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Properties of Plasma Membrane Isolated from Chilling-Sensitive Etiolated Seedlings of *Vigna radiata* L.[1, 2]

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ABSTRACT

Plasma membrane was isolated in a uniform population and with a high purity from chilling-sensitive etiolated young seedlings of *Vigna radiata* (mung bean) utilizing an aqueous two polymer phase separation system and subsequent sucrose density gradient. The isolated plasma membrane was associated with vanadate-sensitive and KNO_3-insensitive ATPase. The ATPase has high specificities both for substrate and Mg^{2+} ion with optimum pH at 6.5. It was slightly stimulated by monovalent anions, especially Cl^-. Proton ionophores such as gramicidin D and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone did not stimulate the enzyme activity. The ATPase is apparently latent and highly stimulated by the addition of detergents such as Triton X-100. A maximum stimulation was achieved by the addition of 0.02% Triton X-100. After treatment with proteinase K in an isotonic buffer solution, the enzyme activity was less affected, whereas the peptides were specifically digested. Based on these facts, the isolated plasma membrane vesicles appear to be tightly sealed and in a right-side-out orientation. The plasma membrane ATPase had two inflection points at higher (18.9°C) and lower (6.7°C) temperatures on the Arrhenius plots of the activity. The lower inflection temperature apparently coincided with that of the anisotropy parameter of embedded 1,6-diphenyl-1,3,5-hexatriene, indicating that the membrane bound ATPase activity was affected by a phase transition of membrane lipids and/or temperature-dependent conformational changes in the enzyme molecules *per se*. Considering the fact that the plant material used here is highly sensitive to chilling temperatures and injured severely by exposure to temperatures below 5°C for a relatively short period, the thermotropic properties of membrane molecules are considered to be involved in the mechanism of chilling injury.

Despite numerous studies on chilling injury of plants in the past decade (6, 14, 15), the mechanism is still obscure. In our previous studies using highly chilling-sensitive, cultured cells derived from *Cornus stolonifera* L. (18, 30), which is a cold resistant woody plant species, a series of sequential ultrastructural changes was demonstrated to occur in the cells immediately after exposure to chilling, *i.e.* structural transformation of proplastids from elipsoidal to stretched and constricted form, a dilation and a vesiculation of ER, an aggregation of inner membrane particles of tonoplasts, and finally a rupture of vacuoles. Along with these time-dependent sequential changes in cell structures, cell leakage of electrolytes and amino acids (29), and a diversion of electron flow to the alternate pathway (31) were observed as the physiological symptoms which might be considered as the cause or the result of chilling injury.

Based on these results, our attention should be focused more or less on the direct or indirect effects of chilling temperatures upon whole structures of cells, including plastids, mitochondria, nuclei, ER, vacuoles, Golgi apparatus, plasma membrane, and cytoskeletons, and on the metabolic processes of membrane biogenesis as influenced by the chill temperatures.

For this purpose, it would be necessary to prepare cell organelles in a high purity and in a large quantity. A series of experiments to establish the isolation techniques are going on in our laboratory using chilling-sensitive etiolated young seedlings of *Vigna radiata*.

In the present study, we will be dealing mainly with the isolation of plasma membrane utilizing the published method of an aqueous two-polymer phase partition system and subsequent sucrose density gradient centrifugation. The isolated plasma membrane was assessed to be highly pure and in a large quantity sufficient for a biochemical and a biophysical analysis. The isolated plasma membrane was partially characterized from viewpoints of sidedness and its thermotropic properties.

MATERIALS AND METHODS

Plant Materials. The seeds of *Vigna radiata* were imbibed and germinated at 26°C in darkness on a stainless steel mesh installed at the one-third depth of a square plastic box (20 × 30 × 16 cm). To this about 3 L of 1 mM $CaSO_4$ solution made up in distilled H_2O were added to the level of the seeds on the stainless steel mesh. After 4 d of germination, hypocotyls (about 6 cm in length) were excised and utilized for the experiments.

Preparation of Membranes. Hypocotyls (140 g fresh weight) were washed once in distilled H_2O and prechilled at 10°C before use. The prechilled tissues were sliced into 180 ml of homogenizing medium prechilled at 0°C and were immediately homogenized with Polytron PT30 at the medium speed setting for 30 s. The medium used for homogenization contained 0.25 M sucrose, 75 mM MOPS[3]-KOH, 5 mM EGTA, 2 mM PMSF, 2 mM SHAM, 2.5 mM potassium metabisulfite, 1.5% PVP (mol wt 24,000), 0.5% BSA (defatted), 10 μg/ml BHT, pH 7.6. The slurry was passed through four layers of gauze and subjected to differential centrifugation at 12,500*g* for 15 min and subsequently at 156,000*g* for 20 min. The 12,500 to 156,000*g* pellets were suspended in 0.25 M sucrose-10 mM K-phosphate buffer (pH 7.8)

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[3] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; SHAM, salicylhydroxamic acid; PMSF, phenylmethylsulfonyl fluoride; BHT, butylated hydroxytoluene; PEG 4000, polyethylene glycol (mol wt 3,340); NPA, naphthylphthalamic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DIDS, 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid; DES, diethylstilbestrol; PTA, phosphotungstic acid; DOC, deoxycholate.

and recentrifuged for 20 min at 156,000*g* for 20 min. The washed pellets were subjected to an aqueous two-polymer phase partition system as reported previously (21, 32). The phase partition system consisted of 5.6% (w/w) each of PEG 4000 and Dextran T500 made up in 0.25 M sucrose-10 mM K-phosphate buffer, pH 7.8, and various concentrations of NaCl (0–30 mM).

Sucrose Density Gradient. The plasma membrane-enriched fraction partitioned into the PEG-enriched upper phase of the phase partition system was diluted with 0.25 M sucrose, 5 mM MOPS-KOH,1 mM EDTA, 10 mM KCl, 0.2 mM PMSF, 10 μg/ml BHT, and 1 mM DTT (pH 7.3) and pelleted by centrifugation at 156,000*g* for 20 min. The pellet was resuspended in the 7 ml of the same buffer system as used above and loaded on a linear sucrose gradient (30 ml, 15–45%, w/w) made up in 5 mM MOPS-KOH, 1 mM EDTA, 10 mM KCl, 0.2 mM PMSF, and 10 μg/ml BHT (pH 7.3). After centrifugation at 85,000*g* for 15 h at 2°C on a Hitachi SW-1 rotor, 1.2 ml aliquots were collected from the top of the gradient.

Enzyme Assay. Activities of Mg^{2+}-ATPase, antimycin A-insensitive NADH-Cyt *c* reductase, and Cyt *c* oxidase and phosphatase were assayed as previously reported (21, 32). UDPase activity was assayed according to the method of Nagahashi and Kane (16). Glucan synthetase I activity was determined by the transfer of radioactivity from [^{14}C]UDP-D-glucose into heat denatured materials. The assay method was essentially the same as the method described by Hall and Moore (7). A naphthylphthalamic acid-binding test was performed as reported elsewhere (21) by using the ^{3}H-labeled compound.

One-Dimensional SDS-PAGE. The membrane pellets were solubilized in 50 mM Tris-HCl (pH 6.8), 3% SDS, 0.5 M sorbitol, 50 mM DTT, and 0.001% bromophenol blue with boiling for 3 min. The discontinuous SDS buffer system of Laemmli (11) was used for the SDS slab-PAGE. After electrophoresis, the slab gels were stained with Coomassie blue according to Fairbanks *et al.* (5). For the determination of glycopeptides, the concanavalin A-peroxidase method was used (23).

Electron Microscopy. Intact tissue sections and membrane materials were fixed in 3%-buffered glutaraldehyde followed by washing in buffer solution, postfixed in 2% OsO_4 solution, dehydrated in an increasing concentration of ethanol, and finally in *n*-butylglycidyl ether as previously reported (21). The dehydrated specimens were embedded in Spurr's (20) epoxy resin. The sections were stained with either saturated uranyl acetate and Reynold's lead solution (19) or periodic-PTA-CrO_3 (17). The stained sections were viewed with a JEM 100C electron microscope.

Fluorescent Polarization Measurement. The fluorescent hydrocarbon DPH was used as a probe to monitor the fluorescent polarization properties of isolated membranes and the liposomes prepared from the extracted lipids (25). The final concentration of DPH was 10 μM/100 μg protein. The steady state fluorescent polarization was measured at various temperatures in an Elscint microvicosimeter MV-1a. The anisotropy parameters calculated from the P values were taken as relative index for membrane fluidity and the logarithms were plotted against reciprocals of absolute temperatures to detect a thermotropic transition of membranes (25).

RESULTS

Plant plasma membranes have been isolated from wide range of plant materials by using an aqueous two-polymer phase system containing NaCl (21, 27, 32). In the present study, the 12,500 to 156,000*g* pellets prepared from hypocotyls of etiolated young seedlings of *V. radiada* were subjected to the phase partition system consisting of 5.6% (w/w) each of Dextran T500 and PEG 4,000 made up in 0.25 M sucrose-10 mM K-phosphate buffer, pH 7.8, and various concentration of NaCl. As shown in Figure 1, with increasing NaCl concentration in the phase system, ER, mitochondria, and Golgi membranes were preferentially partitioned into the Dextran-enriched lower phase as assessed by the activities of marker enzymes; antimycin A-insensitive NADH-Cyt *c* reductase, Cyt *c* oxidase, glucan synthetase I, and UDPase, respectively. Yellow-colored membrane materials, presumably plastid envelopes, were effectively partitioned into the lower phase even at the lower concentration of NaCl (10 mM). The control Mg^{2+}-ATPase activity (assayed in the absence of Triton X-100), however, decreased proportionally with increasing in NaCl concentration. On the other hand, the majority of the Triton X-100-stimulated Mg^{2+}-ATPase activity (assayed in the presence of 0.016% Triton X-100) was recovered in the PEG-enriched upper phase even at the higher NaCl concentration; over 60% of the total activity in the 12,500 to 156,000*g* pellet was recovered in the upper phase at the NaCl concentration of 30 mM. From these results, it appears that plasma membrane can be effectively separated from the crude membranes by using the phase partition system containing 30 mM NaCl.

There existed, however, less consistency in the partition behavior between the Triton X-100-stimulated Mg^{2+}-ATPase and the NPA-binding capacity which is another useful marker for plasma membranes (13). This discrepancy would be due to a heterogeneity of plasma membrane vesicles differing in the capacity of their NPA-binding sites and in their partitioning behavior, especially, at the relatively high concentrations of NaCl. One of the plasma membrane populations would be enriched in the NPA-binding sites and is considered to be partitioned into the lower phase with increasing NaCl concentration.

As shown in Figure 2, the specific activity of the Triton X-

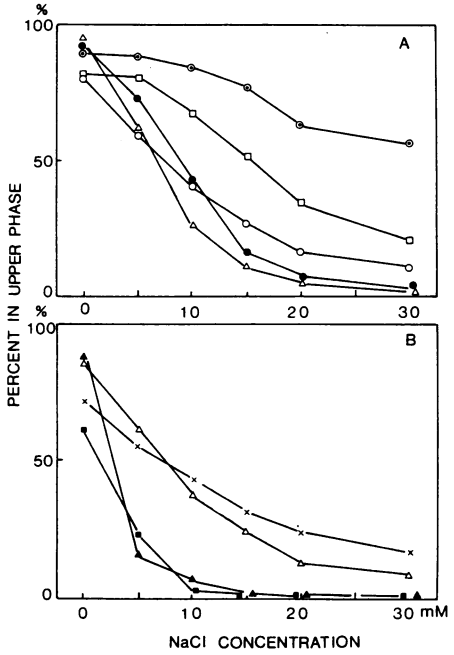

FIG. 1. Effect of NaCl concentration on the partition of microsomal membranes into the upper phase in two-polymer phase system. The phase system contained 5.6% (w/w) each of PEG 4000 and dextran T500 made up in 0.25 mM sucrose-10 mM potassium phosphate buffer, pH 7.8, and various concentration of NaCl as indicated. Partitioning was performed at 0°C. Experimental details for enzyme assays are described in the text. Enzyme activities partitioned into the upper phase were expressed as percentage of the total activities in the microsomal fraction. A, ATPase activities in the absence (○) and presence (⊙) of 0.02% Triton X-100; NPA-binding (□); UDPase (●); glucan synthetase I (△); B, protein (×); acid phosphatase (△); Cyt *c* oxidase (▲); antimycin A-insensitive NADH Cyt *c* reductase (■).

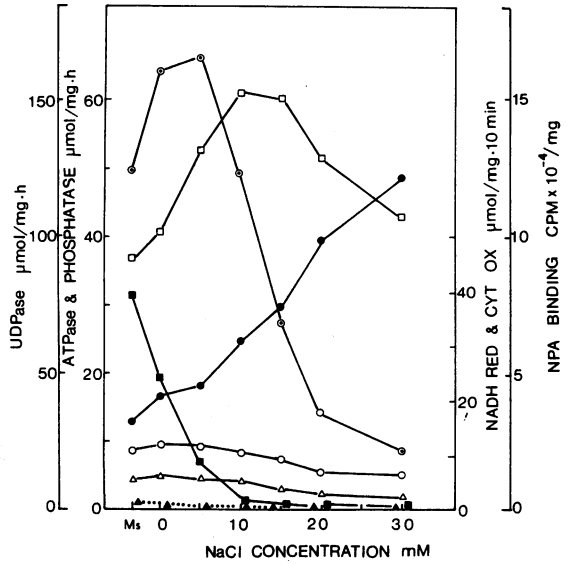

FIG. 2. Specific activities of various marker enzymes partitioned into the PEG-enriched upper phase after the phase partitioning in the presence of various NaCl concentrations. ATPase activities in the absence (○) and presence (●) of 0.02% Triton X-100; NPA-binding (□); UDPase (⊙); Cyt *c* oxidase (▲); antimycin A-insensitive NADH Cyt *c* reductase (■); acid phosphatase (△).

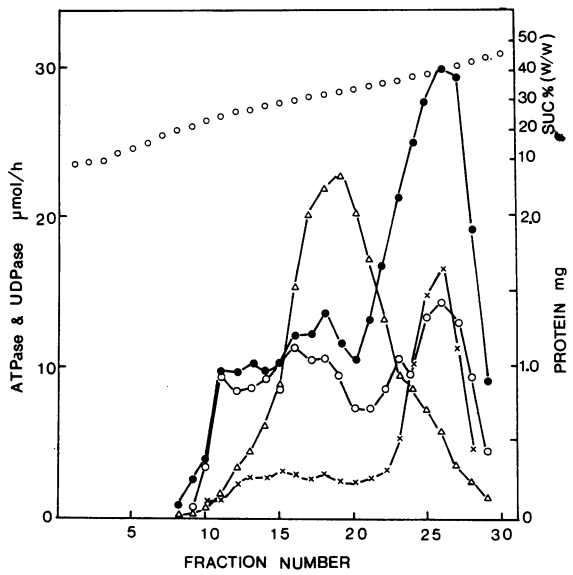

FIG. 3. Linear sucrose density gradient of plasma membrane-enriched upper phase after repeated phase partition in the presence of 10 mM NaCl. After phase partitioning of microsomal fraction in the presence of 10 mM NaCl, the plasma membrane-enriched upper phase was pelleted and resuspended in 7 ml of 0.25 M sucrose, 5 mM MOPS/KOH, 1 mM EDTA, 0.2 mM PMSF, 10 μg/ml BHT, 10 mM KCl, and 1 mM DTT, pH 7.3, and then loaded on 30 ml of a linear sucrose gradient (15–45%, w/w) made up in the same buffer system as above. After centrifugation at 85,000*g* for 15 h at 2°C, 1.2 ml aliquots were fractionated from the top of the gradient. ATPase activities were assayed in the presence of 0.02% Triton X-100 with (○) or without (●) addition of 100 μM vanadate. UDPase (△); Protein content (×). Enzyme assays were done as described in the text.

100-stimulated Mg^{2+}-ATPase increased proportionally with increasing NaCl concentration, whereas the specific capacity of NPA-binding increased about 1.5-fold up to 10 to 15 mM NaCl and then declined at NaCl concentrations higher than 15 mM.

To obtain plasma membranes in a uniform population and in a large quantity, the 12,500 to 156,000*g* particulated fraction was phase-partitioned twice in the presence of 10 mM NaCl and the plasma membrane-enriched fraction recovered in the upper phase was then subjected to a linear sucrose density gradient. The upper phase obtained at 10 mM NaCl phase was essentially free of mitochondria, ER, and unidentified yellow-colored membrane materials, but still partially contaminated with Golgi membranes and acid phosphatase-associated membranes, presumably tonoplast vesicles (Fig. 1). After centrifugation of the sucrose gradient at 85,000*g* for 15 h, two white bands were formed at a lighter (25–30% sucrose) and a heavier (35–40% sucrose) portions of the sucrose gradient. Figure 3 shows the density gradient profiles of marker enzyme activities. The major peak of Triton X-100-stimulated Mg^{2+}-ATPase activity occurred at higher sucrose density and it was highly sensitive to vanadate. A significant amount of Mg^{2+}-ATPase, which was not stimulated by the addition of 0.016% Triton X-100, was associated with the lighter membranes. The ATPase activity was vanadate-insensitive, but effectively inhibited by the addition of 50 mM KNO_3 (data not shown), suggesting tonoplast origin (22). The major peak of UDPase activity as a specific marker enzyme for Golgi membranes (17) occurred at 31% sucrose layer. The activity peak was also coincident with the activity peaks of latent IDPase and glucan synthetase I (data not shown). From these results, tonoplast and Golgi membranes were found to be the major contaminating membranes in the plasma membrane-enriched upper phase after a phase partition and they were easily separated by the subsequent sucrose density gradient.

To shorten the time required for plasma membrane isolation, the pelleted plasma membrane-enriched upper phase was suspended in 0.25 M sucrose, 5 mM MOPS-KOH (pH 7.3), 10 mM KCl, 0.2 mM PMSF, and 10 μg/ml BHT, loaded onto the 32/43% (w/w) discontinuous sucrose density gradient, and centrifuged at 156,000*g* for 1.5 h on a Hitachi angle rotor, RP 50.2. The lighter tonoplast and Golgi membranes and the heavier plasma membranes were banded on the sample/32% and the 32/43% interfaces, respectively.

Table I characterizes each separate membrane from both interfaces. More than 70% of the total UDPase activity applied was recovered in the lighter membrane fraction (sample/32% interface), while over 90% of the total NPA-binding activity was recovered in the heavier membrane fraction (32/43% interface). The ATPase associated with the lighter membranes was inhibited by KNO_3, but less inhibited by vanadate. On the other hand, the ATPase associated with the 32/43% interfacial fraction was highly sensitive to vanadate (about 80% inhibition by 100 μM vanadate), but was insensitive to KNO_3. A remarkable difference was also noted in the stimulation of ATPases by the addition of 0.02% Triton X-100, *i.e.* 4.5-fold stimulation in plasma membrane ATPase and 1.5-fold stimulation in ATPases of tonoplast and Golgi-enriched membrane fraction (sample/32% interface). From these results, plasma membranes are effectively separated from the contaminated tonoplasts and Golgi membranes after the sucrose density gradient of the plasma membrane-enriched upper phase in the phase partition system contained 10 mM NaCl.

Figure 4 shows the electron micrographs of the sample/32% and 32/43% interfacial membrane fractions. The former fraction contained variously sized smooth membrane vesicles (Ia) and they were not stained with PTA-CrO_3 (Ib). The latter fraction (IIa, IIb), on the other hand, was highly enriched in uniformly sized smooth membrane vesicles most of which were positively stained by PTA-CrO_3, indicating a high enrichment of plasma membranes.

As presented in Figure 5, a distinct difference was observed between the two membrane fractions in the SDS-PAGE patterns

Table I. *Distribution of Marker Enzymes between the 32%-Interfacial and the Plasma Membrane Fractions after a Discontinuous Sucrose Gradient of the Upper Phase*

Two hundred and eighty g of hypocotyls of 4-d-old seedlings were used. ATPase activities were assayed in the absence (−TX-100) or presence (+TX-100) of 0.02% Triton X-100

		Interfacial Fraction 32%	Plasma Membrane Fraction
		μmol/h	*μmol/h*
ATPase	Control	23.0 (7.3)[a]	25.2 (5.2)[a]
	−TX-100 + KNO_3 (50 mM)	14.9 (4.8)	25.3 (5.2)
	+ Vanadate (100 μM)	12.9 (4.1)	4.0 (0.8)
	Control	35.1 (11.2)	113.9 (23.4)
	+TX-100 + KNO_3 (50 mM)	18.7 (5.9)	114.0 (23.4)
	+ Vanadate (100 μM)	21.5 (6.8)	15.7 (3.2)
UDPase		464.6 (147.9)	206.8 (42.4)
NPA-binding		0.51 pmol/mg	3.35 pmol/mg
Protein		3.14 mg	4.87 mg
Triton X-100 stimulation		1.52	4.52
KNO_3 inhibition			
−Triton X-100		35.2%	0%
+Triton X-100		46.7%	0%
Vanadate inhibition			
−Triton X-100		43.9%	84.1%
+Triton X-100		38.7%	86.2%
[UDPase]/[ATPase(+Triton X-100)]		13.26	1.81
[Acid Phosphatase]/[ATPase(+Triton X-100)]		0.66	0.13

[a] Specific activity μmol/h·mg protein.

of polypeptides as revealed by either Coomassie blue staining or concanavalin A-peroxidase staining by which glucose or mannose type glycopeptides are specifically stained. The enrichment of tonoplast in the sample/32% interface is also clearly supported by the similarity of polypeptide or glycopeptide composition between the sample/32% interface (lanes A-3, B-3) and the purified tonoplast prepared from the same plant materials (28) as used in the present study. A difference was also remarkable in the polypeptide compositions between the lower phase (endomembranes) and the upper phase (the plasma membrane) in the phase partition.

Figure 6 shows a pH-activity profile of plasma membrane ATPase in the presence or absence of Triton X-100. The optimal pH was located around 6.5 in the absence of the detergent. The activity was slightly stimulated by the addition of 50 mM KCl without any effect on the pH-activity profile. Triton X-100 (0.016%) stimulated the ATPase, especially around pH 6.5 and 7.0. The KCl stimulation, however, was abolished around the optimal pH and salt was observed to be inhibitory at a higher pH. From the pH-activity profile, KCl appeared to shift the pH profile by about 0.5 unit toward acidic region in the presence of the detergent.

Table II shows the effects of various inhibitors on the plasma membrane ATPase. The ATPase was highly sensitive to vanadate and moderately inhibited by DCCD and DES (data not shown). It was also slightly inhibited by DIDS (30 μM) which is an inhibitor for anion transport channels in plasma membranes (4, 9). Azide and KNO_3 exhibited no inhibitory effect. The plasma membrane ATPase showed a high substrate specificity for ATP which could not be replaced by CTP, GTP, and ITP (Table III). However, relatively high activity was observed for UTP, UDP, and IDP. The activity for the latter two substrates must be due to a slight contamination by Golgi membranes. The activity for UTP is unknown as yet. Tables IV and V indicate effects of monovalent and divalent ions on plasma membrane ATPase. The plasma membrane ATPase showed a high specificity to Mg^{2+} and was slightly stimulated by monovalent salts such as KCl, NaCl, NH_4Cl, and choline-Cl, but the stimulation was less in K_2SO_4, KI, and KNO_3. These stimulations, however, were completely abolished by the addition of Triton X-100. Proton ionophores such as gramicidin D and FCCP did not affect the plasma membrane ATPase (data not shown).

The plasma membrane ATPase was stimulated by both nonionic and anionic detergents. Figure 7 shows the effect of detergent concentrations on the ATPase activity. A maximum activity was obtained at the concentration of 0.025% in both detergents. Above 0.025%, the ATPase activity was depressed to some degree. The stimulation was relatively high in Triton X-100 compared with sodium DOC. The observed latency in the plasma membrane ATPase may indicate that the plasma membranes have been isolated in tightly sealed vesicles with a right-side-out orientation and retained the permeability barrier against Mg^{2+} and ATP. In suggesting this notion, the ATPase activity was less sensitive to proteinase K treatment even at higher concentrations where some populations of polypeptides were selectively digested (S. Yoshida, unpublished data). This result may suggest that the isolated plasma membrane vesicles are tightly sealed and oriented right-side-out during preparation, preventing the transmembrane traverse of the added protease and the direct contact with the ATPase located on the cytoplasmic surface of the plasma membranes.

To investigate the effect of temperatures on the plasma membrane ATPase, the activity was assayed simultaneously at different temperatures in a temperature gradient former and the data were arranged as Arrhenius plots. As indicated in Figure 8, two inflections were observed in the slope at 18.9 and 6.7°C. The presence of 0.016% Triton X-100 in the reaction system did not affect the activation energies above, between, and below these inflection points. However, the inflection points slightly shifted to higher temperatures, i.e., from 18.9 to 19.9°C and from 6.7 to 8.9°C, respectively.

The Arrhenius plots of anisotropy parameters of DPH incorporated into plasma membrane vesicles showed a break at 7.4°C (Fig. 9). This break point roughly corresponded to the lower

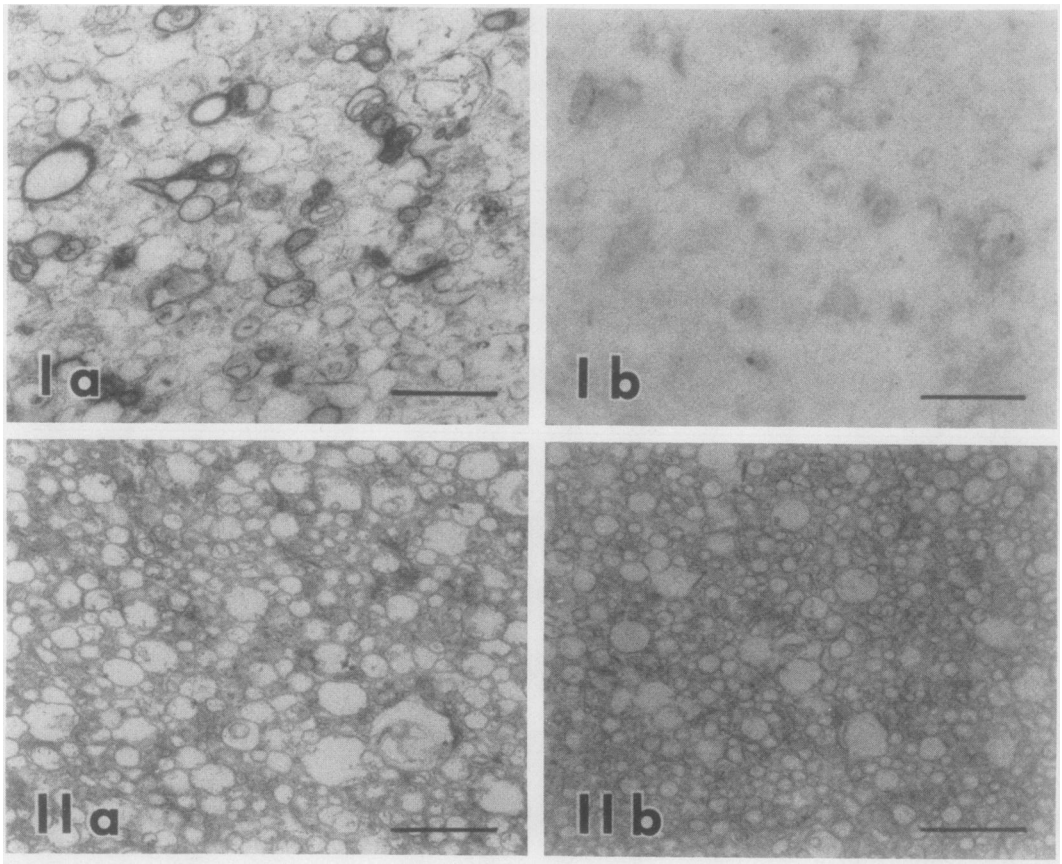

FIG. 4. Electron micrographs of the membrane fractions recovered on interfaces at the sample load/32% and the 32/43% sucrose layers after a discontinuous sucrose density gradient of the plasma membrane-enriched upper phase. The upper phase membrane fraction obtained after repeated phase partitioning in the presence of 10 mM NaCl was resuspended in 20 ml of 0.25 M sucrose, 5 mM MOPS/KOH, 1 mM EDTA, 0.2 mM PMSF, 10 μg/ml BHT, 10 mM KCl, and 1 mM DTT, pH 7.3, and loaded on 10 ml of 32% and 7 ml of 43% sucrose layers made up in the same buffer system as above and centrifuged at 156,000*g* for 90 min on a Hitachi angle rotor (RP 50.2). I, the sample/32% interfacial fraction enriched in tonoplast and Golgi membranes; II, the 32/43% interfacial fraction enriched in plasma membranes. The thin-sections were stained by either uranyl-lead stain (Ia, IIa) or the PTA-CrO_3 stain (Ib, IIb). Bars represent 1 μm. Experimental details are described in the text.

inflection point (6.7°C) on the Arrhenius plots of ATPase activity. Based on these results, it seems likely that the plasma membrane may undergo a thermotropic phase transition around 7°C, below which the etiolated seedlings suffer chilling injury.

DISCUSSION

In the present study, plasma membranes were isolated from chilling-sensitive, etiolated young seedlings of *V. radiata* L. by utilizing an aqueous two-polymer phase partition system containing 10 mM NaCl and by subsequent sucrose density gradient of the plasma membrane-enriched upper phase. The purity of the isolated plasma membranes was fairly high as assessed by the PTA-CrO_3 stain, NPA-binding capacity, and vanadate-sensitive Mg^{2+}-ATPase.

Unlike other plant materials with which we have been concerned (21, 26, 32), the plasma membrane from *V. radiata* showed a different behavior in the phase partition at the higher concentration of NaCl. The specific activity of NPA-binding increased with increasing in NaCl concentration up to 15 mM, and then decreased above 15 mM NaCl, while the specific activity of Triton X-100-stimulated ATPase increased proportionally. In addition, the recovery of plasma membrane in the upper phase was relatively poor at the NaCl concentration of 30 mM. This might indicate that the plasma membrane vesicles consisted of heterogenous populations, *i.e.* a membrane population enriched in the NPA-binding sites, which might have been partitioned into the lower phase at the higher concentrations of NaCl and the other membrane population enriched in Triton X-100-stimulated ATPase, most of which might have been partitioned into the upper phase even at the higher NaCl concentrations. When phase partition was carried out in the presence of 10 mM NaCl, most yellow-colored membranes, ER, mitochondria, and a significant amount of Golgi-derived membrane (more than 75%) were preferentially partitioned into the lower phase, and thus plasma membranes enriched in both of the NPA-binding sites and ATPase were recovered in the whitish upper phase partly contaminated with Golgi membranes and tonoplasts. After sucrose density gradient centrifugation of the plasma membrane-enriched upper phase, the contaminating membranes were effectively separated on a 32% sucrose layer, and the plasma membrane with a high purity was concentrated on an interface of 32/43% sucrose layers.

The ATPase bound to the isolated plasma membrane showed a latent property being effectively stimulated by the addition of detergents such as Triton X-100 and DOC. Although the former was more effective than the latter, the maximum stimulation was observed at the concentrations of 0.02 to 0.025% in both detergents. At similar concentrations, rat liver rough microsomes have been reported to become reversibly permeable to macromolecules without any membrane disassembly (10). Based on these results it might be considered that the stimulation of the ATPase by the detergents is due to the increased permeability of plasma membrane for Mg^{2+}-ATP so that the substrate becomes

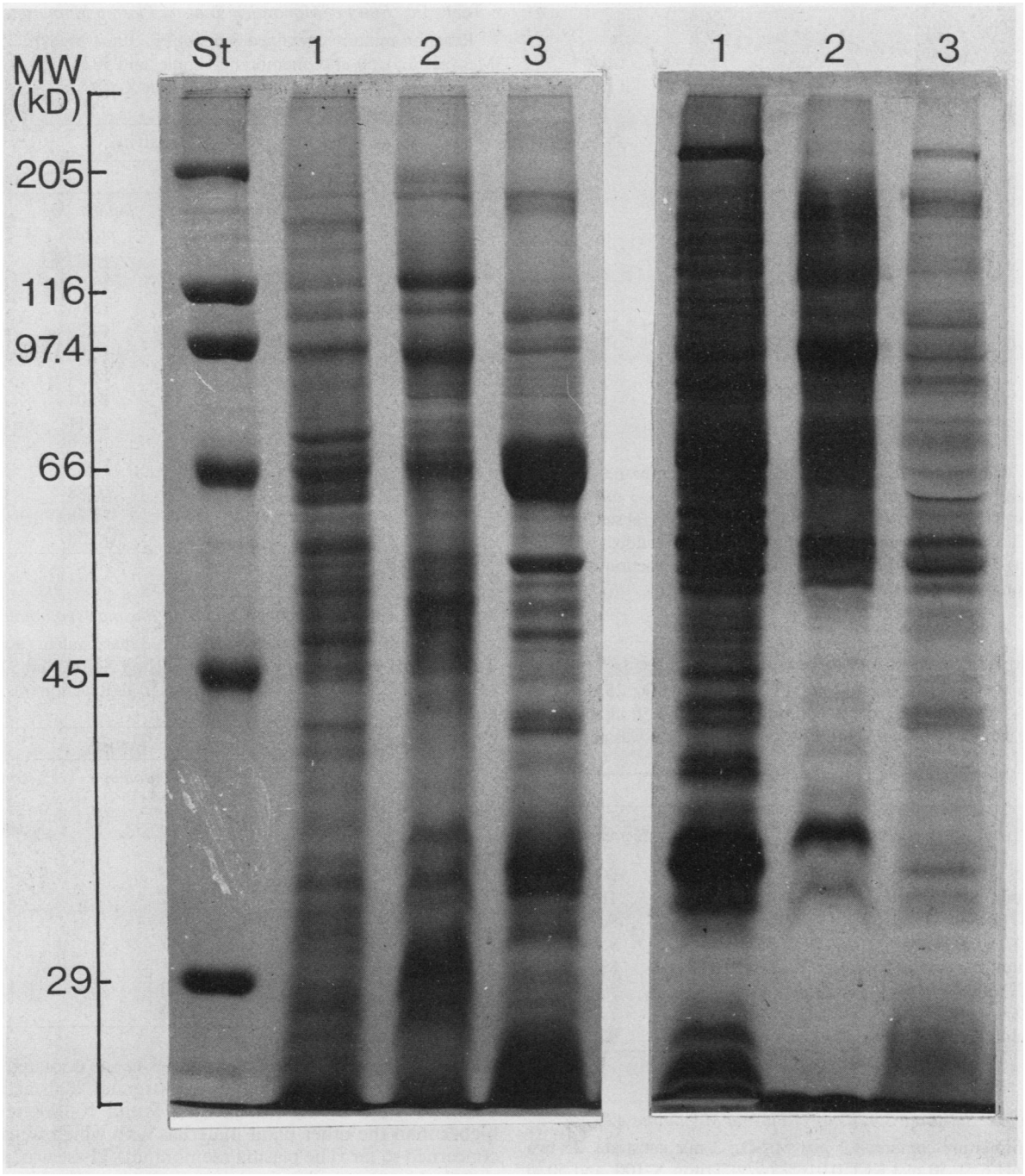

FIG. 5. Slab SDS-PAGE of various membrane fractions. The gels after electrophoresis were stained either with Coomassie blue (left) or concanavalin A-peroxidase stain (right). Lanes 1, 2, and 3 refer, respectively, to membranes partitioned into the dextran-enriched lower phase after a phase partitioning in the presence of 10 mM NaCl, to the 32/43% interfacial fraction (plasma membrane-enriched), and to the sample load/32% interfacial fraction (tonoplast and Golgi-enriched) after a discontinuous sucrose gradient of the upper phase. In each lane, 30 μg protein were applied.

accessible to the enzyme located on the inside of the membrane. This was also supported by the fact that most of the ATPase activity remained intact after treatment of plasma membrane with proteinase K at the concentrations where a great deal of polypeptides were digested (S. Yoshida, unpublished data). From these results, it is conceivable that the plasma membranes have been isolated in sealed and right-side-out vesicles.

In general, plasma membrane vesicles with normal sidedness of a right-side-out are known to partition into PEG-enriched upper phase in an aqueous two-polymer phase partition system (8). According to Larsson *et al.* (12), the plasma membranes isolated from oat roots and cauliflower inflorescences by partition in dextran-polyethylene two-phase system were homogeneous with respect to the sidedness. Most of the vesicles were right-side-out and sealed as assessed by the latent Mg^{2+}-ATPase activity which was dramatically increased by introducing Triton X-100.

Since the pH-activity profile and the monovalent-ion stimulation of plasma membrane ATPase were significantly affected by Triton X-100 in the present study, the detergent may also have a side effect other than the substrate permeation.

The plasma membrane ATPase of etiolated seedlings of *V. radiata* showed high specificities for Mg^{2+} ion and ATP. The relatively high hydrolytic activities for UDP and IDP are presumably due to the slight contamination by Golgi-derived mem-

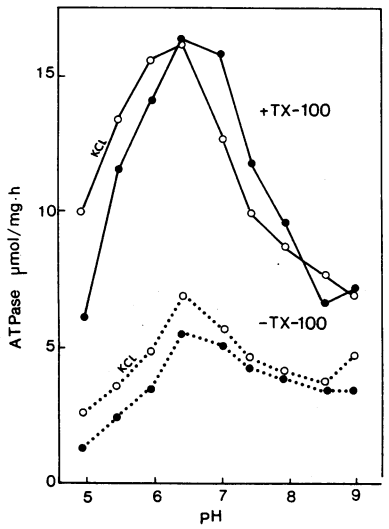

FIG. 6. The pH-activity profiles of plasma membrane ATPase. ATPase assay mixtures contained 3 mM ATP (Tris-salt), 3 mM $MgSO_4$, and 25 mM Tris-Mes in varied pH with presence (○) or absence (●) of 50 mM KCl. Dotted lines (lower part), no detergent was added; solid line (upper part), 0.02% Triton X-100 was added into the reaction mixtures. The reaction was performed at 30°C for 30 min.

Table II. *Effect of Inhibitors on Plasma Membrane ATPase*

Reaction mixture contained 3 mM ATP, 3 mM $MgSO_4$, 25 mM Tris-Mes (pH 6.5), 50 mM KCl, 0.016% Triton X-100, and 50 μl aliquot of membrane sample in a final volume of 250 μl. Reaction was performed at 30°C.

Inhibition	Activity	
	μmol/h·mg protein	*% control*
None	12.51	100
Sodium vanadate (50 μM)	2.26	18
NaN_3 (5 mM)	11.97	96
KNO_3 (50 mM)	11.85	95
Sodium molybdate (1 mM)	10.39	83
DCCD (50 μM)	7.40	59
DIDS (30 μM)	9.13	73
Ethanol (0.1%)	11.54	92

Table III. *Substrate Specificity of Plasma Membrane ATPase*

Reaction mixture contained 3 mM $MgSO_4$, 3 mM substrate, 25 mM Tris-Mes (pH 6.5), 50 mM KCl, 0.016% (w/v) Triton X-100, and 50 μl aliquot of membrane sample in a final volume of 250 μl. Reaction was performed at 30°C.

Substrate	Activity	
	μmol/h·mg protein	*% control*
ATP	13.42	100
ADP	3.32	25
AMP	0.23	2
CTP	2.91	22
GTP	3.99	30
ITP	4.91	37
IDP	12.27	91
UTP	11.70	87
UDP	12.71	94
p-Nitrophenylphosphate	3.89	29

Table IV. *Effect of Monovalent Ions on Plasma Membrane ATPase*[a]

Reaction mixture contained 3 mM ATP, 3 mM $MgSO_4$, 25 mM Tris-Mes (pH 6.5), 50 μl of membrane sample, and 50 mM monovalent ion, with or without addition of 0.016% Triton X-100 in a final volume of 250 μl. Reaction was performed at 30°C.

Monovalent Ion	Activity			
	−TX-100		+TX-100	
	μmol/h·mg protein	*% control*	*μmol/h·mg protein*	*% control*
None	6.45	100	14.82	100
KCl	8.72	135	13.53	91
NaCl	8.17	127	15.08	102
NH_4Cl	8.30	129	14.29	96
LiCl	7.18	113	13.08	88
Choline-Cl	8.66	134	14.74	99
None	6.45	100	14.01	100
KCl	ND	ND	13.11	94
KNO_3	7.45	114	13.70	98
KNO_2	7.24	112	13.33	95
KF	ND	ND	0.88	6
KI	7.63	118	12.78	91
K_2SO_4	7.21	112	13.58	97

[a] ND, not determined.

Table V. *Effect of Divalent Cations on Plasma Membrane ATPase*

Reaction mixture contained 3 mM ATP, 3 mM divalent cation, 25 mM Tris-Mes (pH 6.5), 50 mM KCl, 0.016% Triton X-100, and 50 μl aliquot of membrane sample in a final volume of 250 μl. Reaction was performed at 30°C.

Divalent Cation	Activity	
	μmol/h·mg protein	*% control*
None	2.82	22
Mg^{2+}	13.13	100
Mn^{2+}	6.20	47
Co^{2+}	5.47	42
Ca^{2+}	1.57	12
Zn^{2+}	0.84	6
Cu^{2+}	1.41	11
Cd^{2+}	0	0
Sr^{2+}	1.63	12
Hg^{2+}	0.17	1

branes. The Golgi-derived membranes in the etiolated seedlings of *V. radiata* were found to have extremely high activities for IDP and UDP in the presence of Triton X-100, several-fold higher than the other plant materials with which we have been concerned so far. The plasma membrane ATPase was stimulated slightly by monovalent ions, especially by Cl^-. This seems to correlate with the fact that the ATPase is inhibited by DIDS, which is specific inhibitor for anion channels of plasma membranes as reported (4, 9).

Evidence to show the existence of a H^+-pumping ATPase on plasma membranes as well as on tonoplasts has now accumulated for plants (1–3, 22). The plasma membrane ATPase of *V. radiata* was sensitive to DCCD. However, no stimulation was observed in FCCP and gramicidin D. This fact may indicate either that the plasma membrane vesicles have lost the permeability barrier for H^+ during the preparation or that the plasma membranes have been isolated in right-side-out vesicles where the ATP substrate site is inaccessible. This needs to be studied in future.

The dark-grown young seedlings of *V. radiata* are highly chilling sensitive, suffering injury after exposure to temperatures lower than 5°C. During the chilling treatments under low temperatures, electrolytes were observed to leak out of the tissues,

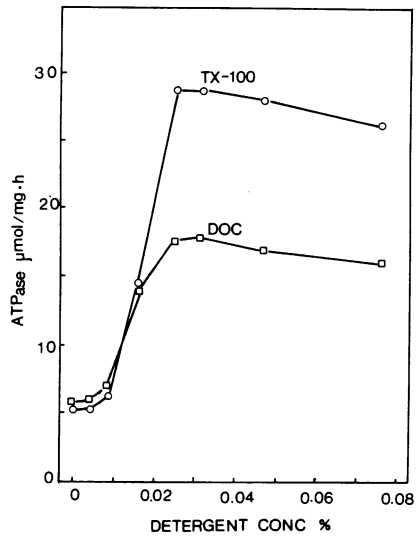

FIG. 7. Effect of detergents on plasma membrane ATPase activity. ATPase assay was performed in a reaction mixture contained 3 mM ATP (Na-salt), 3 mM $MgSO_4$, 25 mM Tris-Mes, pH 6.5, 50 mM KCl, and various concentration of detergents. The reaction was carried out at 30°C for 20 min. Triton X-100 (O), sodium DOC (□).

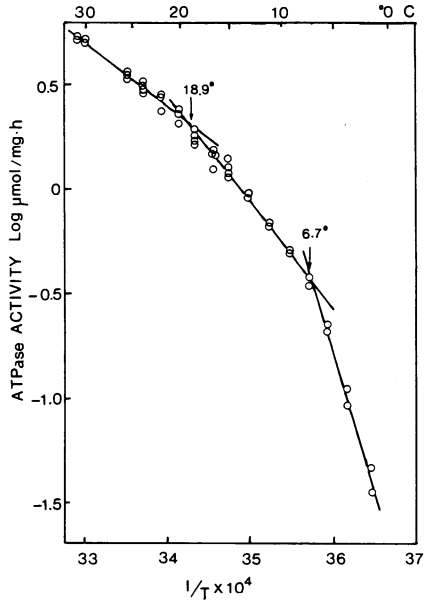

FIG. 8. Arrhenius plots of plasma membrane ATPase activity. ATPase assay mixture contained 3 mM ATP (Tris-salt), 3 mM $MgSO_4$, 25 mM Tris-Mes, pH 6.5, and 50 mM KCl. The reaction was performed simultaneously at various temperatures in a temperature gradient former in which a linear temperature gradient was formed between 0 and 30°C at 2°C intervals. The slopes of the curves were obtained by fitting regression to points derived from the averages of two replications and selecting those giving the best fit. Where discontinuities were suspected, regressions were fitted to all combinations above and below the apparent breaks and the partition with the minimum sum of square was selected. Lines of best fit were then drawn for each partition and the break point was estimated from the intersection of the regression.

and the degree of the leakage was closely associated with the degree of cell injury (S Etani, S Yoshida, unpublished data). This might indicate that a loss of physiological function of the plasma membrane as a result of chilling is involved in the mechanism of the cell injury.

With respect to the temperature dependency of the plasma membrane ATPase, an inflection has been determined on the Arrhenius plots. In orchard grass (S Yoshida, unpublished data), winter rye (M Uemura, unpublished data), Jerusalem artichoke tubers (M Ishikawa, S Yoshida, unpublished data), which are all cold-resistant species, inflections were observed around 15 to 17°C, regardless of season. These inflections, however, were apparently not coincident with a phase transition in the lipids as assessed by fluorescent polarization study using DPH. As has been suggested by Wright *et al.* (24), the temperature-dependency of the enzyme might be ascribable to the intrinsic properties of the enzyme molecules and/or the properties of the boundary lipids. In the present study, plasma membrane ATPase of *V. radiata* showed inflections at higher (18.9°C) and lower (6.7°C) temperatures (Fig. 8) and the latter was apparently coincident with the break point on the Arrhenius plots of anisotropy parameter of DPH incorporated into the membrane (Fig. 9).

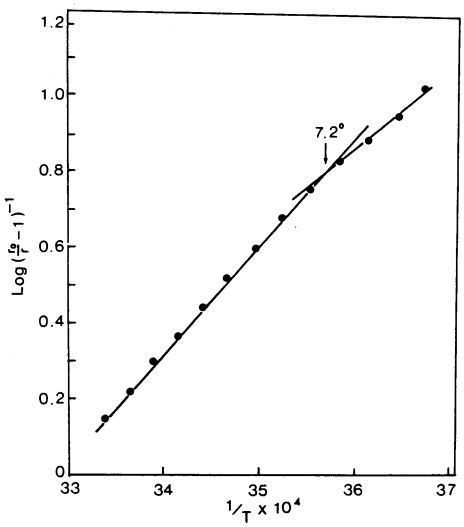

FIG. 9. Arrhenius plots of fluorescent anisotropy parameters of DPH incorporated into hydrophobic core of plasma membranes. The isolated membranes were labeled with DPH and the fluorescence polarization was measured at various temperatures. The anisotropy parameters calculated from the polarization ratio were arranged as Arrhenius plots to detect an inflection.

Fatty acids of plasma membrane phospholipids were less unsaturated (data not shown) than those of cold hardy plant species (26, 27). In liposomes prepared from the total lipid extracts or the separated phospholipids, however, no inflection was detected in the Arrhenius plots of the anisotropy parameters (data not shown). Nearly the same situation is reported for plasma membranes isolated from cold hardy plants (25) and the liposomes prepared from the extracted lipids (25). In the plasma membranes, the inflection on the Arrhenius plots of the anisotropy parameters of DPH occurred at a freezing temperature where the cells sustain frost injury (25). The inflection, however, was not dependent on the membrane lipids *per se*, but primarily on the membrane proteins (25).

Although there still remains uncertainty in the thermotropic behavior of the membrane molecules as far as a steady state measurement of the fluorescent polarization is concerned, it is of interest to note that the inflections of the Arrhenius slopes are well correlated with chilling injury and frost injury of plant cells, whereas the temperature range is quite different. Further detailed studies on the molecular basis of the thermotropic properties of plasma membranes are needed to elucidate the fundamental meaning of these inflections and thereby may provide useful information to explain the mechanisms.

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