147.6	163.2	202.6	42.5	84.7	355.0	265.0	25.7	78.5

C: Cortex, X: Xylem
2 μC_i of glycerol-1-^{14}C was transfused to the cut end of a small piece of poplar twig freshly excised before use and incubated at 20°C. Experimental details are described in the text.
* Overwinterring potted poplars were transferred into a cold room at 4°C in mid-January and left there until June 20. They began to put forth young leaves. They were then taken outdoors and allowed to grow. Sug-P: Sugar phosphates

September, the incorporation of glycerol-1-^{14}C into total lipid was found to increase in both cortex and xylem, and then decrease until mid-winter, especially during October. And the incorporation of glycerol-1-^{14}C again increased from early April to May as budding proceeded. The incorporation of glycerol into the amino acid fraction, however, remained at a relatively low level in autumn, whereas it increased rapidly at the time of budding. In autumn, most of the radioactivity in free sugar fraction was recovered in free glycerol and not, if any, in other sugars, while in spring considerable parts of the radioactivity in this fraction were recovered in glucose, fructose and sucrose (data not presented). These results imply that a great difference in cell metabolism exists between autumn and spring.

Intracellular localization of lipids and their behavior upon hardening and dehardening

To further comprehend the changes in lipid components at a subcellular level during hardening and dehardening, experiments were focussed on intracellular localization of lipid components. Twigs collected in mid-winter were dehardened in a polyethylene bag at room temperature (17–20°C) for a month. After the dehardening, hardiness of the cortical tissues markedly decreased from the winter maximum to –10°C. Control twigs were held at –5°C. Cell fractionation was carried out by differential centrifugation. Each cell fraction was subjected to quantitative analysis of proteins and lipids. The results are presented in Tables

Table 4. Changes in the contents of protein and phospholipid in various fraction of poplar cortical cells during dehardening

Cell fractions	Protein (mg)		Phospholipid (μmoles)		Phospholipid / Protein		D/N
	N	D	N	D	N	D	
200-P	4.99	5.01	1.48	1.21	0.29	0.24	0.83
1,000-P	2.34	1.51	0.76	0.44	0.32	0.29	0.89
12,000-P	3.69	2.53	1.82	0.87	0.49	0.34	0.70
78,000-P	7.94	3.14	5.71	1.59	0.71	0.50	0.70
Sup	59.82	29.84	6.00	1.73	0.10	0.05	0.58
Total	78.78	42.03	15.77	5.87			

Samples were collected on mid-winter and dehardened at room temperature (17–20°C) for 1 month. Hardiness decreased from the winter maximum to –10°C as a result of dehardening
N: Control, D: Dehardened

Table 5. Compositional changes in phospholipids in various fractions of poplar cortical cells during dehardening*

Cell fractions	PC		PI		PE		PG		PA	
	N	D	N	D	N	D	N	D	N	D
200-P	50.3	46.5	7.8	7.7	22.6	24.1	9.8	9.4	9.5	12.4
1,000-P	46.4	35.9	11.3	16.5	22.5	18.0	15.6	18.9	4.1	10.7
12,000-P	43.3	42.8	12.8	12.6	22.4	18.7	12.6	18.6	8.9	7.3
78,000-P	52.6	44.1	6.0	10.6	23.1	22.3	10.8	12.1	7.4	10.8
Sup	50.4	39.0	11.3	17.5	18.6	21.7	14.3	11.2	5.4	10.7

Samples and experimental conditions were the same as described in Table 3
N: Control, D: Dehardened
* Molar percent of total lipid-phosphorus in each cell fraction
Abbreviations are the same as described in Fig. 1

4 to 8. Changes in the contents of proteins and phospholipids in various cell fractions from cortical tissues after dehardening are shown in Table 4. In general, the contents of proteins and phospholipids were observed to increase with a decrease in particle size or density. The content of phospholipids per miligram protein was also found to increase with a decrease in particle size. A significant amount of phospholipids still remained in the supernatant, suggesting that some fraction of light membranes or membrane fragments still remained in the supernatant. When this supernatant was further centrifuged at 170,000×g for 60 min, about 80% of the phospholipids was recovered in the pellet fractions.

Dehardening resulted in a marked decrease both in protein and phospholipids,

Table 6. Changes in the content of protein and phospholipid in various fractions from poplar cortical cells during hardening

Cell fractions	Protein (mg)		Phospholipid (μmoles)		Phospholipid / Protein		H/N
	N	H	N	H	N	H	
200-P	4.20	2.93	0.49	0.58	0.12	0.20	1.66
2,500-P	5.66	7.75	0.91	1.98	0.16	0.26	1.62
12,000-P	4.79	5.70	1.64	2.25	0.34	0.39	1.14
78,000-P	8.22	8.23	4.76	6.43	0.58	0.78	1.34
Sup	53.94	61.83	3.06	3.37	0.05	0.05	1.00
Total	76.81	86.44	10.86	14.61	0.14	0.18	1.28

Samples were collected in the middle of October and hardened at 0°C for two weeks. Hardiness increased from −15 to −30°C as a result of hardening. N: Control, H: Hardened

but the decrease in phospholipids in each cell fraction exceeded that of protein. Thus, the content of phospholipids per miligram protein considerably decreased in each cell fraction after dehardening, especially in the 12,000×g pellet, 78,000×g pellet and supernatant. From these results, it appears that dehardening results not only in the reduction of membranous components in the cells but also in the reduction of phospholipid content per miligram protein in membranes. It seems, therefore, reasonable to assume that membranes may shift from a phospholipid enriched state to a phospholipid depleted state upon dehardening.

The compositional changes in phospholipids in various cell fractions after dehardening are presented in Table 5. Each fraction differs in phospholipid composition to some extent. The 78,000×g pellet fraction was characterized by a high percentage of phosphatidylcholine and low percentage of phosphatidylinositol and phosphatidylglycerol. Upon dehardening, considerable decrease in phosphatidylcholine was observed in most of the cell fractions, and conversely, the percentage in phosphatidylinositol, phosphatidylglycerol and phosphatidic acid, including a trace amount of diphosphatidylglycerol, were increased. Thus, it appears that dehardening may cause some compositional changes in the phospholipids of membranes.

Autumnal poplar twigs hardened at 0°C for 2 weeks increased their hardiness from −15 to −30°C. In the hardened cortex, phospholipid content per miligram protein increased markedly in each cell fraction (Table 6). Compositional changes in phospholipids, especially in the considerable percentage increase of phosphatidylethanolamine, were observed. Thus, it appears that the amount of phospholipids per miligram protein in membranes may vary considerably with changes in hardiness.

Table 7. Compositional changes of galactolipids in various fractions of poplar cortical cells during dehardening

	Cell fractions	MGD	DGD (μmoles)	MGD+DGD	Chlorophyll (O.D 665nm)	MGD+DGD / Chlorophyll	DGD / MGD
Control	200-P	0.23	0.39	0.62	3.42	3.64	1.72
	1,000-P	0.17	0.27	0.44	1.42	6.18	1.61
	12,000-P	0.27	0.43	0.70	3.08	4.65	1.61
	78,000-P	0.37	0.85	1.22	3.94	6.21	2.30
	Sup	0.26	0.47	0.73	1.24	11.93	1.81
	Total	1.30	2.41	3.71	13.10		1.86
After dehardening	200-P	0.46	0.28	0.74	4.36	3.40	0.60
	1,000-P	0.22	0.18	0.40	2.02	4.04	0.83
	12,000-P	0.40	0.31	0.71	4.46	3.20	0.77
	78,000-P	0.33	0.26	0.59	2.74	4.33	0.80
	Sup	0.35	0.24	0.59	0.86	13.58	0.68
	Total	1.76	1.27	3.03	14.44		0.72

Samples and experimental conditions were the same as described in Table 4
MGD: Monogalactosyl diglyceride, DGD: Digalactosyl diglyceride

Table 8. Changes in DGD/MGD ratios in autumnal poplar cortical cells during artificial hardening

Cell fractions	DGD/MGD	
	N	H
200-P	0.87	1.78
2,000-P	0.95	1.53
12,000-P	0.93	1.64
78,000-P	0.97	1.73
Sup	0.90	1.73

N: Control, H: Hardened at 0°C for 2 weeks
Samples were collected in middle of Octover. Hardiness increased from −15 (N) to −30°C (H) as a result of hardening
Abbreviations MGD and DGD are the same as described in Table 7

In galactolipids, noticeable changes were found in every cell fraction; particularly the ratio of digalactosyl diglyceride to monogalactosyl diglyceride decreased considerably as a result of dehardening (Table 7). Also a marked decrease in galactolipids was observed in the 78,000×g pellet and in the supernatant. Upon hardening, on the other hand, the ratio of digalactosyl diglyceride to monogalactosyl diglyceride increased reversely in every cell fraction (Table 8). Thus it appears that the changes in the ratio of digalactosyl diglyceride to monogalactosyl diglyceride in membranes may proceed reversibly in association with hardiness changes. The changes in the ratio of digalactosyl diglyceride to monogalactosyl

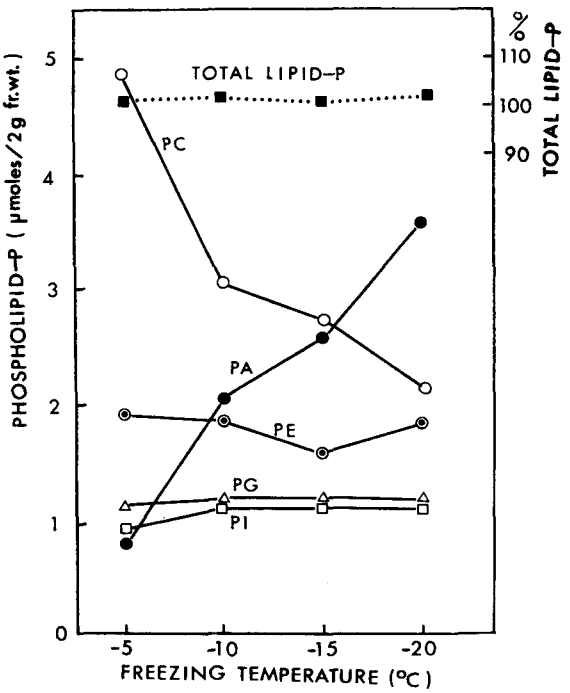

Fig. 11. Changes in phospholipids and total lipid phosphorus in poplar cortical tissues during freezing. The cortical tissues sampled on October 4 resisted freezing to –5°C. The frozen tissues were ground in a motar with cold isopropanol (–10°C) in a cold room at –10°C, then lipids were extracted with chloroform-methanol (2:1, v/v) at room temperature. The amount of total lipid phosphorus is expressed as the percentage of the unfrozen control. PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PI: Phosphatidylinositol, PG: Phosphatidylglycerol, PA: Phosphatidic acid including a trace amount of diphosphatidylglycerol

diglyceride may be due to the transformation of digalactosyl diglyceride into monogalactosyl diglyceride or monogalactosyl diglyceride into digalactosyl diglyceride during dehardening or hardening, respectively.

These galactolipids are known to be concentrated in chloroplast lamellae along with sulfolipids in many plants.[34] With poplar cortical tissues, however, these galactolipids were found not to be specifically located in any particular cell fraction. Also, no relationship was detected between the localization of galactolipids and of chlorophyll in the cell fractions (Table 7). This suggests that in poplar cortical cells galactolipids are also located intracellularly in various membrane systems other than chloroplasts. Thus, it may postulated that the interconversion between monogalactosyl diglyceride and digalactosyl diglyceride in membranes take a role in modifying membrane properties.

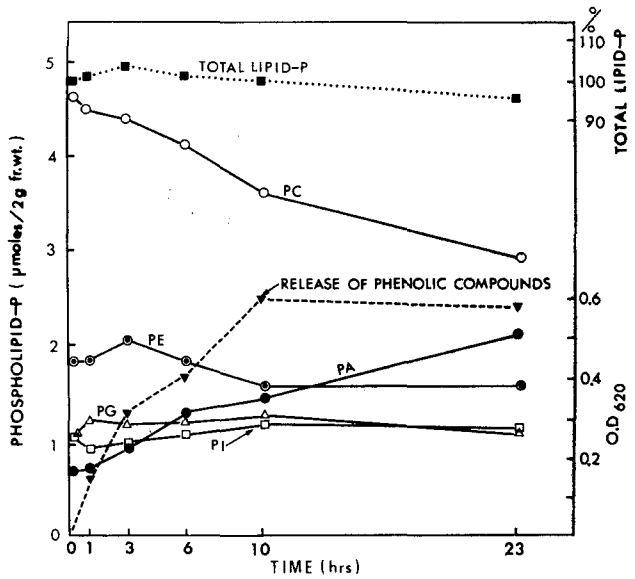

Fig. 12. The time coruse of decrease in phosphatidylcholine during freezing at –10°C. The tissues were sampled on late September. They resisted freezing to –5°C, but injury increased with the length of the time held at –10°C. Freezing injury was determined from the extent of release of compounds reactive with ferric chloride from the frozen thawed tissues and expressed as absorbance at 620 nm. Lipid extractions were performed in the same way as in Fig. 11. The total lipid phosphorus is expressed as the percentage of the unfrozen control. Abbreviations are the same as described in Fig. 11

Degradation of phospholipids during freezing associated with freezing injury

To elucidate the mechanism of frost hardiness of poplar with referenec to membrane lipid changes, special attention was focussed on the degradative changes in membrane phospholipids as a result of freezing at sublethal temperatures.

In the less hardy poplar cortical tissues collected on October 4, no injury was observed after freezing at –5°C for 20 hr, whereas they suffered serious injury by freezing below –10°C. As presented in Fig. 11, in the cortical tissues frozen below –10°C for 20 hr, a remarkable decrease in phosphatidylcholine was observed, while little or no change was observed in phosphatidylinositol and phosphatidylglycerol. A slight decrease was also observed in phosphatidylethanolamine at –15°C. The decrease in phosphatidylcholine was accompanied by a concomitant increase in phosphatidic acid. The total amount of lipid phosphorus, however, showed no significant change during freezing at any temperature. In the less hardy poplar cortical tissues from twigs collected in late September freezing injury increased with the length of time held at –10°C. The time course of the decrease in phospha-

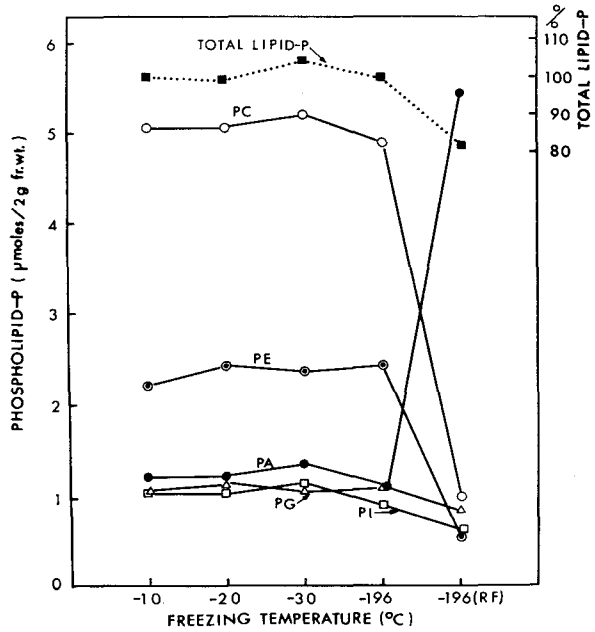

Fig. 13. Changes in phospholipids and total lipid phosphorus in hardy poplar cortical tissues after freeze-thawing. The cortical tissues sampled on early November resisted slow freezing to the temperature of liquid nitrogen (–196°C) as well as –30°C. The tissues directly immersed in liquid nitrogen from room temperature were killed (–196 RF). Lipid extractions were all performed after incubation at 27°C for 2 hr following thawing. The amount of total lipid phosphorus is expressed as the percentage of the unfrozen control. Abbreviations are the same as described in Fig. 11

tidylcholine in the tissues held at –10°C was presented in Fig. 12. Phosphatidylcholine decreased with the length of time held at –10°C, which was also accompanied by a concomitant increase in phosphatidic acid. In this experiment also, only a slight change was observed in the other phospholipids and in the total amount of lipid phosphorus. Thus, the decrease in phosphatidylcholine during freezing seems to be intimately associated with freezing injury of the tissues. To strengthen this notion, some experiments were performed with the hardy poplar cortical tissues collected on November 6. The tissues survived slow freezing to any test temperature down to –30°C or even immersion in liquid nitrogen after prefreezing at –50°C. In those frozen tissues, little or no change in phospholipid components was detected. Even after incubation at 27°C for 2 hr following thawing, almost all of the phospholipid components still remained unchanged (Fig. 13). On the other hand, the tissues frozen rapidly by a direct immersion in liquid nitrogen (–196 RF in Fig. 13) were killed and browning of the tissues was observed immediately after thawing. In these rapidly frozen tissues, however, a drastic change in phospholipid compo-

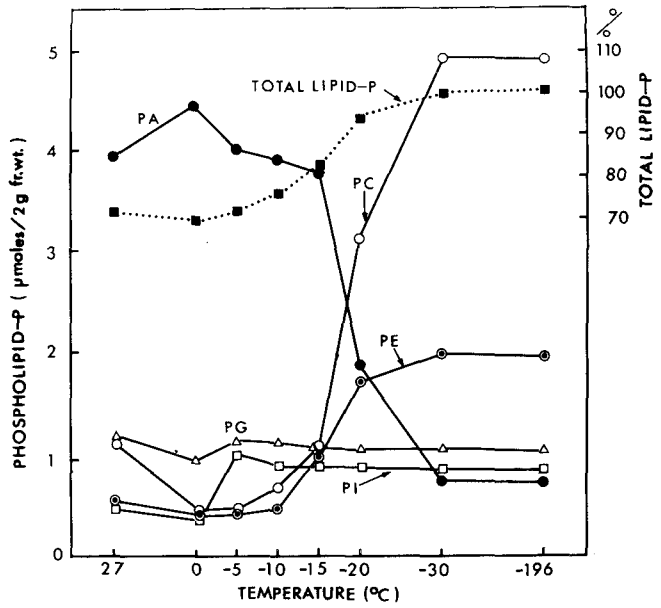

Fig. 14. Temperatures causing phospholipid changes in frozen poplar cortical tissues sustaining injury. Tissues resistant to –5°C were first killed by direct immersion in liquid nitrogen. They were then transferred directly to various temperatures ranging from 0 to –196°C. After 20 hr, the frozen tissues were ground in a motar with cold isopropanol (–10°C), then lipids were extracted with chloroform-methanol (2:1, v/v) at room temperature. Some of the frozen materials were thawed at 0°C and then incubated at 27°C for 1 hr before being extracted. The amount of total lipid phosphorus is expressed as the percentage of the unfrozen control. Abbreviations are the same as described in Fig. 11

nents including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and a slight decrease in the total amount of lipid phosphorus were observed after thawing.

To clarify the subfreezing temperatures under which phospholipid changes proceed, poplar cortical tissues previously damaged by an immersion in liquid nitrogen from room temperature were directly transferred to various temperatures ranging from 0 to –30°C following removal from liquid nitrogen. After standing at these temperatures for 20 hr, phospholipid components were analysed from frozen or thawed samples. As presented in Fig. 14, a significant change in phospholipid components was observed above –30°C. More than 80% of phosphatidylcholine and phosphatidylethanolamine were degraded into phosphatidic acid below –10°C. However, phosphatidylinositol and phosphatidylglycerol remained unchanged below –5°C. A noticeable decrease in phosphatidylinositol occurred after thawing at 0°C. Total lipid phosphorus decreased remarkably above –30°C.

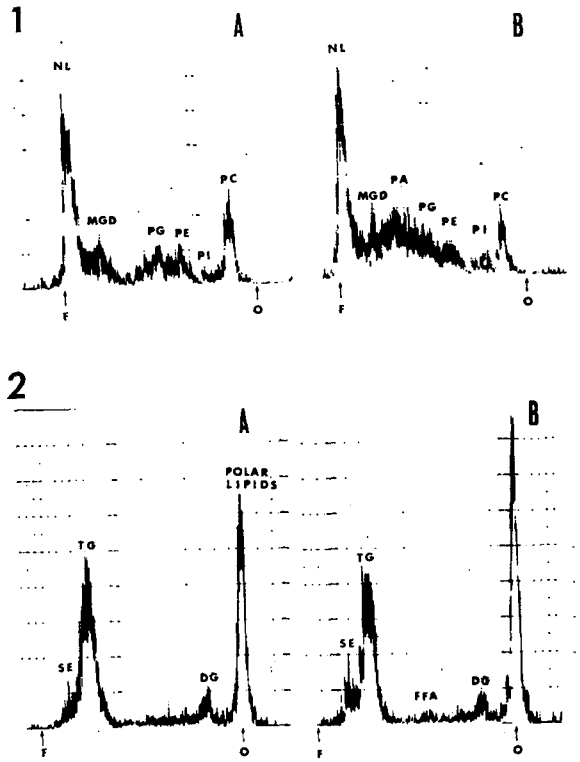

Fig. 15. Radio activity of thin-layer chromatograms of ^{14}C labelled lipids from frozen poplar twig pieces. Lipids were extracted from the whole twig pieces immediately after incorporation (A) and during freezing at −10°C (B). Acetate-1-^{14}C was transfused on one cut surface of the twig pieces and they were incubated at 20°C for 24 hr. Then, the twig pieces were frozen at −5°C for 2 hr, then cooled down to −10°C, which resulted in serious injury. Solvents: Plate 1, chloroform-methanol-acetic acid (64:25:8, v/v/v), Plate 2, petroleum ether-ethyl ether-acetic acid (80:35:1, v/v/v). NL: Neutral lipids, SE: Sterol esters, FFA: Free fatty acids, DG:Diglyceride, TG: Triglyceride, MGD: Monogalactosyl diglyceride. The other abbreviations are the same as described in Fig. 11

In the tissues incubated at 27°C for 1 hr following thawing from liquid nitrogen, some additional changes were observed in phospolipid components. These results indicate that phospholipid degradation may proceed even at subfreezing temperatures above −30°C when the tissues sustained serious injury, but such a degradation may be slowed down at below −30°C. Also it was clearly demonstrated that a decrease in phospholipid components such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol during freezing resulted in a concomitant increase in phosphatidic acid. Thus, it may be postulated that the changes in phospholipid components during freezing are mainly caused by phospholipid

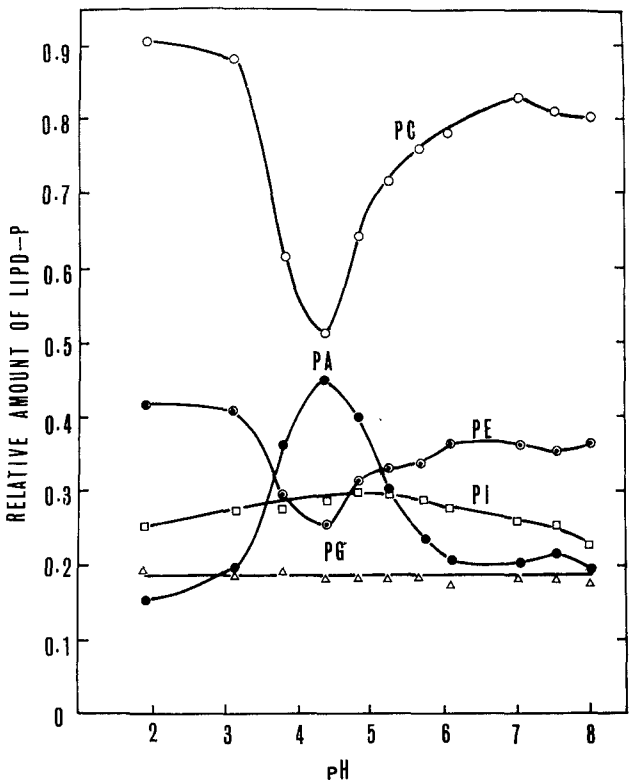

Fig. 16. Effect of pH on degradation of individual phospholipids in pellets prepared from poplar cortical tissues. Tissues were collected in early November. Pellets were prepared by grinding poplar cortical tissues in the medium without EDTA and suspended in 0.01 M tris-HCl buffer (pH 7.8). Reaction mixture consisted of 0.5 ml of the pellet suspension and 0.5 ml of buffer solutions that varied in pH. Incubation was carried out at 27°C for 20 min. Abbreviations are the same as described in Fig. 11

degradation catalysed by phospholipase D. To confirm this, the changes in radioactivity in various lipid fractions treated with acetate-1-^{14}C before freezing were determined during freezing at −10°C with thin-layer chromatography. The twigs were collected in mid-July and they did not resist freezing to −5°C. Any significant change in the total radioactivity in lipids was not observed after freeze-thawing. The majority of the radioactivity was found to be incorporated into fatty acids after mild alkaline hydrolysis. As presented in Fig. 15–1, in the injured twigs frozen at −10°C for 6 hr, the increase or decrease in radioactivity during freezing was found in phosphatidic acid or phosphatidylcholine, respectively. As presented in Fig. 15–2, only a slight change occurred in neutral lipid fractions during freezing at −10°C. These results suggest that phospholipase D may be the

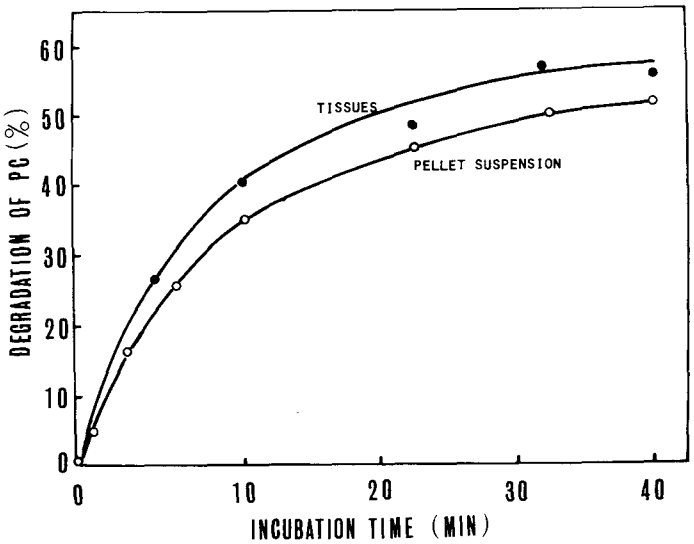

Fig. 17. Time course of degradation of phosphatidylcholine in a pellet suspension and in cortical tissues. Pellet was prepared in the same way as in Fig. 16 and the reaction was started by adding 0.5 ml of 0.2M acetate buffer. The cortical tissue at room temperature was first killed by direct immersion in liquid nitrogen and subsequently rewarmed rapidly in a water bath at 27°C. Incubations were carried out at 27°C

main hydrolysing enzyme of phospholipids in poplar cells during freezing. After thawing, an additional decrease in radioactivity was observed in phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol, and a remarkable increase in radioactivity was observed in the diglyceride fraction (Data not presented). These results suggest that after thawing, other kinds of phospholipases have a role in the degradation of phospholipids.

To better understand the mechanism of activation of phospholipase D and the subsequent degradation of phspholipids caused by freezing at sublethal temperatures, some properties of phospholipase D acting on endogenous phospholipids in membranes were investigated. For this experiment, suspension of 200–78,000×g pelleted fractions from poplar cortical cells was used. This suspension was prepared by homogenizing the cortical tissues in the medium containing 1.0 M sorbitol and 0.2 M tris-HCl buffer solution (pH 8.0), with (pellet+EDTA) or without 10 mM EDTA (pellet−EDTA). As presented in Fig. 16, the activity of phospholipase D on endogenous phospholipids was found to be markedly dependent on pH. The optimum pH for the degradation both of phosphatidylcholine and phosphatidylethanolamine was observed to be near 4.3. The activity of phospholipase D decreased with increasing pH and a slight degree of activity was still

Table 9. Effect of EDTA and divalent cations on the degradation of phosphatidylcholine in vitro

	EDTA or divalent cations added	Percent degradation of phosphatidylcholine	Percent inhibition(−) or promotion(+)
pH 4.8 27°C 20 min	None	50.4	0
	EDTA(20 μmoles)	4.8	-90.4
	EDTA + Ca^{++}(50 μmoles)	69.3	+37.5
	EDTA + Mg^{++}(50 μmoles)	35.4	-29.1
	Ca^{++}(50 μmoles)	71.9	+42.6
	Mg^{++}(50 μmoles)	58.9	+16.8
pH 7.6 27°C 40 min	None	17.1	
	EDTA(20 μmoles)	0	
	Ca^{++}(50 μmoles)	61.2	

The reaction mixture contained 0.5 ml of pellet suspension, 0.5 ml of 0.2 M acetate buffer (pH 4.8) or 0.2 M tris-HCl buffer (pH 7.6), with or without EDTA, $CaCl_2$ and $MgCl_2$. Pellet was prepared in the same manner as described in Fig. 16. Incubation was carried out at 27°C. Reaction was terminated by adding 5 ml of cold isopropanol, and lipids were extracted

observed at neutral pH. The increase in phosphatidic acid was clearly associated with decrease in the amounts of phosphatidylcholine and phosphatidylethanolamine. Little or no change was, however, observed in phosphatidylglycerol at any range of pH. Lipid phosphorus in the phosphatidylinositol fraction was observed to increase to some extent over the broad pH range from 3 to 7. In this experiment, little or no change was observed in the total amount of lipid phosphorus in the pH range from 2.4 to 6.0, although a slight decrease was noted in the pH range from 7 to 8. The time course of the degradation of phosphatidylcholine both in the pellet – EDTA at pH 4.8 at 27°C and in cortical tissue, which was previously killed by direct immersion in liquid nitrogen and subsequently rewarmed rapidly in a water bath at 27°C, are presented in Fig. 17. Within 10 min after incubation at 27°C, the rate of degradation remained at high levels in both pellet suspension and frozen tissue, and then gradually decreased with the length of time. The pH of the cell exudate from the tissue was determined to be 4.8–5.0.

As presented in Table 9, the degradation of phosphatidylcholine in the pellet – EDTA at pH 4.8 was intensively inhibited by the addition of EDTA to the reaction mixture. This inhibition was completely released by the addition of an excessive amount of Ca,$^{++}$ but not completely by the same amount of Mg.$^{++}$ At neutral pH, phosphatidylcholine was also remarkably degraded by the addition of Ca^{++} during incubation at 27°C for 40 min, while degradation was completely inhibited by the addition of EDTA. As presented in Fig. 18, inhibition of phospholipase D

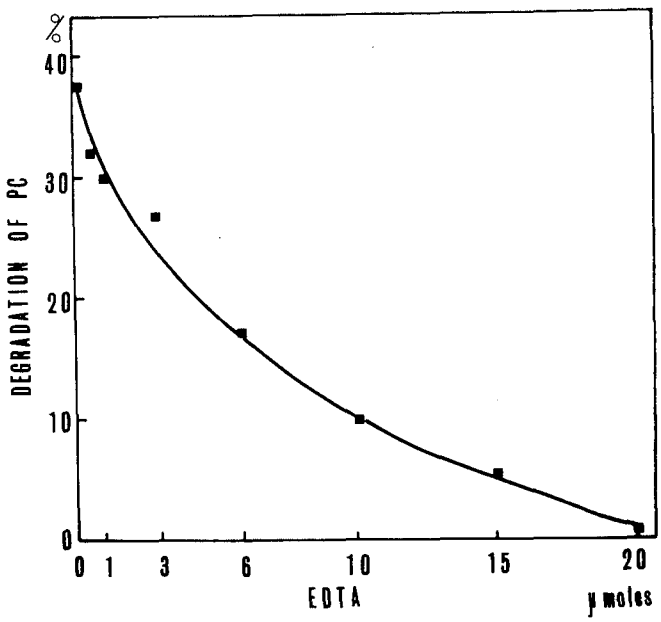

Fig. 18. Inhibition by EDTA of phospholipase D on endogenous phosphatidylcholine. The pellet, prepared in a medium containing 10 mM EDTA, was washed with 0.01 M tris-HCl buffer (pH 7.8). The reaction mixture consisted of 0.5 ml of the pellet suspension, 0.5 ml of 0.2 M acetate buffer (pH 4.8) and various amounts of EDTA. Incubation was carried out at 27°C for 20 min

activity acting on endogenous phosphatidylcholine at pH 4.8 in a pellet derived from cortical tissue homogenized in the medium in the presence of 10 mM EDTA and subsequently washed with tris-HCl buffer solution (pH 7.8), increased with increasing EDTA concentration. From these results, a significant amount of Ca^{++} seems to be tightly bound to membranes. Thus, it may be postulated that Ca^{++} plays an essential role in the degradation of endogenous phospholipids by phospholipase D as in the case of the degradation of exogenous substrates by the enzyme.

As presented in Fig. 19, the pH dependency of phospholipase D was greatly affected by the addition of an excessive amount of Ca^{++} into the reaction system containing the pellet+EDTA. Even in the neutral pH region, a remarkably high level of degradation of phosphatidylcholine was still noted in the presence of an excessive amount of Ca^{++} (C). In the pellet–EDTA a slight degradation of phosphatidylcholine was also noted at neutral pH range (B), but was not in the case of the pellet+EDTA (A).

Table 10 shows the intracellular localization of phospholipase D acting on endogenous phospholipids. Activity of the enzyme is shown as the percent degradation of phosphatidylcholine and phosphatidylethanolamine in various cell

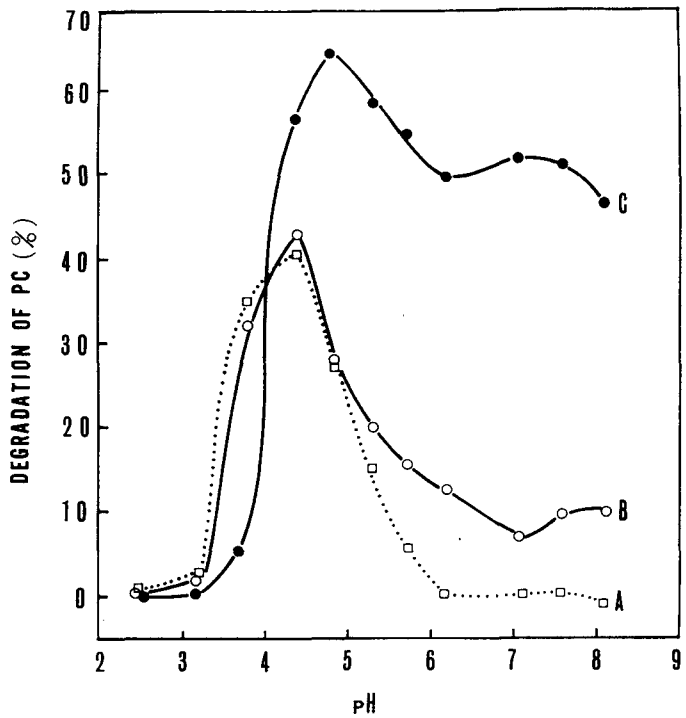

Fig. 19. Effect of Ca^{++} on degradation of endogenous phosphatidylcholine by phospholipase D at varying pH. Pellet were prepared by homogenizing cortical tissue in a medium with EDTA (A, C) or without EDTA (B). Reaction mixtures consisted of 0.5 ml of pellet suspension and 0.5 ml of a 0.2 M buffer solution of varied pH, in the absence (A, B) or in the presence (C) of 50 μmoles of $CaCl_2$. Incubation was carried out at 27°C for 20 min

fractions during incubation with acetate buffer (pH 4.8) at 27°C for 30 min. It appears that phospholipase D is associated with several particulate cell fractions. Phospholipids and the activity of phospholipase D in the supernatant are probably derived from light membranes or membrane fragments and are probably not truly in solution. Thus, the degradation of phospholipids both in vitro and in vivo may occur on various membranes as a result of activation of membrane bound phospholipase D.

Fig. 20 shows autumn and winter seasonal changes in phospholipase D activity catalyzing the degradation of exogenous phosphatidylcholine in cortical tissues of poplar. The specific activity per miligram protein declined only slightly from mid-September to early October, and then remained at nearly the same level until mid-December. The total activity in the crude homogenates remained at a constant level from September to late October, however, and then increased to some extent

Table 10. Intracellular localization of phospholipase D activity

	Relative content before(N) and after incubation (I)						Degradation ratios $\frac{N-I}{N} \times 100$ (%)	
	PC		PE		PA			
Cell fractions	N	I	N	I	N	I	PC	PE
200-P	2.26	1.01	0.73	0.46	0.10	1.56	55.3	37.0
2,500-P	0.95	0.26	0.30	0.19	0.00	0.63	72.6	36.7
12,000-P	1.37	0.43	0.62	0.39	0.31	1.36	68.6	37.1
78,000-P	4.99	1.60	2.54	1.28	0.85	4.35	67.9	50.4
105,000-P	1.59	1.19	0.76	0.62	0.19	0.50	25.2	18.4
Sup	3.22	0.96	0.76	0.29	0.00	2.51	70.2	61.8

Cortical tissues from overwintering poplar, in 0.2 M tris-HCl buffer (pH 8.0), 1.0 M sorbitol and 10 mM EDTA, were ground in a mortar with Polyclar AT and sea sand. The brei was strained through 3 layers of gauze and centrifuged at different speeds. Each pellet was suspended in 0.01 M tris-HCl buffer (pH 7.8). The supernatant was dialysed against 0.01M tris-HCl buffer (pH 7.8). Aliquots of each pellet suspension and its supernatant were each incubated with 0.2 M acetate buffer for 30 min at 27°C

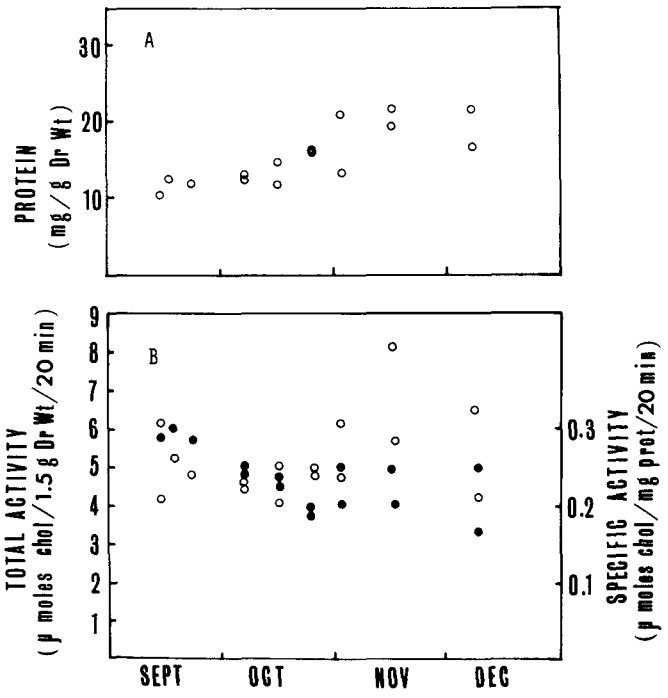

Fig. 20. Seasonal change in phospholipase D activity in cortical tissues of poplar A: Amount of total proteins in crude homogenates. B: Total or specific activity of phospholipase D. The experimental details are described in the text

toward winter. Thus, no inverse relation between frost hardiness and the activity of phospholipase D in the cortical tissues of poplar was observed.

IV. Discussion

When plant tissues are exposed to subzero temperature, ice crystals initially are formed and propagated in the extracellular spaces. If the cells are sufficiently permeable to water or if cooling is sufficiently slow, the cell will be dehydrated to the extent required to maintain equilibrium between the intra and extracellular vapor pressures. The main problems of frost hardiness in plants are concerned with the ability of the cells to withstand dehydration and/or a certain mechanical stress causled by extracellular freezing.

In some microorganisms,[43–45] a decrease in growth temperature has been reported to result in a great change in phospholipids, in both their total amount and in composition as well. In higher plants, a distinct correlation between the growth temperature and the lipid composition was observed in leaves of alfalfa by Kuiper.[28] Alfalfa grown at 15°C was characterized by the presence of large quantities of monogalactosyl diglyceride, digalactosyl diglyceride, phosphatidylcholine and phosphatidylethanolamine, while alfalfa grown at 30°C showed a high percentage of phosphatidylinositol and phsphatidylglycerol, and sulfolipids. However, no relation between cold hardiness and these compositional changes in lipids in alfalfa grown at different temperatures has been reported.

In poplar cortex, phospholipids were observed to consist of 6 different components, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and two unidentified acidic phospholipids. The major part of the seasonal changes in total phospholipids could be attributed to changes in phosphatidylcholine and phosphatidylethanolamine. Thus, some degree of compositional change in phospholipids appears to proceed seasonally in the cortical tissues. It was also confirmed that a hardiness increase is always accompanied by a concomitant increase in phosphatidylcholine and phosphatidylethanolamine. In poplar cortex, the quantities of phosphatidylcholine and phosphatidylethanolamine increased with decreasing temperature from autumn to winter. By contrast, triglyceride decreased abruptly during this time. A similar trend was observed when poplars held at 24–19°C or 15°C for one month were transferred to 0°C. The opposite responses of phospholipid and triglyceride contents to low temperatures imply that there may be an interconversion between them. Similar results were observed with wheat plants in which neutral lipids are converted into polar lipids, particularly into phospholipids, during vernalization[46] or cold hardening.[47] From these results, it appears that environmental temperatures significantly control lipid metabolism and the development of hardiness.

During hardening or dehardening significant changes in the individual particulate cell fractions were found in phospholipids in both their composition and amount on a miligram protein basis. Kahane and Razin[48] observed that membrane lipid synthesis in *Mycoplasma laidlawii* is unaffected by chloramphenicol, and that membranes formed in the presence of the antibiotic had a lower density as a result of increased lipid content. From these results, it is reasonable to assume that protein and phospholipid synthesis in a membrane might not be coordinated and interdependent in poplar cortical cells. Although we have as yet little or no available data as to whether a specific change in the structural proteins in membranes proceeds during hardening or dehardening, it seems likely that changes in phospholipid content per unit membrane area, together with compositional changes, would have an effect on the physical properties of the membranes, and on their functional properties as well. To confirm this, more detailed knowledge on the individual membranes, including plasmalemma, endoplasmic reticulum, and other organelles in which these changes may exclusively proceed, is required.

Recently, an interesting change was found in galactolipids in the chloroplasts from pine needles during a short term dehardening in mid-winter by Bervaes et al.[49] A rapid decrease in hardiness of the dehardened needles was accompanied by a concomitant specific degalactosidation of digalactosyl diglyceride, resulting in a marked increase in monogalactosyl diglyceride. They postulated from this finding that the hydrogen bonding capacity of the membrane surface should decrease upon dehardening as a result of removal of a galactose group from the surface of the lipoprotein membrane. In the present study also, a similar result was obtained with poplar cortical tissues. The molar ratio of digalactosyl diglyceride to monogalactosyl diglyceride in various particulate cell fractions was reversibly changed on hardening or dehardening, while no change was detected in the total amount. In addition, the galactolipids were found to be associated with several particulate cell fractions independent of the chlorophyll contents in poplar cortical cells. Thus, it may be assumed that these qualitative changes in galactolipids occur in some membrane systems other than those of chloroplasts.

Recently, it was reported that sterol compounds are also an essential lipid component in membranes of plant cells[50] and a large amount of sterols was found in plasmalemma from plant cells,[51] as well as in animal cells. In a cell fractionation study with cortical tissues of poplar and black locust trees, free sterols were observed to be located in every cell fraction along with phospholipids, particularly in the 78,000×g pellet fractions.[52] Thus, it appears that sterols are also an important lipid component in membranes in these woody species. But, free sterols and esterified sterols in cortical and xylem tissues of poplar showed only

slight changes through the year, unlike the changes in phospholipids. Further, significant increases in the molar ratios of sterols to phospholipids were observed in every cell fraction from cortical tissues during dehardening of poplar and black locust trees. Thus, the pattern of seasonal changes of sterol compounds appeared to differ considerably from that of other lipids.

The physical properties of membranes in microorganisms are known to be affected by the compositional changes in fatty acid of the constitutive lipids.[53–56] The variation in fatty acid composition observed on lowering the growth temperature of microorganisms has been taken as evidence of the existence of a mechanism regulating the fluidity of the lipid domain in membranes. In higher plants,[29–30,57] the unsaturation of fatty acids in the total lipids has been reported to increase with cold hardening. In poplar cortical tissues, the unsaturation of fatty acids of phospholipids was observed to occur only to a slight extent during hardening.

On the whole, the described results comprise evidence that lipid changes are involved in altering membrane properties in a manner such that protection against freezing is conferred. However, the question remains as to how the lipid changes alter the membrane properties and how the altered membrane properties confer protection against freezing.

Based on an increase in lipid phosphorus and lipoprotein without any change occurring in total lipids during the development of extreme freezing resistance in living bark tissues of black locust tree, Siminovitch et al.[14] postulated that a process of membrane replication that proceeds in conjunction with or as part of the process of total protoplasmic augmentation, occurs as part of the mechanism for developing hardiness. More recently, based on electron microscopic observations of cortical parenchyma cells of black locust tree, Pomeroy et al.[58] clearly demonstrated that seasonal augmentation of total protoplasm, including that of various organelles, particularly, membrane-bound vesicles derived from invaginations and folding of the plasmalemmas, was closely related to the seasonal change in hardiness. Also, Ōtsuka[59], in a study of seasonal changes in hardiness, observed a striking change in the organelles of mulberry cortical parenchyma cells from early winter to late spring, especially in the endoplasmic reticula, vesicles, free ribosomes, plasmalemma and vacuoles. Although the role of an augmentation of organelles per se in frost hardening could not be assessed, Pomeroy et al. concluded that the condition of replication, which led to the folding of the plasmalemma, might have real adaptive significance.

A rapid transformation of ^{14}C in the acyl groups of phosphatidylcholine into triglyceride was demonstrated in cortical tissues of autumnal poplar, when acetate-1-^{14}C was incorporated (unpublished data). Although the physiological function of the observed lipid turnover is obscure, this turnover may throw light on further

studies in which the dynamic state of lipid metabolism or the dynamic state of cellular membranes in plant cells, particularly during the cold acclimation process, may be investigated.

It was demonstrated in the present study that compositional changes in phospholipids in membranes occur in frozen plants that have sustained injury. In less hardy poplar cortical tissues, a striking enzymatic degradation of phosphatidylcholine into phosphatidic acid was observed during freezing at sublethal temperatures. On thawing, the changes were drastically accelerated and resulted in degradation in other phospholipids such as phosphatidylethanolamine and phosphatidylinositol. In well hardened poplar cortical tissues, little or no change was, however, detected during freezing or even after thawing in tissues that had been immersed in liquid nitrogen after prefreezing to -50°C. In the evergreen leaves of *Aucuba japonica*, a similar degradation of phospholipids was also observed, although the degradation pattern differ slightly by species to some extent.[60] It may be concluded that the degradation of phospholipids in poplar cortical tissues was shown to be catalysed by phospholipase D which is known to be widely distributed in plants.[61-64] The enzyme, along with phospholipids, was determined to be associated with several particulate cell fractions from poplar cortical tissues. Accordingly, phospholipase D is assumed to be intercalated or assembled in membrane structures as a functional protein in poplar cortical cells. However, no distinct relationship between seasonal changes in specific activity of phospholipase D and the susceptibility to freezing of poplar cortical cells was discovered. Nor did any of the observed data suggest the presence of an inhibitory factor for the enzyme in poplar cortex. Moreover, no release of enzyme from particulate fractions into the supernatant could be detected after a freeze-thawing cycle of the cells. Basing on these facts, it seems likely that the control system of the phospholipase D activity is present in the integrity of the membranes.

In yeast cells sustaining injury by a rapid freezing in liquid nitrogen or a rapid rehydration after freeze-drying, Souzu[65] demonstrated that the amount of total lipid and lipid phosphorus extracted was markedly increased, unlike in yeast cells cooled or rehydrated slowly. He also observed a marked degradation of phospholipids in rapidly frozen or freeze-dried yeast cells after a period of incubation following thawing or rapid rehydration. On the basis of these findings, he postulated that the rapid removal or addition of water molecules, which may take part in hydrophobic bonding between lipids and proteins in cellular membranes, may be a dominant factor in rupturing of the membranous lipoprotein structures and in subsequent degradation of the phospholipids. In slowly frozen and thawed plant tissues as in the present study, such a rapid removal or addition of water molecules during freezing or thawing seems unlikely to occur. However, severe

dehydration and/or a mechanical stress produced on membranes as a result of extracellular freezing may cause a modification in the molecular association between proteins and lipids in them.

In general, ethyl ether and Ca^{++} are known to be essential factors for the enzymatic degradation of exogenously added phospholipids by phospholipase D from plant sources.[62-64] It has also been found that ethyl ether can be replaced by an anionic detergent such as dodecylsulfate, but Ca^{++} cannot be replaced by any other divalent cation.[66] On the other hand, for the degradation of endogenous phospholipids in pellet fractions from poplar cortical tissues, ethyl ether or an anionic detergent was found not to be required. Merely a reduction in pH of the cell free reaction system resulted in a marked degradation of the endogenous phospholipids. The phospholipase D reaction was strongly inhibited by the addition of EDTA. Even when the pellet, prepared by homogenizing the cortical tissues in the presence of EDTA, was repeatedly washed with tris-HCl buffer solution (pH 7.8), the degradation at acidic pH still remained unaffected. These facts suggest that a considerable amount of Ca^{++} is probably bound to membranes. On the other hand, addition of excessive amounts of Ca^{++} to the cell free reaction system resulted in a marked acceleration of the degradation of phosphatidylcholine and in a considerable degradation of phosphatidylcholine even at neutral pH. Thus, it now appears that the degradation of the endogenous phospholipids by phospholipase D in membranes is strongly affected by both pH and Ca^{++} concentration.

In general, in plant cells, the pH is known to be markedly lower in vacuoles than in other parts of the cytosol region.[67] In numerous species of plants, seasonal fluctuations in the pH have been reported to be rather small.[67] In poplar cortical tissues, the pH of cell exudates was determined to range from 4.6 to 5.2 through the year. In summer, the pH shifted to a slightly lower value. Accordingly, after freezing plant cells at sublethal temperatures, enzymatic degradation of membrane phospholipids would be caused by an abrupt change in the permeability of the tonoplast, which then results in a release of acidic substances and/or Ca^{++} into the cytosol region. It may also be postulated that such changes would probably occur concomitantly in other, including plasmalemma, membranes.

With respect to the functional basis of damage to cell membranes, Heber[68] presented evidence to show that the loss of semipermeability of membranes during freezing is the primary factor in cell injury, based on the fact that the activity of cyclic photophosphorylation of isolated chloroplast membrane fragments is impaired by a freeze-thawing cycle. The "membrane-hole" hypothesis proposed by Levitt[1,22] basing on the SH theory, also essentially points to the reversible or

irreversible changes in the semipermeability of membranes. Gusta and Weiser[69] reported that a rapid destruction of nucleic acids by nucleases occurred in plant cells following a killing frost or freeze-drying. They postulated from this finding that freezing injury in plant cells may result from rapid enzymatic degradation of cell constituents following destruction of intracellular compartmentalization,[70] as reported in animal cells.[71-72]

The seat of the primary chemical changes involved in freezing injury appears to be in the membranes of plant cells. However, the nature of the deleterious changes induced in cellular membranes by freezing remains obscure. The particular difficulty is that we do not know enough concerning the effects of freezing at a molecular level. If we did understand these effects at this level, some reasonable explanation could be made from the observed changes as to the manner in which protection is conferred.

V. Summary and Conclusion

Experiments were designed to elucidate the relationship between lipid changes and temperature-induced hardiness in poplar. The results obtained are summarized as follows:

1. In poplar cortex, phospholipids were observed to consist of 6 different components, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and two unidentified acidic phospholipids. The major part of the seasonal changes in total phospholipids could be attributed to changes in the former two.

2. It was confirmed that a hardiness increase is always accompanied by a concomitant increase in phosphatidylcholine and phosphatidylethanolamine. Their content was inversely related to the environmental temperatures. Thus, quantitative and compositional changes in phospholipids appear to play an important part in increasing the hardiness of cortical cells.

3. An inverse response in the content of phospholipids and triglyceride at low temperatures was also observed. This fact may imply that there is an interconversion between these lipids. From this finding, it appears that environmental temperature significantly controls lipid metabolism and the development of hardiness in the cortical cells.

4. Almost all of the phospholipids were associated with particulate cell fractions. Upon hardening or dehardening, significant changes were found in phospholipids in both their composition and amount on a miligram protein basis in the individual cell fractions.

5. Hardening or dehardening resulted in significant, inverse changes in the molar ratio of digalactosyl diglyceride to monogalactosyl diglyceride in the indivi-

dual cell fractions.

6. A striking degradation of phosphatidylcholine into phosphatidic acid was observed in the cortical tissues of less hardy poplar, when they were frozen below a lethal temperature. No change in phospholipids was, however, detected in winter cortical tissues during freezing or even after immersion in liquid nitrogen after prefreezing to –50°C. These facts suggest that some deleterious changes that occur in cellular membranes are associated with freezing injury.

7. The degradation of phospholipids in poplar cortical tissues was shown to be catalyzed by phospholipase D which is associated with several particulate cell fractions along with phospholipids. Solely a reduction in the pH of cell free reaction systems also resulted in a marked degradation of the endogenous phospholipids. From this fact, it may be postulated that acid leakage from vacuoles into the cytosol, produced by freezing is involved in a sequence of deleterious processes in the cells.

From the results described above, it may be concluded that the seat of the principal chemical changes involved in both hardiness and freezing injury is in the membranes of plant cells. That lipid changes proceeded in the cortical cells during hardening or dehardening can be taken as evidence that membrane properties are altered in a manner such that protection against freezing injury is conferred. However, the question remains as to how the lipid changes serve to alter membrane properties, and how the altered membrane properties confer protection against freezing. Moreover, no distinct answer can be given as to the primary nature of the deleterious change induced in cellular membranes by freezing. These are problems for future studies to resolve.

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