Isolation and Identification of Plasma Membrane from Light-Grown Winter Rye Seedlings (*Secale cereale* L. cv Puma)[1]

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ABSTRACT

An effective method for the isolation of plasma membrane from light-grown winter rye seedlings (*Secale cereale* L. cv Puma) was established using a liquid two-polymer phase separation. The conditions for the specific partition of plasma membrane into the polyethylene glycol-enriched upper phase were examined, including variations in the polymer concentration, buffer system, pH, and NaCl addition in the phase partition system. The most effective phase partition system for the isolation of plasma membrane from winter rye consisted of 5.6/5.6% (w/w) polyethylene glycol 4000/dextran T500 in 0.25 molar sucrose-10 millimolar potassium phosphate-30 millimolar NaCl (pH 7.8), repeated once. When the isolated plasma membrane was centrifuged on a linear sucrose density gradient, a single band was found at the 34% (w/w) sucrose layer (1.141 grams per cubic centimeter) which co-fractionated with the pH 6.5-ATPase.

Identification of plasma membrane was performed by the combination of phosphotungstic acid-chromic acid stain and specific binding of N-1-naphthylphthalamic acid. Based on morphometrical observations after phosphotungstic acid-chromic acid stain, the isolated plasma membrane consisted mostly of vesicles of high purity. The isolated plasma membrane also showed extremely high specificity for N-1-naphthylphthalamic acid-binding, 10-fold higher than other membranes. It was also confirmed that there is a distinct difference in properties between plasma membrane and other membranes. The endomembranes such as from chloroplasts, mitochondria, and endoplasmic reticulum were observed to be highly sensitive to Zn^{2+} ion and lower pH, which resulted in an abrupt aggregation of membranes. On the contrary, plasma membrane was very stable to these treatments and no aggregation was observed. These unique properties of isolated plasma membrane are generally observed in a wide variety of plant species and can be utilized for the assessment of the purity of preparations of isolated plasma membranes and for their identification.

A growing interest has emerged on the biochemical and physiological functions of plant plasma membranes (11–14, 17–19, 22). According to recent literature, there is a consensus that cellular membranes, especially plasma membranes, are of primary importance to understand the mechanism of freezing injury of plant cells (20). There seem to be mainly two routes to be approached to the mechanism of freezing injury. First, it is essential to clarify the nature of the membrane damage caused by the stress. Second, it is also important to know the changes in the nature of the plasma membrane to be responsible for the development of freezing tolerance during the cold acclimating

process.

A major impediment to these approaches, however, has been in the difficulties of isolating plasma membranes in both a sufficiently pure form and a state identical with their *in vivo* state in plants. In most cases, plasma membrane-enriched fractions have been isolated mainly from tender and non-photosynthesizing tissues such as roots (3, 12, 28), etiolated seedlings (15, 21), and protoplasts (5, 9, 20) by the combination of mechanical disruption and sucrose density gradient centrifugations. One of the major problems encountered by investigators attempting to isolate plasma membranes from differentiated green tissues with thick cell walls has been the cross-contamination of fragmented chloroplast membranes, which are very difficult to separate from other membranes by differences in density or sedimentation characteristics. Furthermore, the sucrose density gradient centrifugations are lengthy, and the medium *per se* creates an osmotic gradient which may affect, to some extent, the intactness of the isolated membranes.

A phase partition of membranes between immiscible aqueous solutions of two polymers, developed by Albertsson (1), would be one of the most promising methods to separate plasma membranes from light-grown green tissues. The partition is known to be dependent on membrane surface properties rather than their size and density (1). Recently, pure plasma membranes have been successfully isolated from green tissues of orchard grasses in our laboratory (31) and from etiolated oat seedlings and their roots in another laboratory (33), using the phase partition system.

The aim of the present study was to establish a method for isolating plasma membrane from light-grown winter rye seedlings using a phase partition system as the preliminary step to examining the biochemical changes of the plasma membrane during the cold acclimating process. We also describe here new methods of identifying the plasma membrane isolated from plant tissues, using zinc and low pH.

MATERIALS AND METHODS

Plant Material. Seeds of winter rye (*Secale cereale* L. cv Puma) were germinated and grown in vermiculite supplemented with Hoagland solution in the greenhouse at a 20°C day (16 h) and 15°C night (8 h). Plants were harvested when they reached the three- or four-leaf stage, after about 20 d. The crown tissues at the basal part of seedlings, about 10 cm in length, were used as the experimental materials.

Homogenization and Differential Centrifugation. The crown-tissues, washed with cold tap water and then deionized H_2O, were cut into small pieces with a razor blade and homogenized with a Polytron PT20 at the medium speed setting for 90 s. The homogenizing medium consisted of 0.5 M sorbitol, 50 mM

Table I. *Cellular Distribution of Membrane Marker Enzyme Activities, Protein, and Chl between Particulate Fractions after Differential Centrifugation*

	Fractions					
	4,000g Pellet		14,000g Pellet		156,000g Pellet	
	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
Marker enzymes						
ATPase (pH 6.5)[a]	65.03	7.97	139.35	7.16	38.46	10.28
ATPase (pH 9.0)[a]	40.26	4.93	79.74	4.10	20.52	5.47
IDPase[a]	58.71	7.19	116.04	5.96	35.55	9.49
Acid phosphatase[a]	122.74	15.04	203.77	10.47	39.31	10.51
Cyt c oxidase[b]	0.28	0.03	2.63	0.14	0.40	0.11
NADH Cyt c reductase[b] (+ antimycin A)	0.21	0.03	0.62	0.03	0.42	0.11
Protein (mg)	8.16		19.46		3.74	
Chl (mg)	9.89		27.67		1.69	

[a] Total activity and specific activity were expressed as μmol Pi/h (equivalent to 10 g fresh weight) and μmol Pi/mg protein·h, respectively.

[b] Total activity and specific activity were expressed as μmol Cyt c/min (equivalent to 10 g fresh weight) and μmol Cyt c/mg protein·min, respectively.

MOPS^2-KOH, 10 mM EGTA-neutralized with KOH, 2.5 mM potassium metabisulfite, 4 mM SHAM, 1 mM PMSF, 5% (w/v) soluble PVP (mol wt 24,500), and 1% (w/v) defatted BSA (pH 7.6). Four ml of the homogenizing medium were used per g fresh weight of tissues. The brei was successively squeezed through two layers of gauze and two layers of Miracloth, and then passed through Amberlite XAD-4 column (5 × 13 cm) for removal of phenolic compounds. The effluent was successively centrifuged at 1,500g for 7 min, 14,000g for 15 min, and 156,000g for 20 min. The 14,000g pellet and the 156,000g pellet were designated as crude mitochondrial and microsomal fractions, respectively. All procedures were performed at 0 to 5°C.

Equilibrated Linear Sucrose Density Gradient Centrifugation. The crude mitochondrial and microsomal fractions were washed once by suspending in 0.5 M sorbitol-15 mM Tris-maleate (pH 7.3) and by centrifugations at 14,000g for 15 min and 156,000g for 20 min, respectively. Each washed pellet was resuspended in 6 ml of 10% (w/w) sucrose-15 mM Tris-maleate-1 mM EDTA, (pH 7.3) and loaded onto 30 ml of linear density gradient of sucrose, 15 to 50% (w/w) in 15 mM Tris-maleate-1 mM EDTA (pH 7.3). After centrifugation at 96,000g for 13 h at 2°C on a Hitachi SW27 rotor, 1.2-ml aliquots were collected from the top of the gradient with a ISCO fractionator model 640.

Enzyme Assays. Antimycin A-insensitive NADH Cyt c reductase and Cyt c oxidase were assayed spectrophotometrically at 25°C by following the reduction and oxidation of Cyt c at 550 nm as described previously (32). Acid phosphatase was assayed in sodium acetate buffer at pH 5.0 with p-nitrophenyl-P as the substrate as reported previously (33). ATPase was assayed by the method of Hodges et al. (12), with slight modification. Latent IDPase, after storage of fractions at 0°C for 5 d, was assayed according to the method of Ray et al. (23), with slight modification. Total Chl content was assayed spectrophotometrically after extraction with 80% (v/v) acetone by the method of Arnon (2). Protein content was determined by the method of Broadford (6), with slight modification. BSA was used as the standard.

Partition in Aqueous Two-Phase Polymer System. The crude microsomal fraction obtained from differential centrifugation

was washed once by suspending in 0.25 M sucrose-10 mM K-phosphate (pH 7.8) and by centrifugation at 156,000g for 20 min. The washed pellet was suspended in the same buffer system as used for the washing. Aliquots of membrane suspension were applied to an aqueous two-phase polymer system. The aqueous two-phase polymer system consisted of an aliquot of the membrane suspension and PEG 4000/dextran T500 in final concentrations of 5.6/5.6, 5.9/5.9, 6.2/6.2, 6.5/6.5, and 6.8/6.8% (w/w) in the same buffer system as that used for the washing, with or without the addition of NaCl at the indicated concentrations. In some cases, the pH of the buffer system was changed. After temperature equilibration at 0°C, each phase system was thoroughly mixed by several inversions and was centrifuged at 400g for 3 min to decrease the time for phase setting. The upper phase was carefully removed from the lower phase with a Pasteur pipette without disturbing the materials at the interface. The materials at the interface were collected with the lower phase. Both phases were diluted with 0.5 M sorbitol-3 mM Tris-maleate (pH 7.3) and then pelleted down by centrifugation at 156,000g for 20 min. The pellets were washed again in the same buffer system as above to remove polymers thoroughly, and finally suspended in 0.5 M sorbitol-3 mM Tris-maleate (pH 7.3).

Batch Procedure for Phase Partition. To achieve a further purification of the plasma membrane, the washed crude microsomal fractions was subjected to the batch procedure as outlined in Figure 6. An aliquot of the membrane suspension was added to the polymer mixture consisting of 5.6/5.6% (w/w) PEG 4000/ dextran T500 in 30 mM NaCl, 0.25 M sucrose, and 10 mM K-phosphate (pH 7.8). After temperature equilibration at 0°C, the phase system was thoroughly mixed by several inversions and centrifuged at 0°C as described above. The upper phase (U$_1$) was repartitioned with the newly synthesized lower phase. The upper phase (U$_2$) and the combined lower phases (L$_1$ + U$_1$L) were diluted and pelleted down as described above. The pellets were washed again and finally suspended in 0.5 M sorbitol-3 mM Tris-maleate (pH 7.3).

The crude mitochondrial fraction (1,500–14,000g) was also subjected to the same batch procedure as used for the crude microsomal fraction.

NPA Binding Assay. [2,3,4,5-^{3}H]-N-1-Naphthylphthalamic acid ([^{3}H]NPA; 30 Ci/mmol) was purchased from CEA-France, Gif-sur-Yvette, France. The capacity of NPA binding was tested with the crude mitochondrial fraction and the U$_2$ and L$_1$ + U$_1$L

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^{2} Abbreviations: MOPS, 3-[N-morpholino]propanesulfonic acid; SHAM, salycylhydroxamic acid; PMSF, phenylmethylsulfonyl fluoride; PEG 4000, polyethylene glycol 4000 (mol wt 3,350); NPA, N-1-naphthyl-phthalamic acid; PTA-CrO$_3$, phosphotungstic acid-chromic acid.
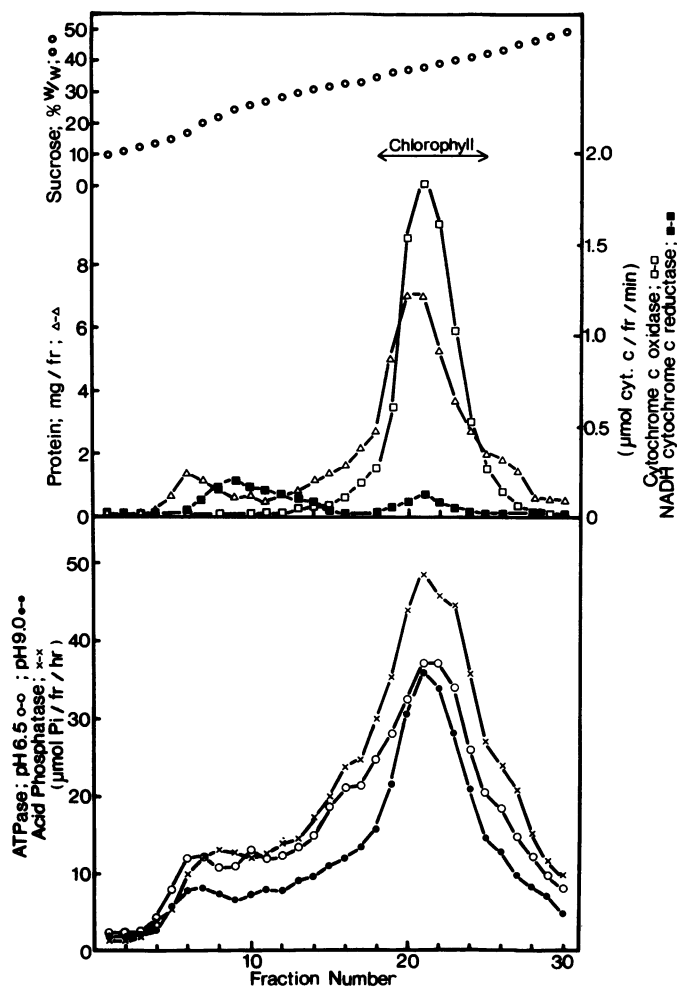
FIG. 1. Equilibrated sucrose density gradient of the mitochondrial fraction. The crude mitochondrial fraction (4,000–14,000g pellet) was suspended in 6 ml of 10% (w/w) sucrose containing 15 mM Tris-maleate-1 mM EDTA (pH 7.3) and loaded onto a 30-ml linear gradient of sucrose (15–50%, w/w) in 15 mM Tris-maleate-1 mM EDTA (pH 7.3). After centrifugation at 96,000g for 13 h at 2°C, 1.2-ml aliquots were collected from the top of the gradient. Upper (A): (O), sucrose; (Δ), protein; (■), antimycin A-insensitive NADH Cyt c reductase; (□), Cyt c oxidase. Lower (B): ATPase activity assayed at pH 6.5 (O) and at pH 9.0 (●); (×), acid phosphatase. Chl mostly distributed between fractions 18 and 23.
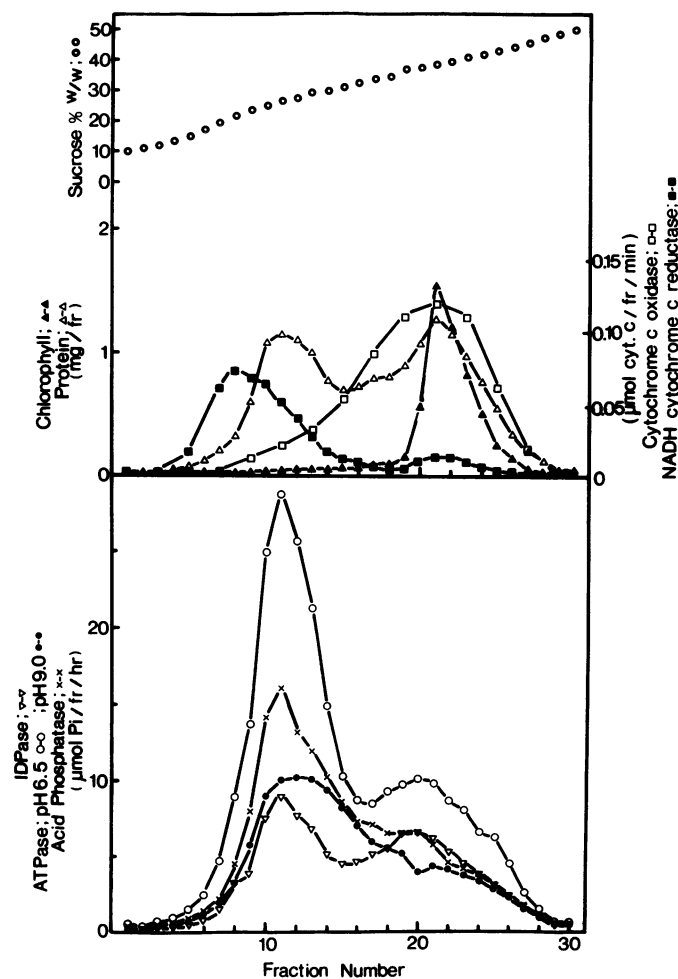
FIG. 2. Equilibrated sucrose density gradient of the microsomal fraction. The crude microsomal fraction (14,000–156,000g pellet) was suspended, loaded onto the sucrose gradient, centrifuged, and collected as described in legend for Figure 1. Symbols except Chl (▲) and latent IDPase (∇) were referred to legend for Figure 1.

fractions after batch procedure of the microsomal fraction. Each membrane was suspended in 0.25 M sucrose-10 mM sodium citrate buffer-5 mM MgSO₄ (pH 5.0). To 1.8-ml aliquots of the membrane suspension, [3H]NPA in a final concentration of 1 nM was added with or without the addition of the unlabeled NPA in a final concentration of 10 μM. The final volume was 2 ml. The mixtures were incubated at 20°C for 30 min and then pelleted at 100,000g for 40 min. The supernatant was decanted and the pellet was suspended with 0.5 ml of 0.15% (w/v) deoxycholic acid. Aliquots (0.2 ml) of the supernatant or of membrane suspension were transferred to vials containing 4 ml of a scintillation solution, ATOMLIGHT (New England Nuclear), and radioactivity was monitored in a Packard liquid scintillation counter, model Tri-carb, 3330. Specific NPA binding was calculated by the method of Thompson et al. (27). The pH dependency of the specific NPA binding was determined in the same reaction mixture as described above, except the replacement of the buffer varied in pH.

Electronmicroscopy. Membrane samples of the crude mito-

chondrial fraction and of the U_2 and L_1 + U_1L of the microsomal fraction after the batch procedure were suspended in 3% (v/v) glutaraldehyde in 0.5 M sorbitol-3 mM Tris-maleate (pH 7.3) and fixed for 2.5 h at 0°C. Fixed membrane fractions were pelleted by centrifugation at 14,000g for 20 min and the pellets were embedded in agar. The agar blocks were rinsed through subsequent changes of cold buffer solution and post-fixation was done in 2% buffered osmium tetroxide solution for 3 h at 0°C and additionally for 3 h at room temperature, and then they were dehydrated in an ethanol series and n-butylglycidyl ether. The dehydrated agar blocks were embedded in Spurr's resin (25). Intact tissues were also fixed, dehydrated, and embedded as described for membrane pellets but omitting the process of embedding in agar. Sections were stained with PTA-CrO₃. The sections of the gold grids were placed in 10% (w/v) periodic acid for 30 min followed by thoroughly washing them in distilled H₂O with three time-changes. The grids were then transferred to 1% (w/v) PTA in 10% (w/v) CrO₃ for 20 min before thoroughly washing. All procedures for PTA-CrO₃ staining were performed at room temperature. Some sections were also stained with uranyl acetate and lead citrate (24) as the standard procedure. These sections were examined using a JEOL 100C electron microscope. The proportion of plasma membrane vesicles stained positively with PTA-CrO₃ was determined morphometrically.
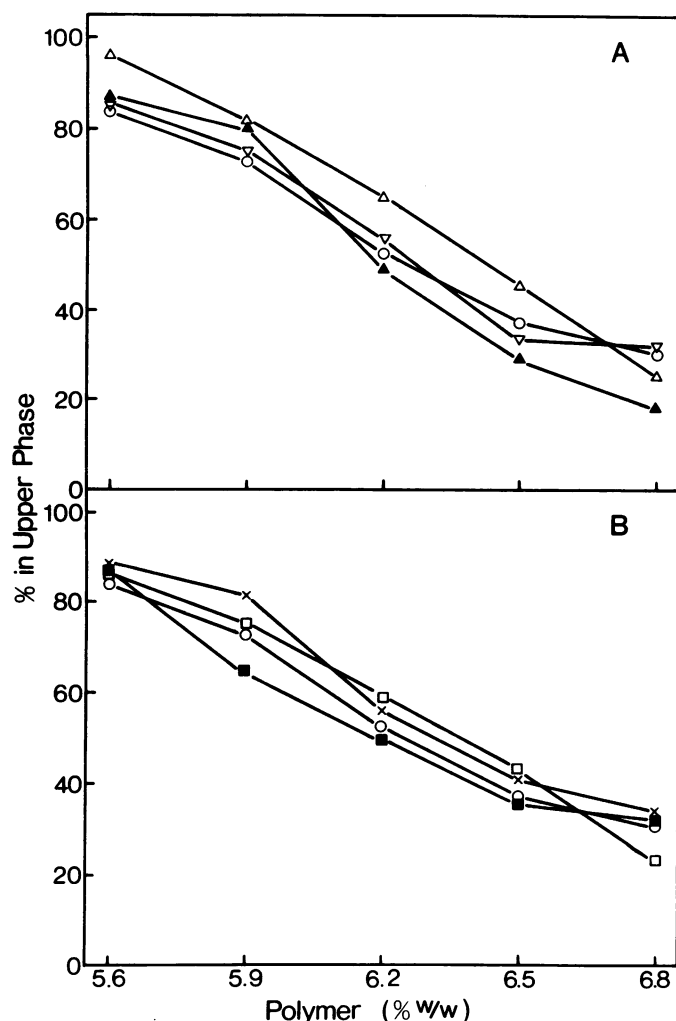
FIG. 3. Effect of polymer concentration on the partition of the crude microsomal membranes in a two-phase system containing 0.25 M sucrose-10 mM K-phosphate (pH 7.8). The two-phase system consisted of various concentrations of each polymer, PEG 4000 and dextran T500, at indicated concentration. Partition was performed at 0°C. The results included in this figure (A and B) were all taken from the same experiment. Symbols are the same as those in legend of Figures 1 and 2.
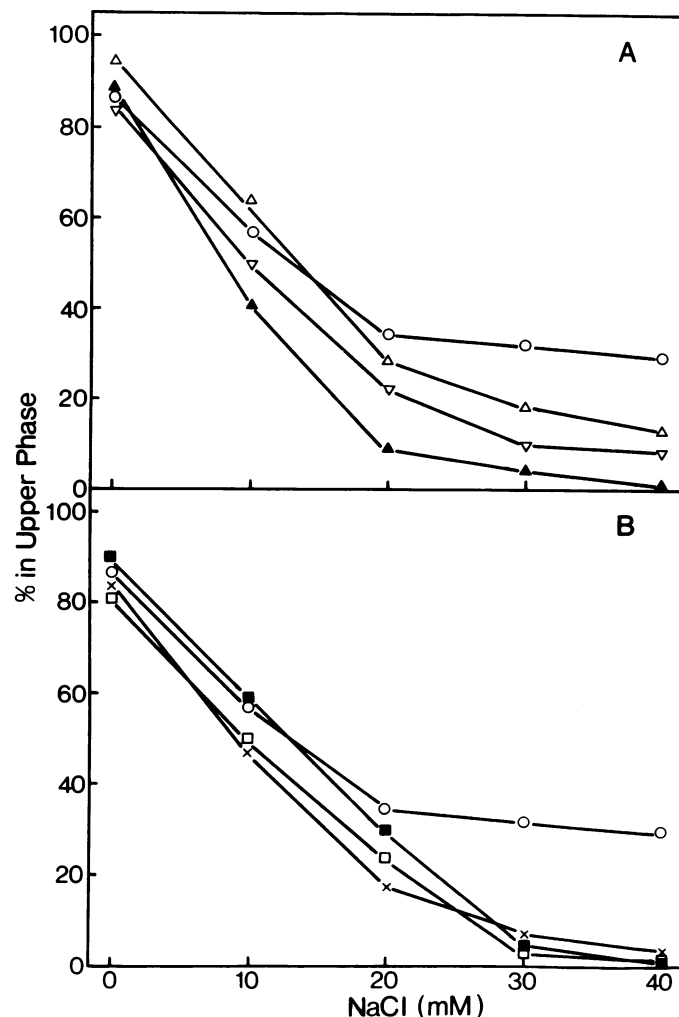
FIG. 4. Effect of NaCl concentration on the partition of crude microsomal fraction in a two-phase system containing 5.6/5.6% (w/w) PEG 4000/dextran T500 in 0.25 M sucrose-10 mM K-phosphate (pH 7.8). NaCl was added at the indicated concentration. Partition was performed at 0°C. The results included in this figure (A and B) were all taken from the same experiment. Symbols are the same as those in legend of Figures 1 and 2.

Effects of Zinc Ion and Low pH on Aggregation of Membrane Particles.

To determine the effects of zinc ion and low pH on the aggregation of various membranes, an aliquot (0.1 ml) of the U_2 and $L_1 + U_1L$ fractions of the microsomal fraction were incubated either with 1.9 ml of 0.5 M sorbitol-3 mM Tris-maleate (pH 7.3) containing $ZnCl_2$ at a desired concentration or with 1.9 ml of 0.5 M sorbitol-10 mM sodium citrate buffer adjusted to a desired pH at 20°C for 30 min. Membrane aggregation was monitored by the change of A at 510 nm spectrophotometrically and/or visually by observation under phase-contrast microscope.

RESULTS

Cellular Localization of Various Marker Enzymes.

The cellular distribution of membrane marker enzyme activities among particulate fractions after differential centrifugation is presented in Table I. The 14,000g pelleted fraction contained the highest total activity of any marker enzyme and also was enriched in its content of protein and Chl. On the other hand, specific activities of ATPase (pH 6.5), latent IDPase, and antimycin A-insensitive NADH Cyt c reductase were observed to be relatively high in

the 156,000g pellet. The KCl stimulation of ATPase at pH 6.5 was found to be very low in every fraction, ranging from 5% to 10% (data not shown).

Sucrose Density Gradient Centrifugation of Crude Mitochondrial and Microsomal Fractions.

Activity profiles of the mitochondrial (4,000–14,000g) and microsomal (14,000–156,000g) fractions are presented in Figures 1 and 2, respectively. In the mitochondrial fraction, a major peak of pH 6.5 ATPase activity occurred at fraction 21. Cyt c oxidase activity and fragmented chloroplast membranes were co-fractionated around fraction 21. In the microsomal fraction, a major peak of the pH 6.5 ATPase occurred at fractions 10 to 13 (equivalent to 26–29%, w/w, sucrose) and a relatively small peak of ATPase activity occurred at fractions 18–21 (equivalent to 34–38%, w/w, sucrose). The former major peak of ATPase activity was heavily overlapped with pH 9 ATPase, IDPase, and acid phosphatase activities and the later small peak of ATPase activity was overlapped with Cyt c oxidase, acid phosphatase, IDPase, pH 9 ATPase activities, and fragmented chloroplast membranes. Based on these results, it seems likely that there exist, at least, three different types of membranes associated with pH 6.5 ATPases in rye cells, i.e. one
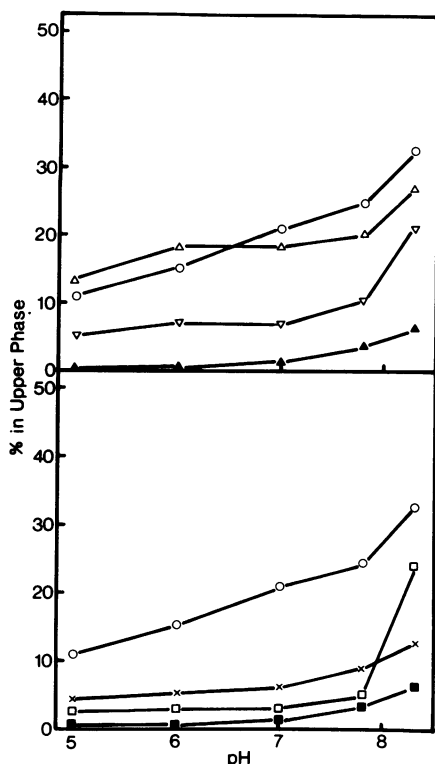
FIG. 5. Effect of pH on the partition of crude microsomal fraction in a two-phase system containing 5.6/5.6% (w/w) PEG 4000/dextran T500, 30 mM NaCl in 0.25 M sucrose-10 mM K-phosphate at various pH values. The results included in this figure (A and B) were all taken from the same experiment. Symbols are the same as those in legend of Figures 1 and 2.
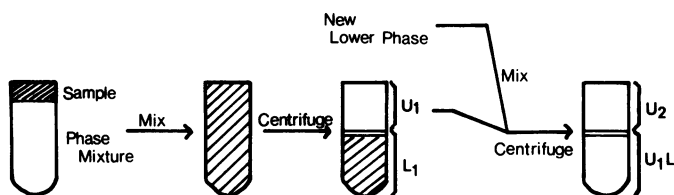

FIG. 6. Batch procedure for plasma membrane isolation in a PEG 4000/dextran T500 two-phase system. An aliquot of the microsomal suspension of 0.25 M sucrose-10 mM K-phosphate (pH 7.8) was added to a phase mixture with a final composition of 5.6/5.6 (w/w) PEG 4000/ dextran T500, 30 mM NaCl, 0.25 M sucrose, and 10 mM K-phosphate (pH 7.8). The phase system was thoroughly mixed by several inversions and centrifuged at 400g for 3 min to decrease the time for phase separation. The upper phase (U_1) was removed from the lower phase (L_1) and transferred into a newly prepared lower phase. After mixing and centrifugation as above, the upper phase (U_2) was removed from the lower phase (U_1L). The combined lower phases (L_1 and U_1L) were enriched in endomembranes. The repartitioned upper phase (U_2) was highly enriched in plasma membrane.

is sedimented at a lower speed (14,000g) and the other two are sedimented at a higher speed (156,000g) during differential centrifugation.

Phase Partition of Membranes. In order to separate plasma membrane, microsomal membranes were subjected to a phase partition in dextran T500 and PEG 4000. The effect of polymer concentration on the partition of the microsomal membranes in 10 mM K-phosphate (pH 7.8) and 0.25 M sucrose is presented in Figure 3. As the polymer concentration increased, most of the membranes were preferentially partitioned into the lower phase

and no selective partition was observed among various membranes. As presented in Figure 4, however, addition of NaCl to the phase partition system of 5.6% (w/w) polymer concentration has a remarkable effect on the partition of membranes. By increasing NaCl concentration up to 40 mM, a fraction of the pH 6.5 ATPase-associated membrane, i.e. about 30% of the total activity in the microsomal fraction, was preferentially partitioned into the upper phase. Most of the other membranes, such as chloroplast fragments, ER, mitochondria, Golgi membranes, and acid phosphatase-associated membranes were preferentially partitioned into the lower phase. The effect of pH on the recovery of the pH 6.5 ATPase-associated membrane in the upper phase of the phase partition system containing NaCl is presented in Figure 5. As the pH increased, the recovery of the ATPase-associated membrane into the upper phase also increased. However, at pH 8.3, other membranes, especially mitochondria, were partitioned into the upper phase. Based on this result, the optimum pH for the specific partition of the pH 6.5 ATPase-associated membrane into the upper phase seems to be around pH 7 to 7.8. When the buffer concentration was varied between 5 and 10 mM, no difference in the partition of membranes was observed (data not shown).

To achieve further purification of the pH 6.5 ATPase-associated membrane partitioned preferentially into the upper phase, the microsomal membranes were subjected to a batch procedure (Fig. 6). After repartition of the upper phase at 30 mM NaCl with a newly synthesized lower phase, a further reduction in chloroplast fragments and enzyme activities, such as acid phosphatase, IDPase, Cyt c oxidase and antimycin-A-insensitive NADH Cyt c reductase, was achieved (Table II). On the other hand, reduction in protein content and the pH 6.5 ATPase-associated membrane in the upper phase were slight. As a result, the specific activity of the ATPase in the upper phase after the repeated partition increased to some extent, while the specific activities of the other enzymes decreased significantly. Thus, the batch procedure was found to be very effective for separating the pure pH 6.5 ATPase-associated membrane.

The mitochondrial fraction (4,000-14,000g) was also subjected to the phase partition system employed for the microsomal fraction. Only 2% of the total activity of the pH 6.5 ATPase in the mitochondrial fraction was recovered in the upper phase after the batch procedure and the upper phase was relatively contaminated with ER and acid phosphatase-associated membranes (Table III).

After phase partition of the microsomal membranes by the batch procedure, the ATPase-associated membrane in the upper phase was centrifuged on a linear sucrose density gradient (15–50%, w/w). A discrete white band was formed at fractions 15 to 19, centered at fraction 17, equivalent to 1.141 g/cm³ (Fig. 7). It was co-fractionated with pH 6.5 ATPase. A trace amount of IDPase activity was co-fractionated with the ATPase, but the other marker enzyme activities were hardly detectable.

NPA Binding Test. The specific binding capacity of NPA was tested with membranes partitioned into the upper phase, U_2, and the lower phase, $L_1 + U_1L$, and the crude mitochondrial membranes. Although the optimum pH for the specific NPA binding was observed to be around 4.0 (Fig. 8), the pH was set at 5.0 to avoid a secondary disturbance of the normal functions of membranes at too low a pH. As summarized in Table IV, a significant amount of total specific NPA-binding activity was located in the 14,000g pellet, whereas the specific activity on protein basis was quite low. As to the 156,000g pellet, the membrane partitioned into the upper phase had the highest capacity for specific NPA binding on protein basis, whereas the membrane partitioned into the lower phase showed quite a low specific NPA binding capacity on protein basis.

PTA-CrO$_3$ Staining. The specificity of the PTA-CrO$_3$ stain for

Table II. *Distribution of Membrane Marker Enzyme Activities, Protein, and Chl between Fractions Obtained by the Batch Procedure for the Microsomal Fraction as Shown in Figure 6*

The values in parentheses indicate percent activity of each marker enzyme and percent content of protein and Chl for the initial microsomal sample.

	Fractions					
	U_1		U_2		$L_1 + U_1L$	
	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
Marker enzymes						
ATPase (pH 6.5)[a]	0.97 (22.8)	13.67	0.75 (17.6)	14.42	3.31 (77.5)	10.54
IDPase[a]	0.35 (9.6)	4.93	0.15 (4.1)	2.88	3.11 (85.4)	9.90
Acid phosphatase[a]	0.31 (7.9)	4.37	0.09 (2.3)	1.73	3.39 (86.9)	10.80
Cyt c oxidase[b]	0.00 (3.6)	0.02	0.00 (0.9)	0.01	0.04 (87.3)	0.13
NADH Cyt c reductase[b] (+ antimycin A)	0.00 (3.2)	0.02	0.00 (0.7)	0.00	0.03 (92.7)	0.11
Protein (mg)	0.07 (17.1)		0.05 (12.5)		0.31 (75.7)	
Chl (mg)	0.01 (4.0)		0.00 (0.2)		0.17 (95.7)	

[a] Total activity and specific activity were expressed as μmol Pi/h (equivalent to 1 g fresh weight) and μmol Pi/mg protein·h, respectively.

[b] Total activity and specific activity were expressed as μmol Cyt c/min (equivalent to 1 g fresh weight) and μmol Cyt c/mg protein·min, respectively.

Table III. *Percent Distribution of Protein and Membrane Marker Enzyme Activities between Fractions Obtained by the Batch Procedure of the Crude Mitochondrial Fraction as Shown in Figure 6*

Total activity of each membrane marker enzyme and protein content in crude mitochondrial fraction were taken as 100%.

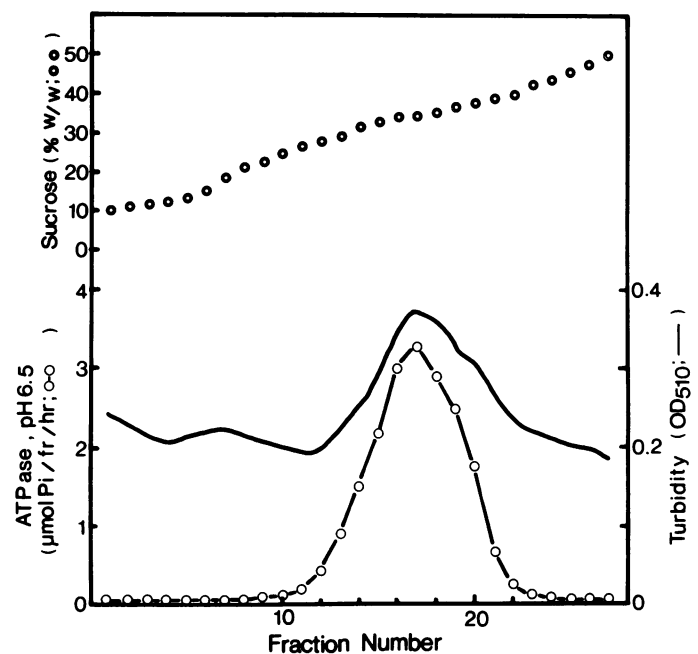|	Fractions	
	U_2	$L_1 + U_1L$
	%	
ATPase (pH 6.5)	1.9	98.1
ATPase (pH 9.0)	2.0	98.0
IDPase		
Acid phosphatase	0.8	99.2
Cyt c oxidase	0.1	99.9
NADH Cyt c reductase (+ antimycin A)	1.1	98.9
Protein	1.2	98.8

FIG. 7. Equilibrated sucrose density gradient of the ATPase-associated membrane purified after batch procedure of phase partition. The ATPase-associated membrane (the upper phase, U_2) was isolated according to the protocol indicated in Figure 6. The membrane was suspended in 3 ml of 10% (w/w) sucrose containing 15 mM Tris-maleate-1 mM EDTA (pH 7.3) and loaded onto a 13-ml linear gradient of sucrose (15–50%, w/w) in 15 mM Tris-maleate-1 mM EDTA (pH 7.3). After centrifugation at 96,000g for 13 h at 2°C, 0.6-ml aliquots were collected from the top of the gradient. (——), A at 510 nm; (O——O), ATPase activity at pH 6.5; (●), density of sucrose. The activities for other membrane marker enzymes were negligible.

the plasma membrane was determined on parenchymal cells of the middle part of light-grown rye seedlings (about 10 cm above the ground). These sections were treated with the conventional uranyl acetate-lead citrate procedure which stained the cellular membranes and organelles (Fig. 9A). In comparison, when sections were treated with PTA-CrO₃, not only the plasma membrane, but also some portions of the thylakoid lamellae membrane were positively stained (Fig. 9B). However, the other membranes or organelles including mitochondria, tonoplast, chloroplast envelope, and ER were hardly stained with the PTA-CrO₃. The staining capacity of the thylakoid membranes in intact cells was not dependent on the conditions of the staining process, *i.e.* the length of time and the temperature of preincubation with periodide solution, the treatment with phosphotungstate-CrO₃ and the post-rinsing with distilled H₂O. On the other hand, the thylakoid membranes both in the crude mitochondrial fraction (Fig. 9, G and H) and in the lower phase L_1 + U_1L after the batch procedure (Fig. 9, E and F) were hardly stained with the PTA-CrO₃. When the staining procedure was also applied to the isolated chloroplasts and the ruptured chloroplast membranes prepared by sonication, the intact chloroplasts were positively stained with PTA-CrO₃, but the ruptured chloroplasts were not stained (unpublished data). Thus, the staining capacity of the thylakoid membranes *in situ* may require the intactness of the

membrane structure. Based on these facts, the PTA-CrO₃ stain may be applied to isolated membrane vesicles to identify the plasma membranes. Further, the upper phase membrane fraction (U_2) after the batch procedure contained no detectable amount of thylakoid membranes as judged from the Chl content.

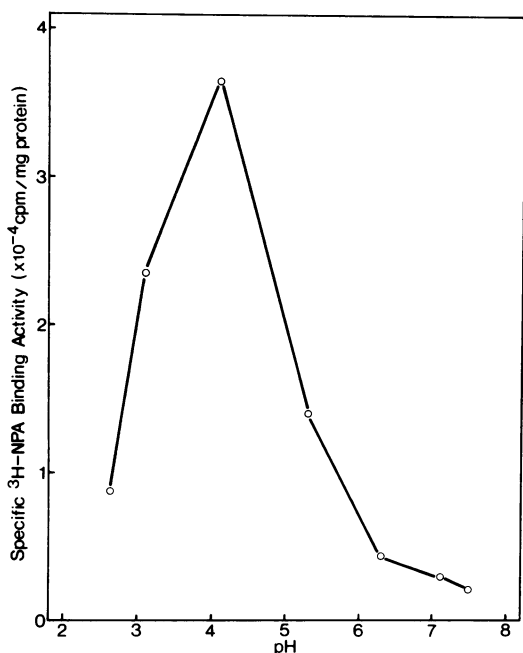A very high proportion of the membranes that partitioned into

FIG. 8. The pH profile of the specific NPA binding activity of plasma membrane. Reaction mixture consisted of 1 nM ^{3}H-NPA, 0.25 M sucrose-10 mM sodium citrate buffer-5 mM MgSO$_4$ at the indicated pH, 10 μM unlabeled NPA if added and an aliquot of plasma membrane sample (U$_2$) prepared by the batch procedure. Reaction was performed at 20°C for 30 min. The calculation method for specific NPA binding activity and other experimental details were described under "Materials and Methods."

the upper phase (U$_2$) was positively stained with the PTA-CrO$_3$ (Fig. 9, C and D). The PTA-CrO$_3$-stained membrane vesicles in the upper phase (U$_2$) were relatively homogeneous in size, ranging from 0.2 to 0.5 μm in diameter. The lower phase fraction (L$_1$ + U$_1$L) contained a very low proportion of PTA-CrO$_3$-stained membranes and some of them were fragmented. From these results, it is quite likely that the upper phase fraction (U$_2$), after phase partition twice in the presence of NaCl, is highly enriched in membrane vesicles originating from plasma membranes.

Effect of Zinc Ion and Low pH on the Properties of the Isolated Plasma Membrane. Zinc chloride in various concentrations was added to the various types of membranes suspended in 0.5 M sorbitol-10 mM Tris-maleate (pH 7.3), and the degree of aggregation of the membrane vesicles was monitored by the A change at 510 nm. Essentially no aggregation was observed in the purified plasma membrane vesicles (U$_2$), up to 20 mM of Zn^{2+} ion (Fig. 10). In contrast, the membranes recovered from the lower phase (L$_1$ + U$_1$L) were severely aggregated by the addition of Zn^{2+} ion even at 5 mM or lower. When Zn^{2+} ion was added to the crude mitochondrial membranes, almost all of the membranes were severely aggregated at less than 5 mM (data not

shown). Thus, it appears that the plasma membrane has a unique property in terms of the sensitivity to Zn^{2+} ion, which allows it to be distinguished from the other membranes. Only the divalent cations belonging to the group II-b in the periodic table such as Zn^{2+}, Cd^{2+}, and Hg^{2+} are effective; other divalent cations such as Ca^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}, Cu^{2+}, and Co^{2+} ions and also monovalent cations are ineffective (data not shown).

The other obvious properties to distinguish the plasma membrane from other membranes was its stability against low pH treatment. When the pH of the membrane suspension was lowered from 7.5 to 2.5, a drastic aggregation was observed in the lower phase membranes below pH 6.0 as shown by an increase in A at 510 nm (Fig. 11). On the other hand, with plasma membrane, only a slight aggregation was observed below pH 4.0. The crude mitochondrial membranes were also easily aggregated into large clusters by lowering pH below 5.0 (data not shown). The specific aggregation of membranes either with Zn^{2+} or by low pH is easily observed under a phase-contrast microscope (Fig. 12). These unique properties of plasma membranes should be utilizable as a simple and reliable tool to identify plasma membranes isolated from plant cells.

DISCUSSION

In 1971, Albertsson developed a method for the separation of membrane particles based on their partition between two immiscible aqueous polymers (1). The partition is dependent on membrane surface properties rather than their size and density. If plasma membranes from plant cells have such unique properties, the specific partition into either of two phases would be expected under selected conditions. Recently, Widell et al. (31) separated plasma membranes from etiolated young oat seedlings and their roots using a batch procedure of an aqueous polymer two-phase system. They used dextran T500 (6.3%, w/w) and PEG 4,000 (6.3%, w/w) as the polymers and 0.25 M sucrose-5 mM K-phosphate (pH 7.8) as the solvent. We tried the same system as above for separation of plasma membrane from light-grown rye seedlings, but failed to achieve any specific partition of plasma membrane into the PEG-enriched upper phase. The membranes recovered from the upper phase were always contaminated with significant amounts of chloroplast fragments, mitochondria, Golgi membranes, and other membranes. Based on this fact, it seems likely that the method presented by Widell et al. (31) is not applicable for separation of plasma membrane from a light-grown and differentiated green tissue.

Recently, we developed a method for separating plasma membranes from green tissues of orchard grasses using a phase partition system contained NaCl (33). In orchard grass tissues, a phase system consisting of 5.6% (w/w) each of Dextran T500 and PEG 4000, 0.5 M sorbitol, 15 mM Tris-maleate (pH 7.3), and 30 mM NaCl was most effective in separating plasma membrane with high purity. In the present study, the recovery of plasma membrane from green rye seedlings was relatively low in the same phase partition system as described above (data not shown). Thus, the selection of suitable conditions for the phase partition

FIG. 9. Electron micrographs of intact cells and isolated membrane fractions from light-grown winter rye seedlings. A, B: Intact parenchymal cells stained with uranyl acetate and lead citrate (A) and with PTA-CrO$_3$ (B). Cell components such as cell wall (CW), endoplasmic reticulum (ER), mitochondria (MT), chloroplasts (CH), vacuoles (V), and plasma membrane (PM) were uniformly stained with uranyl acetate-lead citrate. After PTA-CrO$_3$ staining, the plasma membranes and a portion of the thylakoid membranes were stained. C, D: Plasma membrane fraction (U$_2$), after batch procedure. Membranes were stained with uranyl acetate-lead citrate (C) and with PTA-CrO$_3$ (D). Vesicles were mostly observed in a smooth form, with a high proportion of PTA-CrO$_3$-stained vesicles. E, F: Microsomal membranes partitioned into lower phases, L$_1$ + U$_1$L, after batch procedure. Membranes were stained with uranyl acetate-lead citrate (E), and with PTA-CrO$_3$ (F). Various types of membranes were found in this fraction. Fragmented chloroplasts containing thylakoid membranes were dominantly observed but none of them was stained with PTA-CrO$_3$. PTA-CrO$_3$-positive-stained vesicles or membrane fragments are only scarcely observed. G, H: Crude mitochondrial membranes (1,500–14,000g pellet). Membranes were stained with uranyl acetate-lead citrate (G) and with PTA-CrO$_3$ (H). Membranes were mostly negative to PTA-CrO$_3$ stain. Bars represent 1 μm. Experimental details were described under "Materials and Methods."
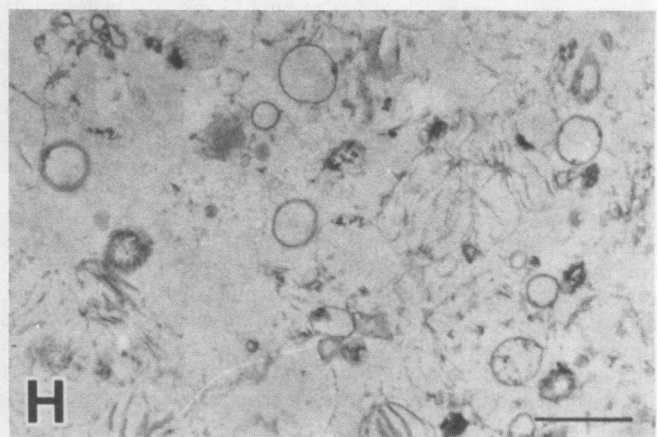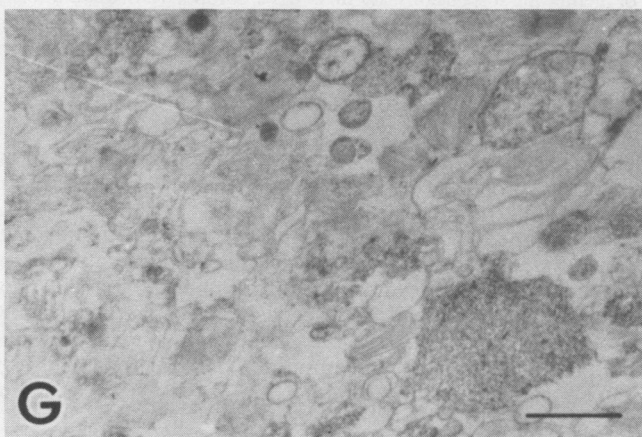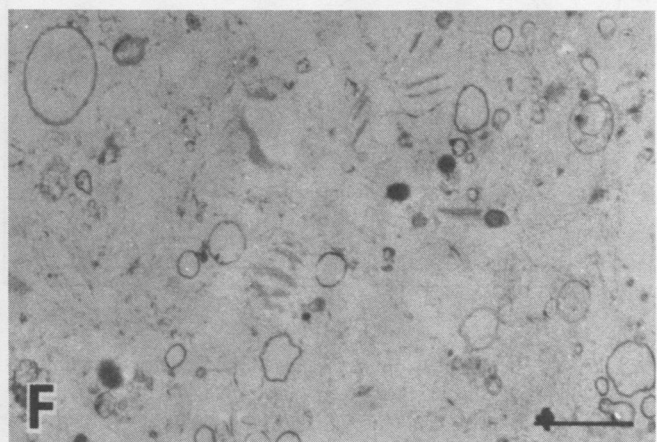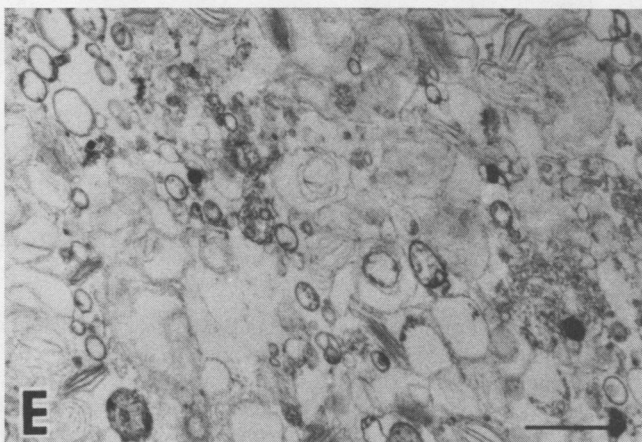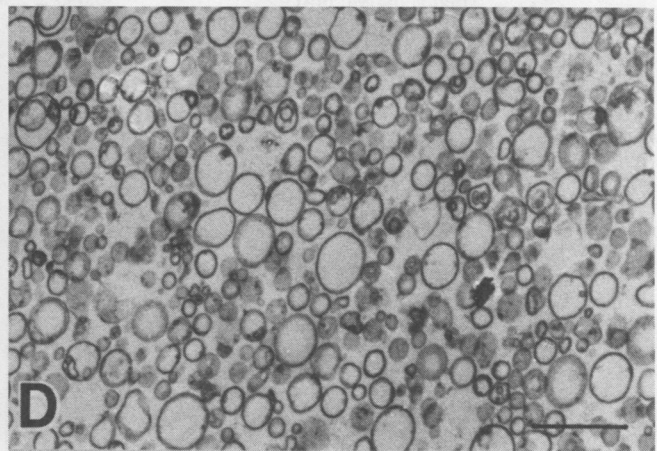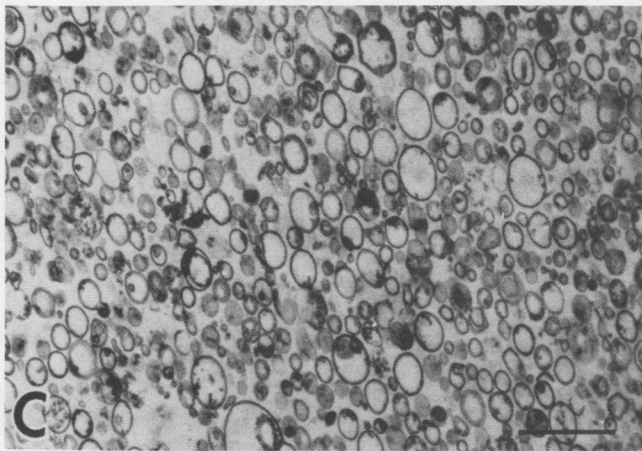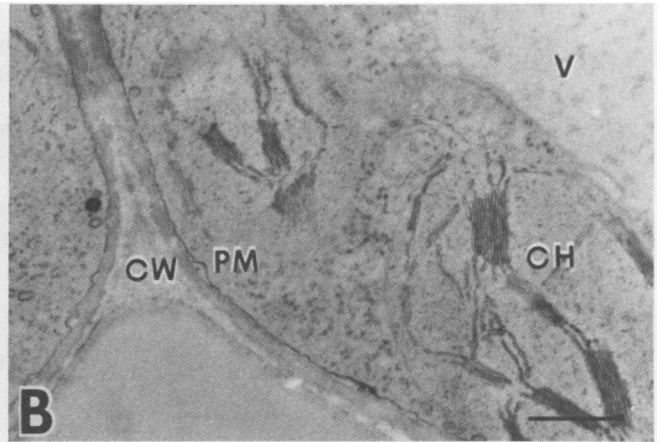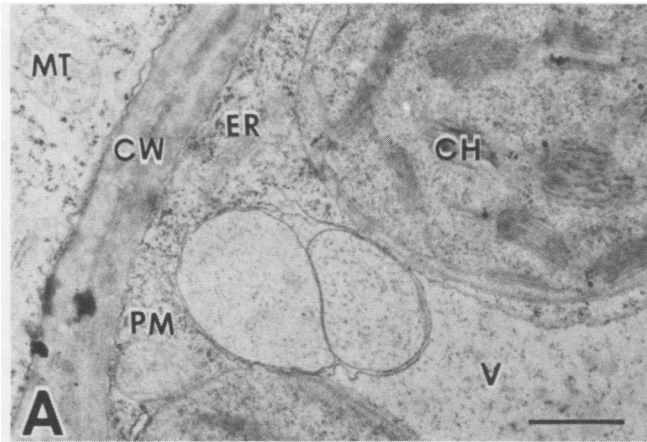
Table IV. *Distribution of Specific NPA-Binding Membranes and Protein between Cell Fractions from Winter Rye Seedlings*

Twenty g of tissues were used in each experiment. Specific binding activity was determined as the difference between the total bound radioactivity and nonspecifically bound radioactivity. Total specific binding and specific binding on mg protein basis were represented. Two different experiments are shown and each experiment was replicated three times. Values are shown as the average ± SD.

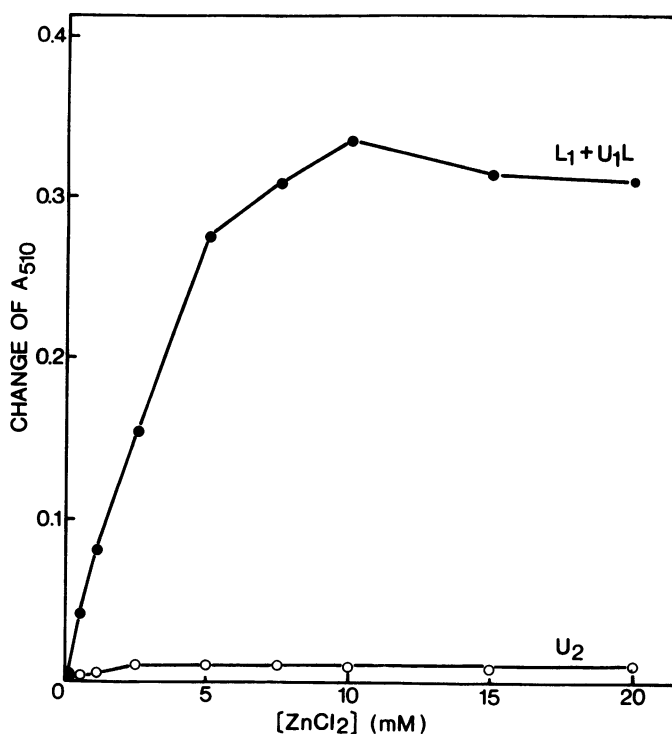|	14,000g Pellet (Crude Mitochondria)	156,000g Pellet	
		Lower phase ($L_1 + U_1L$)	Upper phase (U_2)
Exp. 1			
Protein (mg)	44.95	6.82	1.28
Total activity (cpm)	60,143 ± 18,562	17,159 ± 1,360	33,156 ± 1,282
cpm/mg protein	1,338 ± 413	2,516 ± 200	25,903 ± 1,001
Exp. 2			
Protein (mg)	33.71	5.38	1.03
Total activity (cpm)	30,035 ± 2,865	9,431 ± 216	30,303 ± 1,072
cpm/mg protein	891 ± 85	1,753 ± 40	29,418 ± 1,039

FIG. 10. Effect of $ZnCl_2$ on the aggregation of plasma membranes and endomembranes isolated from the microsomal fraction of rye seedlings. The plasma membranes (U_2) and the endomembranes ($L_1 + U_1L$) were isolated using the batch procedure as described in the text. Each membrane was suspended in 0.5 M sorbitol-15 mM Tris-maleate (pH 7.3). To 0.1-ml aliquot of membrane suspensions, 1.9 ml of $ZnCl_2$ solution in 0.5 M sorbitol-15 mM Tris-maleate (pH 7.3) at a desired concentration were added. The mixture was incubated at 20°C for 30 min before measuring the A at 510 nm to monitor the degree of membrane aggregation. The initial absorbance of each membrane suspension without the addition of $ZnCl_2$ was adjusted at A_{510} = 0.25 and taken as 0.

system is an important factor in adapting the method for various plant materials. In our system, selection of the buffer systems to be used for the phase partition, *i.e.* Tris-maleate or K-phosphate, was the only variable factor for isolating plasma membranes from a wide variety of plants. We have already succeeded in isolating plasma membranes from light-grown green seedlings of winter wheat and timothy, Jerusalem artichoke tubers, and living
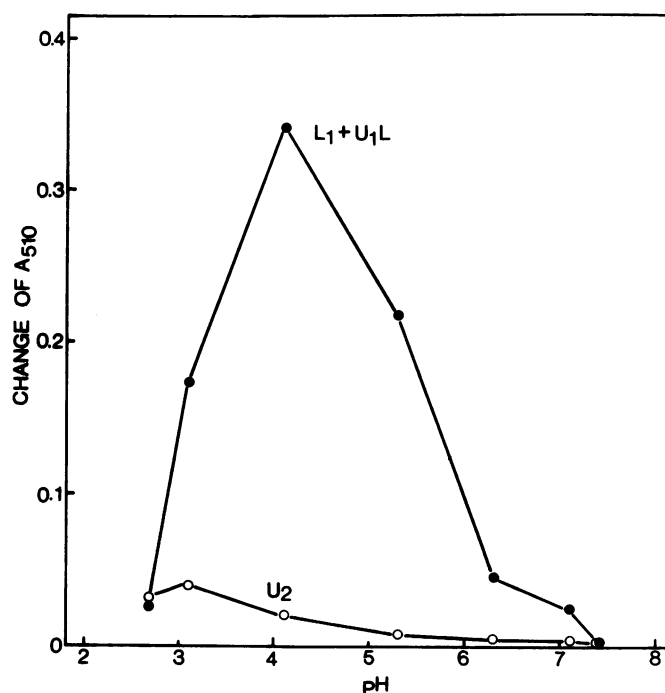
FIG. 11. Effect of pH on the aggregation of plasma membranes and endomembranes isolated from the microsomal fraction of rye seedling. The plasma membranes (U_2) and the endomembranes ($L_1 + U_1L$) were isolated as described in Figure 10. Each membrane pellet was suspended in 0.5 M sorbitol-1 mM sodium citrate buffer (pH 7.3). To 0.1-ml aliquot of membrane suspensions, 1.9 ml of 0.5 M sorbitol-10 mM sodium citrate buffer at the indicated pH were added and incubated at 20°C for 30 min before measuring the A at 510 nm to monitor the degree of membrane aggregation. The initial absorbance of each membrane suspension at pH 7.3 was adjusted at A_{510} = 0.25 and taken as 0.

bark cells of black locust trees and mulberry trees by utilizing the same phase partition system as used in the present study (manuscript in preparation).

As reported in the previous paper (33), the homogenizing process is also critical. We tried to control degradative enzyme activities involved in plant cells, such as phospholipase D, phenol oxidase, proteases, and lipoxygenase during the homogenizing process. For this purpose, we used a homogenizing medium containing a relatively high amount of defatted BSA (0.5-1.0%), EGTA as specific chelator for Ca, 1 mM PMSF, and 4 mM
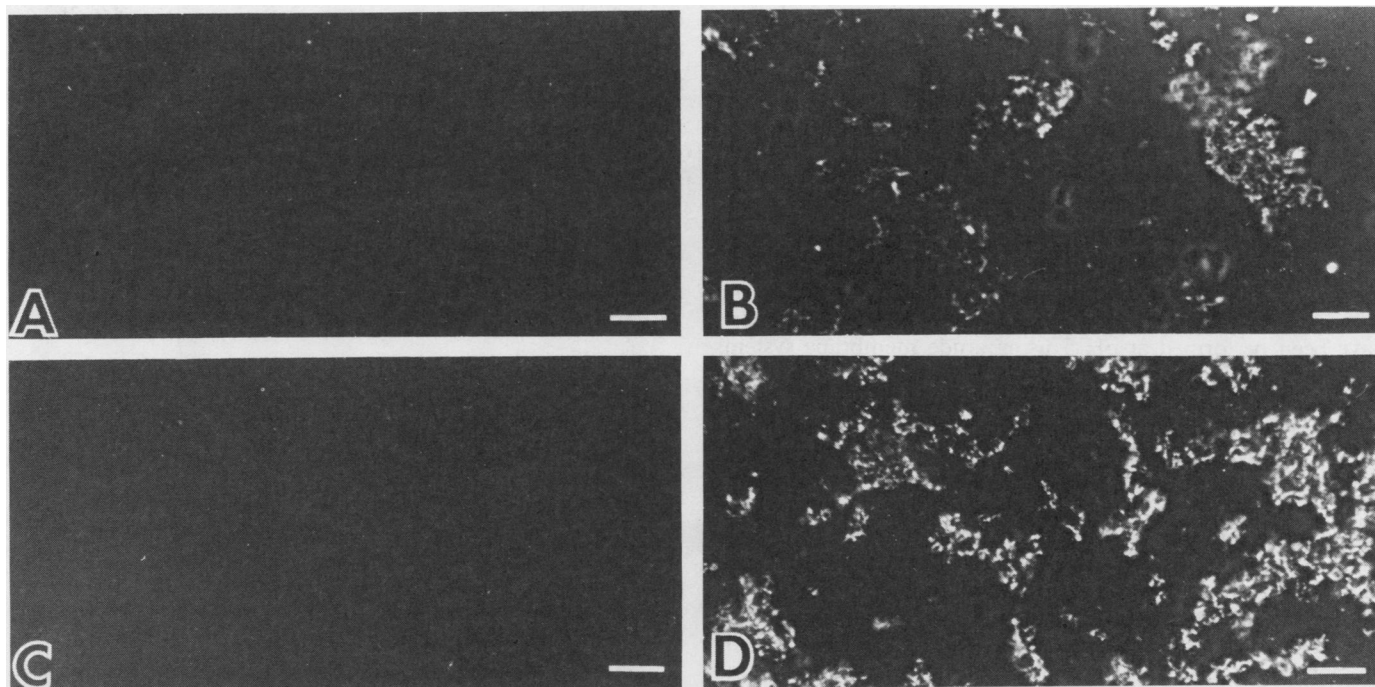
FIG. 12. Phase-contrast microscopic photographs demonstrating the effect of ZnCl₂ and low pH on the aggregation of the membranes. A, B: Effect of ZnCl₂ (10 mM) on the aggregation of plasma membranes, U_2 (A) and the endomembranes, $L_1 + U_1L$ (B). C, D: Effect of low pH (pH 4.2) on the aggregation of plasma membranes, U_2 (C) and the endomembranes, $L_1 + U_1L$ (D). Experimental details are described under "Materials and Methods." Bars represent 10 μm.

SHAM. We also used 2.5 mM potassium metabisulfite instead of DTT as the reducing chemical to protect membranes from oxidative reactions. The phenolics were neutralized with 5% soluble PVP during homogenization and, further, the remaining phenolics were effectively removed from the homogenates by passing through an Amberlite XAD-4 column (5 × 13 cm). In our preliminary experiments, we used a rather simple homogenizing medium consisting of 0.5 M sorbitol, 50 mM MOPS-KOH (pH 7.6), 5 mM EDTA, 5 mM DTT, and 5% PVP, but failed to achieve any specific partition of plasma membrane from rye plants (data not shown).

A sucrose density gradient may be utilizable for plasma membrane preparation from tender and non-chlorophyllous tissues only after a gentle homogenization procedure. In the present study, however, an equilibrated sucrose density gradient was of no use for separating plasma membrane from light-grown rye seedlings which required a harsh procedure to be disrupted. The second peak of the pH 6.5 ATPase activity at fraction 20 (equivalent to 1.141 g/cm³), although enriched in plasma membrane, was heavily contaminated with fragmented chloroplast membranes and mitochondrial membranes (Fig. 2).

In rye cells, it seems likely that there exist at least three different types of membranes associated with ATPases having nearly the same optimum pH around 6.5 (manuscript in preparation). One of the ATPase-associated membranes is co-sedimented with mitochondria after a differential centrifugation at 14,000g (type I). It was co-banded with mitochondria and fragmented chloroplast membranes on a linear sucrose density gradient (Fig. 1). The high proportion of the total ATPase activity in the crude mitochondrial fraction is mainly ascribable to this ATPase-associated membrane (type I). The second one (type II) is co-sedimented with plasma membrane (type III) in the microsomal fraction after a differential centrifugation at 156,000g. The type II membrane equilibrated at lighter density (1.100–1.105 g/cm³) on a linear sucrose density gradient. The total ATPase activity of the type II membrane was quite high in comparison with that of the

plasma membrane equilibrated at 1.141 g/cm³ (Fig. 2). Based on these facts, more than 65% of the total ATPase activity in the total particulate fraction of rye cells may be due to the activity of the types I and II ATPases other than plasma membrane ATPase (type III). These type I and II ATPases were quite different in their specificities both for the substrate and the divalent cation from those of plasma membrane ATPase. The former two showed no specificity for the substrate and for Mg²⁺, whereas plasma membrane ATPase was highly specific for the substrate and Mg²⁺ ion. Vanadate and diethylstilbestrol reported as inhibitors for plant plasma membrane-ATPase (3, 7) exhibited only partial inhibition for the ATPases for type I and II membranes, whereas the plasma membrane ATPase was significantly inhibited by these chemicals. The KCl stimulation was observed to be quite low in every ATPase from those three types of membranes (manuscript in preparation).

In the present study, plasma membrane was identified by the combination of the PTA-CrO₃ stain and the specific binding of NPA. The plasma membrane in situ in intact cells and also the separated membrane vesicles were positively stained with the reagent. Taylor and Hall (26) reported that silicotungstate-CrO₃(STA-CrO₃) stained chloroplast grana lamellae not only in situ but also in the isolated membranes. In our present study, the chloroplast, once disrupted and isolated, became inaccessible to the PTA-CrO₃ stain. Although we can not explain the reason at the moment, the PTA-CrO₃ stain should be a useful tool for identifying isolated plasma membrane vesicles. In the present study, fortunately, the plasma membrane isolated by means of phase partition is essentially devoid of any contamination by chloroplast fragments.

As reported (10, 11, 14, 16), the NPA binding site appears to be specifically located on plasma membranes. The specific binding capacity of the isolated plasma membrane on protein basis was 10-fold higher than that of the other membranes, such as the lower phase membrane fraction and the crude mitochondrial fraction (Table IV). The crude mitochondrial fraction, however,

contained a relatively high amount of total specific NPA-binding activity, indicating the co-existence of plasma membrane. After a phase partition of the crude mitochondrial fraction, plasma membrane was recovered in the upper phase (Table III). The recovery of plasma membrane, however, was relatively low. Presumably, the co-existed plasma membrane in the crude mitochondrial fraction may have been mostly in a fragmented form or in inside-out large vesicles which would be preferentially partitioned into the lower phase with endomembranes.

Thompson et al. (20) reported that the optimum pH for the specific binding in the plasma membrane-enriched fraction from corn coleoptiles was pH 5. In prior works, NPA binding tests have been performed at pH 5 using crude membrane systems from various plant materials (10, 14). However, no information has been presented so far concerning the optimum pH for purified plasma membranes. In the present study, the optimum pH for the specific binding of NPA to plasma membrane from rye seedlings was observed to be around pH 4 (Fig. 8). Narayanan et al. (18, 19) reported that the optimum pH for auxin binding of a plasma membrane-enriched fraction was well below pH 4.5. Thus, the optimum pH for auxin per se or of structural analogs such as NPA may be variable, depending on the plant materials. In the present study, however, the NPA binding assay was performed at pH 5 to prevent the deleterious effect of a low pH on membranes, especially on endomembranes which were found to be highly sensitive to pH values below 5.

Other distinctive features of plasma membranes isolated from rye seedlings appeared in their sensitivities to Zn^{2+} ion and low pH. Addition of Zn^{2+} ion or lowering the pH below pH 5 resulted in an abrupt and dramatic aggregation both of crude mitochondrial membranes and the lower-phase membranes after a phase partition of the microsomal fraction. On the other hand, no aggregation was detected in the isolated plasma membrane vesicles with either treatment. The Zn-method for isolation of pure plasma membrane with a large sheet form from cultured chicken embryo fibroblasts has been reported by Warren et al. (29). It was also reported that FMA (fluorescein mercuric acetate) is effective for the isolation of plasma membrane (30). Zn^{2+}, Hg^{2+}, and Cd^{2+} ions belong to group II-b in the periodic table and these divalent cations were highly specific for the aggregation of membrane other than the plasma membranes of plant cells. Warren et al. (29) suggested that these divalent cations might have a stabilizing effect on the structure of plasma membrane. Barland et al. (4) noted that Zn^{2+} ion would be capable of reacting with a wide variety of ligands in the cells. Although the detailed role of Zn^{2+} ion on plant membranes is obscure now, there may exist a specific site for Zn^{2+} binding in membranes other than plasma membrane and vice versa. The specific effect of Zn^{2+} ion was also conformed in the plasma membranes isolated from a wide variety of plant materials such as living bark cells of black locust trees and mulberry trees, orchard grasses, winter wheats, and Jerusalem artichoke tubers (data not shown).

Fuhrmann et al. (8) reported that there exists a marked difference in the sensitivity to low pH between plasma membrane and mitochondria prepared from yeast cells. When the pH of the membrane suspensions was lowered to 4.0, a drastic aggregation was observed in mitochondria, whereas no aggregation was observed in the plasma membrane vesicles. These differences in the low pH sensitivity of the two membranes coincided with the differences in their isoelectric points, i.e. pH 3 for plasma membrane and pH 4.5 for mitochondria. In the present study, the plasma membrane vesicles showed only slight aggregation below pH 4.0; on the other hand, the other membranes such as crude mitochondrial membranes and the membranes recovered from the lower phase showed a drastic aggregation even at pH 5.0. Below pH 4.0, the aggregated membranes formed large clusters and were easily sedimented at $1g$. These characteristic features

of membranes either at low pH or in the presence of Zn^{2+} ion are easily observable under a phase-contrast microscope (Fig. 12) and are utilizable for the assessment of the purity of plasma membranes isolated from various plant materials.

Biochemical analysis of plasma membrane isolated from cold-acclimated winter rye seedlings is now in progress in our laboratory.

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