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Isolation of Highly Purified Fractions of Plasma Membrane and Tonoplast from the Same Homogenate of Soybean Hypocotyls by Free-Flow Electrophoresis[1]

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ABSTRACT

A procedure is described whereby highly purified fractions of plasma membrane and tonoplast were isolated from hypocotyls of dark-grown soybean (*Glycine max* L. var Wayne) by the technique of preparative free-flow electrophoresis. Fractions migrating the slowest toward the anode were enriched in thick (10 nanometers) membranes identified as plasma membranes based on ability to bind *N*-1-naphthylphthalamic acid (NPA), glucan synthetase-II, and K^+-stimulated, vanadate-inhibited Mg^{2+} ATPase, reaction with phosphotungstic acid at low pH on electron microscope sections, and morphological evaluations. Fractions migrating farthest toward the anode (farthest from the point of sample injection) were enriched in membrane vesicles with thick (7–9 nanometers) membranes that did not stain with phosphotungstic acid at low pH, contained a nitrate-inhibited, Cl-stimulated ATPase and had the *in situ* morphological characteristics of tonoplast including the presence of flocculent contents. These vesicles neither bound NPA nor contained levels of glucan synthetase II above background. Other membranous cell components such as dictyosomes (fucosyltransferase, latent nucleosidediphosphate phosphatase), endoplasmic reticulum vesicles (NADH- and NADPH-cytochrome *c* reductase), mitochondria (succinate-2(*p*-indophenyl)-3-*p*-nitrophenyl)-5-phenyl tetrazolium-reductase and cytochrome oxidase) and plastids (carotenoids and monogalactosyl diglyceride synthetase) were identified on the basis of appropriate marker constituents and, except for plastid thylakoids, had thin (<7 nanometers) membranes. They were located in the fractions intermediate between plasma membrane and tonoplast after free-flow electrophoretic separation and did not contaminate either the plasma membrane or the tonoplast fraction as determined from marker activities. From electron microscope morphometry (using both membrane measurements and staining with phosphotungstic acid at low pH) and analysis of marker enzymes, both plasma membrane and tonoplast fractions were estimated to be about 90% pure. Neither fraction appeared to be contaminated by the other by more than 3%.

With elongating plant cells, the total membrane surface contributed by tonoplast surrounding the central vacuole and the plasma membrane of the cell surface are nearly equal (20). Studies of membrane dynamics in plants during the growth process, as well as various transport and assignment studies would be facilitated if procedures for simultaneous isolation of these two membrane components from the same homogenates were available.

In initial reports for isolation of plasma membranes from plant cells (6, 11), identification was based on the use of phosphotungstic acid at low pH to specifically and characteristically stain plasma membrane vesicles in electron microscope sections of fixed and embedded pellets of membrane fractions (27). Numerous reports followed with purification primarily by sucrose gradients (see Refs. 8–10, for review) or by aqueous two phase systems consisting of Dextran T 500 and PEG 3350 (13). Other techniques such as isoelectric focusing also have been applied (4).

Two enzyme markers including a K^+-stimulated, vanadate-inhibited increment in Mg^{2+}-ATPase activity (8) and a glucan synthetase exhibiting a high K_m for UDP-glucose (33), the so-called glucan synthetase II activity (26) have been associated with plasma membranes of plants.

Approaches to the isolation of vacuoles and/or tonoplast membranes have been through the use of low-shear tissue slicing methods or preparation of protoplasts followed by controlled osmotic lysis, mechanical breakage, or by rupture by treatment with polybases (32, 34). A nitrate-inhibited, anion-stimulated Mg^{2+} -ATPase was assigned to the tonoplast as a marker based on studies with latex vacuoles of *Hevea brazilianensis* (17) and using flotation on dextran gradients (32).

Despite this considerable progress, neither preparations of plasma membrane nor of tonoplast have been demonstrated unequivocally to be free of contamination of one by the other. More important from the standpoint of studies of membrane dynamics and biogenesis, is the need to obtain isolated plasma membrane and tonoplast fractions of high degree of purity from the same homogenate. The latter would greatly facilitate direct comparisons in short turn labeling and turnover studies, for example.

The present report describes the application of preparative free-flow electrophoresis (5) to the isolation of plasma membrane and tonoplast fractions from the same homogenate in good yield and excellent fraction purity. In this procedure, a resuspended crude fraction enriched in plasma membrane plus tonoplast was introduced as a fine jet into a separation buffer moving perpendicular to the field lines of an electric field. Membrane vesicles

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bearing different charge-densities migrated at different velocities across the separation chamber and thus were resolved.

Identification of both plasma membrane and tonoplast was based on three criteria: (a) independent morphological identification based on thickness of membranes and staining with phosphotungstic acid at low pH that could be related unequivocally to the positional relationships of these same membranes in the intact cell (*cf.* 25), (b) confirmation by currently available and accepted biochemical markers for both plasma membrane and tonoplast of plants, and (c) by the absence of established markers for Golgi apparatus, mitochondria, plastids, and ER. At least two biochemical markers plus morphological evaluation were utilized for each cell component, such that assignment can now be made for membranes from plant cells with a degree of confidence equal to or exceeding that possible a few years ago only with membrane fractions from rat liver (18).

MATERIALS AND METHODS

Plant Material. Seeds of soybean (*Glycine max* [L.] Merr., var Wayne) were soaked 4 to 6 h in deionized H_2O, planted in moist vermiculite, and grown 4 to 5 d in darkness. Two cm long segments, cut 5 mm below the cotyledons, were harvested under normal laboratory light conditions and used for the isolation of membranes.

Membrane Isolation. Hypocotyl segments (35–50 g) were vacuum infiltrated for 5 min in homogenization medium (0.3 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 25 mM Tris/Mes [pH 7.5]). Ten to 12 g portions were homogenized in 10 ml of fresh ice-cold chopping medium, with or without the addition of 1 mM PMSF[3] and 0.1% (w/v) BSA, using a mechanized razor blade chopper. The homogenates were filtered through one layer of Miracloth (Chicopee Mills, New York) and rinsed with 10 ml of homogenization medium each. The combined filtrates were centrifuged for 10 min at 6,000g_{max} (6,000 rpm, Sorvall, HB-4 rotor), and the resulting supernatant was centrifuged for 30 min at 40,000g_{max} (Beckman, SW-28 rotor). The pellets (40,000g_{max}) were resuspended in electrophoresis chamber buffer (see below), combined, and again centrifuged for 30 min at 40,000g_{max} (Beckman, SW-28 rotor). The resulting pellets was resuspended in electrophoresis chamber buffer, using approximately 1 ml per 10 g starting fresh weight of hypocotyls used.

Free Flow Electrophoresis. The electrophoresis medium (electrophoresis chamber buffer) contained 0.25 M sucrose, 2 mM KCl, 10 μM $CaCl_2$, 10 mM triethanolamine, and 10 mM acetic acid (pH 7.5) (NaOH). The electrode buffer contained 100 mM triethanolamine and 100 mM acetic acid (pH 7.5) (NaOH). The electrophoresis equipment was a VaP-5 continuous free-flow electrophoresis unit (Bender and Hobein, Munich, FRG). The conditions for the electrophoretic separations were as follows. The separations were carried out under constant voltage 800 V/9.2 cm field, 165 ± 5 mamp, buffer flow 1.7 ml/fraction·h, sample injection 2.7 ml/h, and constant temperature of 6°C. The distribution of membranes in each separation was monitored from the *A* at 280 nm. The membranes were collected from individual fractions or from pooled fractions by centrifugation for 30 min at 110,000g_{max} (Beckman, SW-28 rotor) and fixed directly for EM or resuspended in appropriate buffer for assays of marker enzymes.

Electron Microscopy. Membrane fractions or whole tissue were fixed in a mixture of 2% buffered glutaraldehyde + 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.3) for 2 h at 4°C (3). This fixation procedure accentuated morphological differences between tonoplast and plasma membrane and between these two membranes and other endomembranes to permit their recognition and resolution in isolated fractions. The material was rinsed in the same buffer, dehydrated through a graded acetone or ethanol series, and embedded in Luft's Epon. Thin sections were post-stained with uranyl acetate or with alkaline lead citrate.

Phosphotungstate at Low pH to Stain Plasma Membrane. Thin sections of fractions or whole tissues prepared for EM as above were collected from a water surface with a plastic loop and transferred to nickel grids. To stain the plasma membrane, sections were first transferred to 1% aqueous periodic acid for 30 min to completely destain the sections. This was followed by five changes of distilled H_2O for 10 min each and treatment for 5 min with 1% phosphotungstic acid in 10% chromic acid (27). After 5 washes of 10 min each with distilled H_2O, the sections were transferred from the plastic loop to parlodion-coated-copper grids. Specimens were viewed and photographed with a Philips EM 200 or EM 300 electron microscope.

Electron Microscope Morphometry. Measurements were on negatives photographed at a primary magnification of 50,000 and enlarged 20 times with a calibrated enlarging projector to give a final magnification of 1,000,000 times so that the measured membrane diameters were in the range of 6 to 12 mm. Measurements of the magnified images were made between the outer edges of the stained portions of the membrane profiles to the nearest 0.5 mm with a transparent ruler held perpendicular to the tangent of the vesicular profiles.

Three to four electron micrographs from each of three different preparations were measured. Ten measurements were taken from each vesicle where the images were sharp and the vesicle membrane was nearly perpendicular to the plane of the section. Factors, including bias, that may affect measurement had been evaluated previously in another study (19) and were taken into consideration in the present evaluations. A minimum of 300 measurements were averaged for each determination.

Binding of *N*-1-Naphthylphthalamic Acid. The binding assay described by Lembi *et al.* (15) as modified by Ray (26) was used. The membranes from the electrophoretic separations were suspended in 10 mM sodium citrate (pH 5.5) containing 250 mM sucrose and 5 mM $MgCl_2$. Two series of determinations were carried out in parallel. One series (*A*) contained 0.1 μCi [^{3}H]-NPA plus 10 μl ethanol and the second series (*B*) contained 0.1 μCi [^{3}H]NPA plus 0.1 mM unlabeled NPA added in 10 ml ethanol. The tubes were mixed by inversion, incubated at 4°C for 30 min, and centrifuged for 15 min at 25,000*g* (Sorvall SS34 rotor) to pellet the membranes. Supernatants were decanted and the tubes inverted to drain. For determination of radioactivity, the bottoms of the tubes were cut off 1 mm above the pellets and transferred to counting vials. Specific radioactivity was taken as the radioactivity from tube A minus that from tube B.

Glucan Synthetase II. The assays were as described (26). Fifty μl of membrane fraction resuspended in 0.25 M sucrose, 10 mM KCl, 25 mM Tris/Mes (pH 8.0), were added to 50 μl of the same buffer containing UDP-glucose and cellobiose, giving the final concentrations of 0.5 mM UDP-glucose (25 μCi UDP-[U-^{14}C]-glucose [ICN] added) and 0.15 mM cellobiose. The reactions were for 30 min at 25°C in Eppendorf tubes. The reactions were terminated with the addition of 25 μl 100 mM $MgCl_2$, 50 μl boiled microsomal membranes (approximately 0.5 mg protein), and 700 μl ethanol followed by boiling for 1 min. The tubes were left overnight at 4°C and then centrifuged for 2 min in an Eppendorf centrifuge. The supernatant was removed, and the pellets were washed 4 times with 70% ethanol. After the last wash, the tubes were drained, and the bottom parts were cut off with a razor blade and transferred to scintillation vials as de-

[3] Abbreviations: PMSF, phenylmethylsulfonyl fluoride; Bis-Tris propane, 1, 3-bis[tris(hydroxymethyl)methylamino]propane; IDP, inosine-5′-diphosphate; INT, 2-(*p*-indophenyl)-3-*p*-nitrophenyl)-5-phenyl tetrazolium; MGDG, monogalactosyldiglyceride; NPA, *N*-1-naphthylphthalamic acid.

scribed for NPA binding.

Sterolglucoside Synthesis. Assays were as described by Hartmann-Bouillon and Benveniste (7). Membrane fractions were suspended in 25 mM Tris/HCl (pH 8.0). The assay medium contained 0.1 μmol cholesterol, 8 mM $MgCl_2$, 0.08% (by volume) Triton X-100, 0.3 mM UDP-glucose (0.1 μCi [U-^{14}C]glucose, 304 mCi/mmol, Amersham), 50 mM Tris/HCl (pH 8.0) in a volume of 450 μl. The reaction was started by the addition of 50 μl of suspended membranes (50–150 μg proteins). The reactions were for 30 min at 30°C and were terminated by addition of 2 ml ice-cold chloroform-methanol 1:2 (v:v). After at least 30 min on ice, the lipids were extracted according to Bligh and Dyer (1). The radioactivity incorporated into organic solvent-soluble material was determined by liquid scintillation methods in ACS (Amersham).

Table I. *Membrane Dimensions in Soybean Tissue, Fixed Simultaneously with Glutaraldehyde and Osmium Tetroxide*

Cell Component	Membrane Thickness	Class
	nm ± SD	
Plasma membrane	10.1 ± 0.7	I
Tonoplast membrane	7.2 ± 0.8	II
Endoplasmic reticulum	5.7 ± 0.7	III
Nuclear envelope		
Outer membrane leaflet	5.8 ± 0.8	III
Inner membrane leaflet	6.5 ± 0.6	III
Mitochondria		
Outer membrane	5.0 ± 0.6	III
Inner membrane	6.2 ± 0.7	III
Etioplast		
Outer envelope membrane	5.0 ± 0.6	III
Inner envelope membrane	6.1 ± 0.8	III
Thylakoid	8.0 ± 1.0	II
Peroxisome	5.5 ± 0.6	III
Golgi apparatus	6–9	Mixed

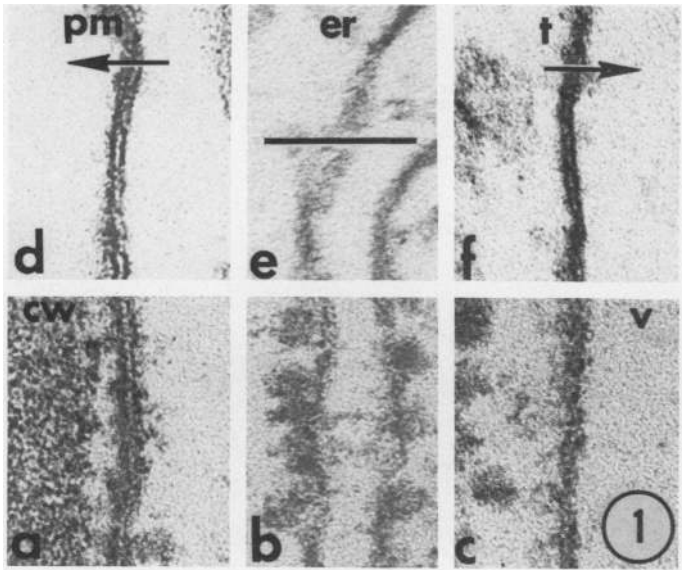

FIG. 1. Soybean membranes after fixation by the simultaneous glutaraldehyde plus osmium tetroxide method of Franke *et al.* (3) *in situ* (a–c) and as identified from free-flow electrophoresis fractions (d–f). a to c, Plasma membranes (pm), endoplasmic reticulum (er), and tonoplast (t) in the intact cells where membranes were identified from positional relationships relative to cell walls (cw) and the central vacuole (v), for example. Comparisons of these same membranes are shown in d to f with corresponding free-flow electrophoresis fractions. Plasma membrane (pm) is thick (about 10 nm) and shows the dark-light-dark pattern clearly. Tonoplast (t) also is thick but shows a less distinct dark-light-dark pattern. Endoplasmic reticulum (er), as representative of the class of thin membranes, is only about 6 nm thick and has the least distinct dark-light-dark pattern. In d and f, arrows point to the interiors of the vesicles from which membrane regions illustrated were derived. Scale bar = 0.1 μm.

Latent IDPase. The procedure for latent IDPase was modified from that of Morré *et al.* (21). Fractions were resuspended in 50 mM Tris-Mes (pH 6.5) containing 100 mM KCl and 5 mM $MgSO_4$. The assay medium contained, in a final volume of 1 ml, 50 mM Tris-Mes (pH 6.5), 100 mM KCl, 5 mM $MgSO_4$, and 2.5 mM IDP. Incubations were for 20 min at 37°C and were stopped by addition of 300 μl of 20% SDS and 3 ml 1% ammonium molybdate in 2 N H_2SO_4. Phosphate released was estimated by the procedure of Fiske and Subbarow. The latent activity was measured upon 4 d of storage at 4°C with subtraction of the activity measured in the same fraction freshly prepared.

Fucosyltransferase. To measure fucose transfer of endogenous membrane acceptors, reactions contained in a total volume of 0.2 ml, sugar nucleotide (0.03 μCi of GDP-[U-^{14}C]fucose, 217 mCi/mmol; New England Nuclear), $MnCl_2$ (1.0 μmol), 2-mercaptoethanol (1 μmol), Mes (10 μmol) (pH 6.5), Triton X-100 (0.3 mg) and protein (50–200 μg). Components were preincu-

Table II. *Composition of Free-Flow Electrophoresis Fractions Based on Morphometry*

Free-Flow Fraction	Percent of Membranes in Fraction			
	9–11 nm	7–9 nm	5–7 nm	PTA-stained
	profiles/100 profiles			
A	2 ± 3	88 ± 2	10 ± 4	1 ± 1
B	7 ± 8	55 ± 6	37 ± 19	9 ± 2
C	18 ± 9	18 ± 14	59 ± 30	19 ± 4
D	46 ± 12	11 ± 11	43 ± 22	33 ± 8
E	92 ± 6	4 ± 3	6 ± 5	97 ± 1

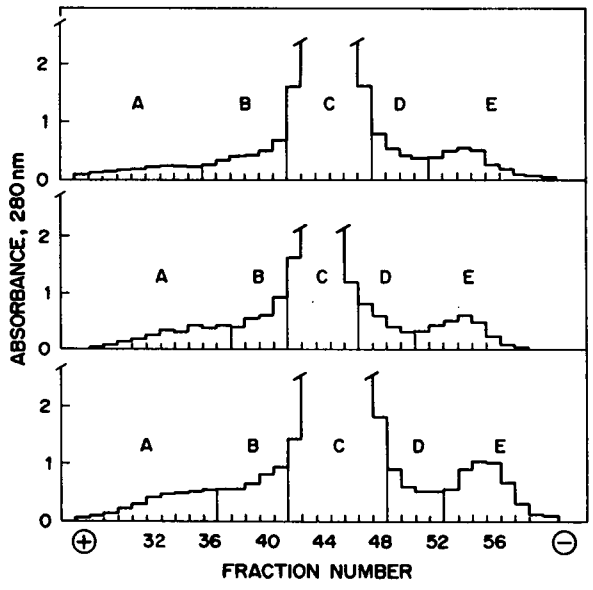

FIG. 2. The *A* at 280 nm of fractions from three representative free-flow electrophoresis separations. In all experiments, A_{280} was used only as a basis for pooling the fractions. For determinations of specific activities, proteins were estimated subsequently for each fraction in every experiment. A to E, pooled fractions. With the midpoint of the separation at fraction 45, the actual fractions sampled were A, 27–36; B, 37–41; C, 42–48, D, 49–52; E, 53–60. The composition of these fractions based on morphology and biochemical markers is summarized in Tables II and III.

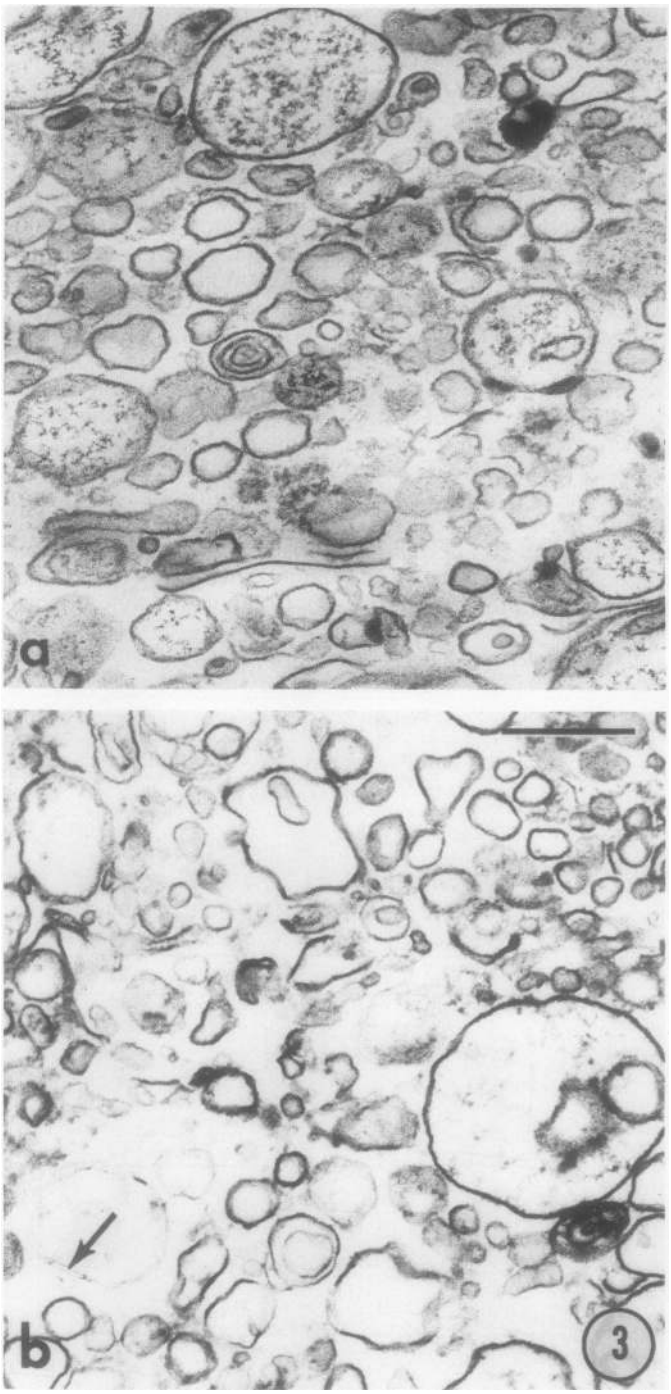

FIG. 3. Electron micrograph of fraction E (plasma membrane) from soybean. In a, the sections were conventionally stained with alkaline lead citrate. The bulk of the vesicles were bounded by membranes 9 to 11 nm in thickness (Table II) that stained with phosphotungstic acid (b). In b, the sections were destained and restained with phosphotungstic acid at low pH to accentuate plasma membranes (27). More than 95% of the membranes attracted the stain. Arrow denotes an unstained vesicle. Scale bar = 0.5 μm.

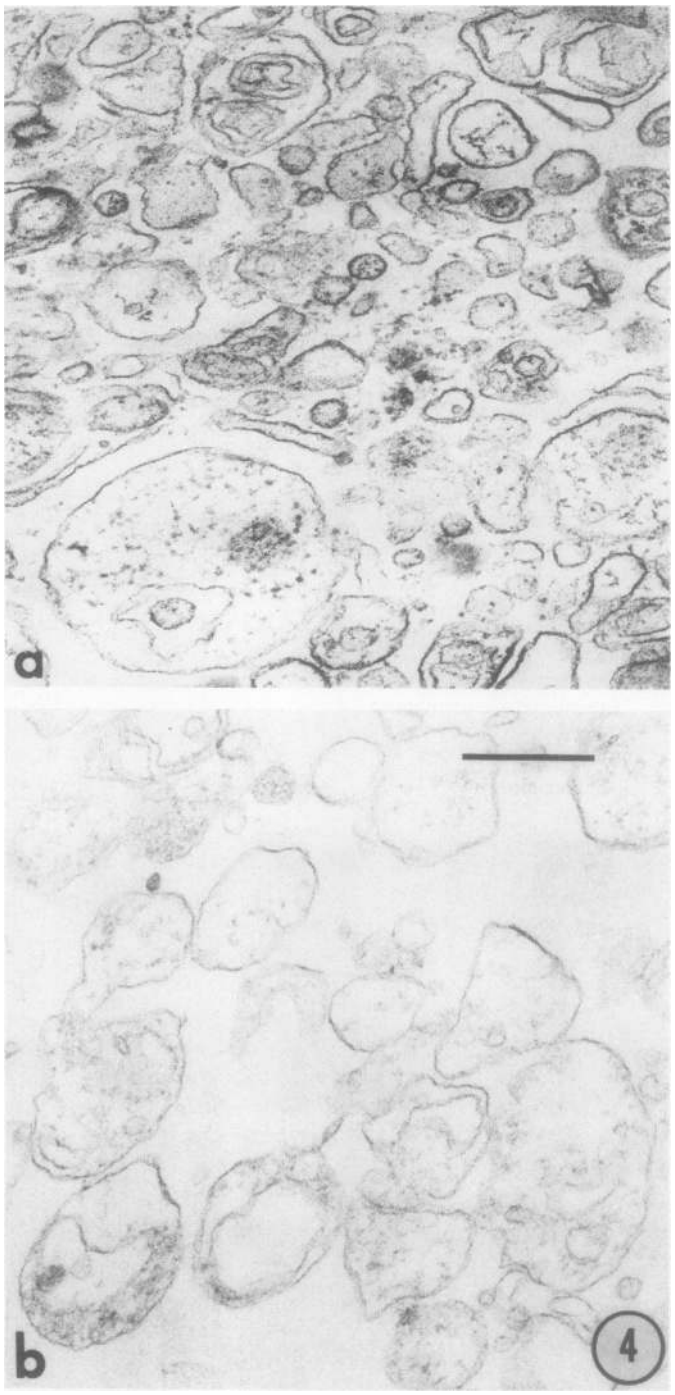

FIG. 4. Electron micrograph of fraction A (tonoplast) from soybean. In a, the sections were stained with alkaline lead citrate and the bulk of the vesicles were bounded by membranes 7 to 9 nm thick (Table II). The membranes did not stain with phosphotungstic acid (b). The procedure for staining with phosphotungstic acid was identical to that for Figure 3 and plasma membranes of tissue sections and from fraction E stained at the same time were reactive. Scale bar = 0.5 μm.

bated at 37°C in the presence of Triton, and the reactions were initiated with labeled substrate. After incubation at 37°C for 45 min, the reactions were terminated with 1 ml of cold 10% TCA. The precipitates were collected on glass fiber filters, using a multifiltration apparatus (Millipore). One ml wash of the incubation tube was filtered, and the total precipitate was washed sequentially with 6 ml of 5% TCA containing 0.5% unlabeled fucose, 4 ml ethanol/ether (1:1, v/v) and 4 ml diethylether. The filters were dried and radioactivity determined in a toluene-based scintillation fluid.

For glucan synthetase II, sterolglucoside synthesis, and fucosyl transferase, specific activities denote endogenous acceptor dependent activities corrected for bound radioactivity by means of

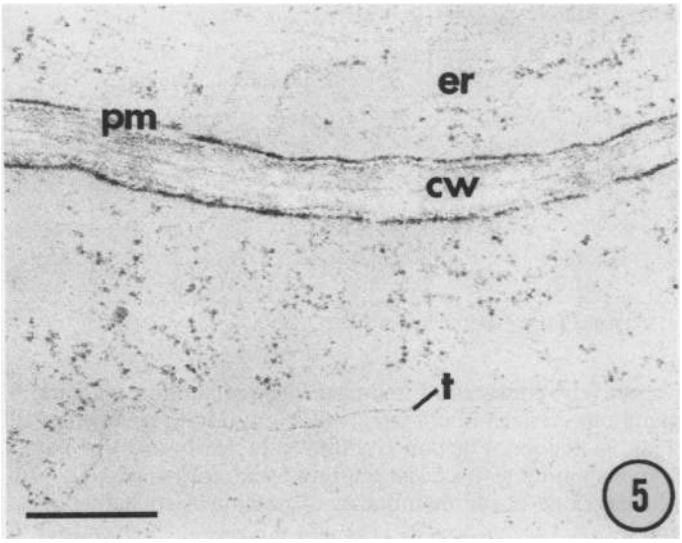

FIG. 5. Portion of a cortical cell of etiolated soybean hypocotyl segment fixed for EM using simultaneous osmium tetroxide plus glutaraldehyde (7) and then destained and restained with phosphotungstic acid at low pH according to Roland *et al.* (27). The specificity of the staining procedure is shown by plasma membrane (pm) being the only membrane structure that attracted stain. Tonoplast (t), ER, and other internal membranes were unstained. Ribosomes and cell walls (cw), while not membranous, did stain. Scale bar = 0.5 μm.

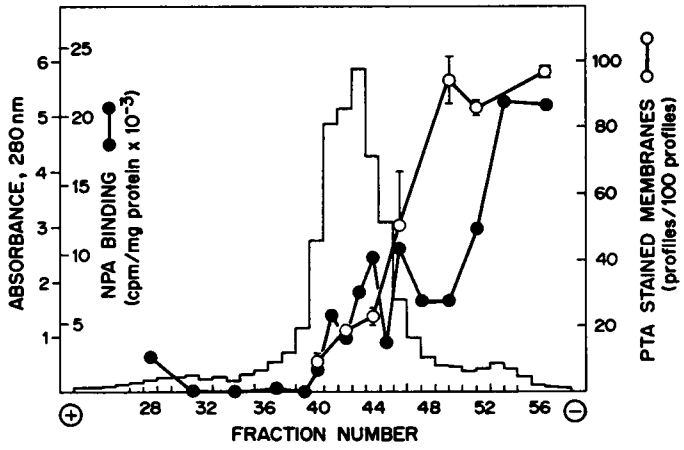

FIG. 6. Free-flow electrophoresis separation showing the location of two plasma membrane markers, binding of NPA (●) and reaction with phosphotungstic acid (PTA) at low pH (○). The deviation bars give the variation between three different sampling regions within the same pellet for PTA staining. The NPA binding was from a single electrophoretic separation. Values are for individual rather than pooled fractions. Additional data from pooled fractions are given in Table II for PTA staining and in Table III for NPA binding.

a blank containing all reaction components in which the reaction was terminated at time zero.

Other Marker Enzymes and Constituents. NADPH Cyt *c* reductase was measured according to Hodges and Leonard (10). Succinate-INT-reductase was estimated as described by Pennington *et al.* (see Ref. 20) but at 30°C and Cyt oxidase was as described by Sun and Crane (30).

Assays for carotenoids were as described by Hurkman *et al.* (12) and for activity of UDP-galactose diacylglycerol galactosyltransferase (MGDG synthetase) according to Sandelius and Selstam (28) except that only radioactive substrate was used. Protein determinations were by the Lowry *et al.* method with BSA as standard.

Gel Electrophoresis. Electrophoresis was on SDS-PAGE. Slab gels (11 × 14 × 0.3 cm) were electrophoresed at 25 to 30 mamp for 4 h (12.5% gels). Proteins were visualized by staining with Coomassie blue and destained through an isopropanol/acetic acid/water (120/40/340) series. The gels were fixed and dried using a Bio-Rad slab gel drier (model 1125B).

RESULTS

Membrane Measurements and Identification of Tonoplast. Based on measurements of membranes of tissues after simultaneous fixation with glutaraldehyde plus osmium tetroxide, the membranes of soybean hypocotyls fell into three classes based on thickness and reactivity with phosphotungstic acid at low pH (Table I; Fig. 1). Class I membranes were 10 nm thick, showed the dark-light-dark pattern most clearly, reacted with phosphotungstic acid at low pH, and consisted of plasma membrane and occasional elements of the Golgi apparatus derived from the mature face. Class II membranes were on the average 7.5 nm thick (range 6–9 nm), showed the dark-light-dark pattern poorly, and did not react with phosphotungstic acid at low pH. The principal membrane in this class was tonoplast although some Golgi apparatus membranes from the mature pole and thylakoid membranes of proplastids could not be distinguished from tonoplast on the basis of these criteria. In the etiolated soybean tissue prior to homogenization, thylakoid membranes accounted for an amount of membrane nearly equivalent to the inner envelope membrane or about 2% of the total membrane. Class III membranes were thinner than 7 nm, showed the dark-light-dark pattern little if at all, and did not react with phosphotungstic acid at low pH. Membranes in this category included all of the remaining internal membranes of the cell (nuclear envelope, ER, inner and outer mitochondrial membranes, inner and outer plastidal envelope membranes, peroxisome membranes, and Golgi apparatus membranes of the immature or forming face). These results summarized in Table I provided one approach to identification of plasma membrane and tonoplast independent of assumptions as to distributions of biochemical markers. The assignment (Table II) from initial identification based on positional relationships of the membranes within the tissue (Fig. 1) was subsequently confirmed and extended by findings based on analyses of biochemical markers (Table III).

Morphology of Starting Material. Homogenates were centrifuged for 10,000*g* for 10 min to remove the bulk of the mitochondria, intact plastids, nuclei, cell fragments, and debris. The 10,000*g* supernatant, then centrifuged for 30 min at 40,000g_{max} to concentrate smooth membrane vesicles, provided the starting material for the free-flow electrophoretic separations. The fractions consisted of 30% vesicles staining with phosphotungstic acid at low pH and identified as plasma membrane. Of the total membranes, 31% was identified as tonoplast based on measurements of membrane thickness following simultaneous glutaraldehyde-osmium tetroxide fixation.

Distribution of Membrane Protein Within Free-Flow Separations. Measurements of *A* at 280 nm for each individual electrophoretic separation revealed a broad distribution with two shoulders, one toward the anode and one toward the cathode (Fig. 2). The distribution was qualitatively reproducible, but the midpoint of the fractionation did vary one to several free-flow electrophoresis fractions among different separations. From a total of more than 60 separations none deviated from the general pattern as illustrated in the figures. Either all fractions (with some minor fractions combined) of a separation were analyzed, or the fractions of a free-flow electrophoretic separation were pooled into five fractions (A–E) following measurement of *A* at 280 nm (Fig. 2). From starting material of 35 to 50 g of soybean hypocotyls, fraction A (tonoplast) contained 0.8 to 1.0 mg membrane protein

Table III. *Specific Activities and Distribution of Total Activities of Marker Enzymes Among Free-Flow Fractions*

Fraction	Glucan Synthetase II		Sterol Glucoside Synthesis		NADPH Cyt *c* Reductase		Succinate-INT Reductase		Latent IDPase		MGDG Synthetase		NPA Binding		Protein
	pmol/ μg·h	%[a] *(n = 3)*	*pmol/ μg·h*	% *(n = 3)*	*pmol/ μg·h*	% *(n = 3)*	*pmol/ μg·h*	% *(n = 3)*	*N mol/ μg·h*	*(%) (n = 3)*	*cpm/ μg·h*	%	*cpm/ μg*	*(%) (n = 2)*	% *(n = 8)*
A	5	0.8 ± 0.3	10	9 ± 1	0.5	0.8 ± 0.5	204	1 ± 1	3.0	(3 ± 3)	144	1	3.0	2 ± 2	7 ± 2
B	4	3 ± 2	7	14 ± 9	2.3	2 ± 1	352	3 ± 1	15.0	(17 ± 9)	372	4	0.0	1 ± 1	8 ± 2
C	14	39 ± 13	2	21 ± 17	4.5	89 ± 1	1436	92 ± 2	7.0	(78 ± 11)	881	89	9.4	46 ± 5	71 ± 4
D	37	25 ± 14	7	7 ± 4	4.4	4 ± 1	284	2 ± 1	0.2	(0.2 ± 0.2)	321	4	16.0	9 ± 5	6 ± 2
E	37	32 ± 1	20	50 ± 6	2.3	4 ± 0.3	48	1 ± 1	0.0	(0 ± 0)	272	2	37.0	40 ± 8	8 ± 2

[a] The percent is the distribution of total activity (specific activity × protein) averaged for *n* experiments ± SD.

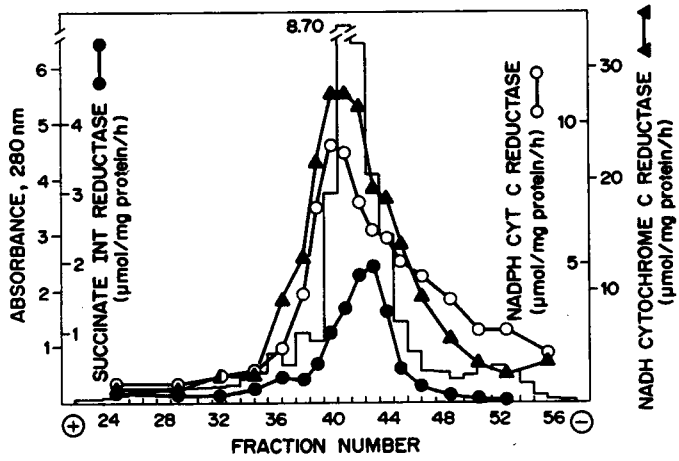

FIG. 7. Free-flow electrophoresis separation showing the location of succinate INT reductase (●), a mitochondrial marker, and of NADPH Cyt *c* reductase (○), an ER marker, as well as NADH-Cyt *c* reductase (▲). The separations given are representative and comparable. Each of these markers has been measured on several electrophoretic separations. Additional data from pooled fractions are given in Table III.

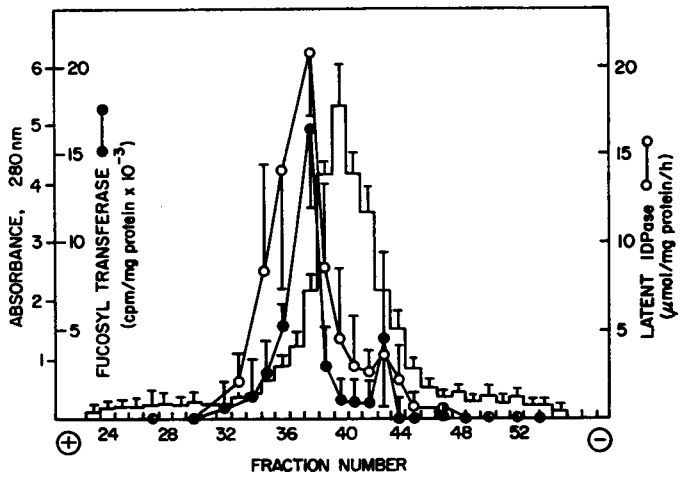

FIG. 8. Free-flow electrophoresis separation showing the location of the Golgi apparatus marker, latent nucleoside diphosphate phosphatase (○) and of a putative Golgi apparatus marker, fucosyltransferase (●). Results are analyses from three different preparations ± SD. Additional data for latent IDP_{asc} are given in Table III.

and fraction E (plasma membrane) contained 1.0 to 1.3 mg membrane protein.

Electron Microscopy of Free-Flow Electrophoresis Fractions. Electron microscopic examination revealed that fractions of the left shoulder (*e.g.* fractions 24–34) and fractions of the right shoulder (*e.g.* fractions 48–54) (electrophoretic zones A and E, respectively) consisted of homogeneous preparations of smooth membrane vesicles of potentially different origins within the cell (Figs. 3a and 4a). Fraction E consisted of nearly 100% of membranes staining with phosphotungstic acid at low pH (Fig. 3b), whereas none of the membranes of fraction A so stained (Fig. 4b).

Following simultaneous glutaraldehyde-osmium tetroxide fixation (Fig. 5), as with glutaraldehyde fixation followed by postfixation with osmium tetroxide, the plasma membrane was the only membrane structure heavily stained. Tonoplast and all internal membranes were unstained although cell walls and ribosomes were reactive (Fig. 5). Measurements of membrane thickness revealed that 95% of the membranes contained in fraction E exhibited a clear dark-light-dark pattern (Fig. 1, a and d) and membrane thickness of about 10 nm (Table I) as was characteristic of the plasma membrane *in situ* (Table II). The small numbers of thin membranes (Table III) and of membranes not staining with phosphotungstic acid at low pH (Figs, 3b, 6) suggested a low level of contamination of vesicles from any source not derived from the plasma membrane, an observation subsequently confirmed from analyses of marker activities.

In contrast to fraction E, fraction A showed few vesicles reactive with phosphotungstic acid at low pH (Fig. 4b) and the vesicles exhibited the dark-light-dark pattern less clearly (Fig. 1, c and f) than did those vesicles identified as plasma membrane (Fig. 1, a and d). Thus, fraction A was apparently not contaminated to any significant degree by vesicles derived from class I (plasma) membranes.

To identify the dominant membrane of origin of vesicles of fraction A, membrane thicknessess were measured. About 90% of the vesicles had membranes with thickness in the range of 7 to 9 nm (Table II) suggesting an origin from tonoplast (Table I). The remaining 15% of the membranes were thinner than 8 nm and may have been derived from contaminating internal membranes on this basis.

Evaluation of Putative Plasma Membrane Markers. Confirmation of a plasma membrane origin of the membranes of fraction E was provided by measurements of NPA binding and its correlation with the distribution of PTA-reactive membrane vesicles (Fig. 6). NPA binding increased with higher fraction number (*e.g.* between fractions 40 and 56) as did the percentage of vesicles that stained with phosphotungstic acid at low pH. For assays of other plasma membrane activities, fractions were pooled to increase the amount of starting material and to reduce the necessity for assaying the large number of fractions provided from each free-flow electrophoresis separation. Fraction E, the putative plasma membrane-rich fraction, contained the highest specific activity of glucan synthetase II (Table III). Glucan synthetase II was low or absent from the putative tonoplast-rich fraction (fraction A, Table III). Sterol glucoside synthetase, while concentrated in plasma membrane, appeared also to be present in tonoplast vesicles (Table III).

Table IV. *Potassium Stimulation of Mg^{2+}-ATPase in Soybean Fractions Prepared by Free-Flow Electrophoresis and Effect of Vanadate, Nitrate, and Chloride*

Assays were conducted according to the general procedure of Scherer (29). Inhibitors and activators were added to the preparation in buffer as outlined below. Results are from a single experiment in which all treatments were assayed on all fractions. Individual treatments were repeated three or more times to verify major trends.

	Additions	Total Microsomes	Free-Flow Electrophoresis Fractions				
			A	B	C	D	E
			μmoles/h·mg protein				
A.	None (50 mM Tris-Mes [pH 6])	0.79	0.18	0.75	0.91	0.40	0.49
B.	A + 3 mM $MgSO_4$	0.73	0.32	1.12	1.17	1.18	1.28
	B-A: Effect of Mg^{2+}	−0.06	0.14	0.37	0.26	0.78	0.79
C.	B + 50 mM K_2SO_4	1.54	0.35	1.59	1.72	1.64	2.10
	C-B: Effect of K^+	0.81	0.03	0.47	0.55	0.46	0.72
D.	C + 125 μM Na_2VO_4	0.49	0.13	0.81	0.85	0.64	1.16
	D-C: Effect of vanadate	−1.05	−0.22	−0.78	−0.87	−1.00	−0.94
E.	50 mM Bis-Tris propane buffer + 3 mM $MgSO_4$ to pH 7 with HCl[a]	1.01	0.64	1.45	1.21	1.12	1.16
	E-B: Effect of Cl^-	0.28	0.32	0.33	0.04	−0.06	−0.12
F.	E + 50 mM HNO_3 to pH 7 with Tris	0.94	0.13	0.58	0.90	0.80	1.39
	F-E: Effect of nitrate	−0.07	−0.51	−0.87	−0.22	−0.32	0.23

[a] The final Cl^- concentration was 75 mM.

Contamination by Endoplasmic Reticulum, Mitochondria, and Plastids. The bulk of the ER (and nuclear envelopes), mitochondria, and plastids in the starting fraction were concentrated in the center of the electrophoretic separation as revealed by EM (not illustrated) and assays of marker activities (Fig. 7). Distributions of mitochondria and ER overlapped each other from activities of succinate-INT reductase (Fig. 7) and Cyt oxidase for mitochondria and NADH- and NADPH-Cyt *c* reductases (Fig. 7) for ER as well as from morphology. The distribution of Cyt oxidase was similar to that of succinate-INT reductase with a maximum specific activity at about fraction 40 of 15 μmol/min·mg protein. Although concentrated in fraction C, the recovery of carotenoids from the free-flow electrophoresis fractions was very poor (separations were carried out in the light) and another marker for etioplasts, monogalactosyldiglyceride synthetase was monitored. Nearly 90% of the latter activity was found in fraction C in the midpoint of the separation with only 1 and 2% of the total activity in fraction A (tonoplast) and fraction E (plasma membrane), respectively.

Contamination by Golgi Apparatus. As an assay for Golgi apparatus contamination, latent IDPase was used and compared to fucosyl transferase (Fig. 8). Both activities were concentrated to the left of the midpoint of the separations between ER and tonoplast. These fractions had only low neutral IDPase activities when freshly prepared but upon storage for several days at 4°C a neutral IDPase activity appeared exclusively in these fractions. The Golgi apparatus, for the most part, appeared to unstack prior to the electrophoretic separation and were present in the starting fraction dominantly as single cisternae or as swollen vesicles.

ATPase Activities. Further confirmation of the identity of fraction A as tonoplast and fraction E as plasma membrane came from measurements of ATPase activities and the differential responses to inhibitors. Fraction E contained dominantly, although not exclusively, a K^+-stimulated, vanadate-inhibited Mg^{2+}-ATPase activity not sensitive to nitrate (Table IV) while a nitrate-inhibited activity was associated dominantly with fraction A (Table IV). These activities further demonstrated the uniqueness of the two fractions and confirmed the low level of cross-contamination indicated from morphological measurements (Table II).

PAGE Patterns of Membrane Proteins. As a final criterion for identification of fraction E as plasma membrane and contrast with fraction A, plasma membranes of greater than 90% purity as verified by staining with phosphotungstic acid at low pH were prepared by two phase partition according to Kjellbom and Larsson (13) and the pattern of electrophoretic bands compared on 12.5% SDS-polyacrylamide gels (Fig. 9). The plasma membranes from the 2-phase separation (lane B) yielded a pattern identical with that of plasma membranes prepared by free-flow electrophoresis (lane C) and quite different from that of tonoplast (lane D). The tonoplast and plasma membrane had a few major polypeptide bands with similar mobilities. Yet, many differences were evident in both the high and low mol wt regions of the gels.

DISCUSSION

While numerous fractions from plant sources have been reported which are enriched in either plasma membrane or tonoplast, few procedures have been described where both membranes have been derived in pure fractions, one resolved from the other, from the same homogenate. Standard analytical sucrose gradient procedures, *e.g.* Chadwick and Northcote (2), have been useful for assignment studies but have only limited value for preparative work because of overlap with other contaminating membranes. The technique of preparative free-flow electrophoresis appears to accomplish this by providing a major protein peak which contained the bulk of the cytoplasmic membranes (ER, mitochondria, plastids, Golgi apparatus, nuclear envelope) as identified by morphology and biochemical markers, and two additional peaks, one to the right and one to the left of the major protein distribution. These two latter peaks consisted of plasma membrane and tonoplast, respectively, in approximately equal amounts (8 and 7% of the total protein applied, respectively) and equal fraction purities (>90%).

To identify plasma membrane, and especially tonoplast, a strategy was followed in which identity could be related to the positional relationships of these membranes within the tissue, *i.e.* plasma membrane lying adjacent to the cell wall at the cell surface and tonoplast surrounding the large central vacuole within the cell's interior. For this purpose, tissues and fractions

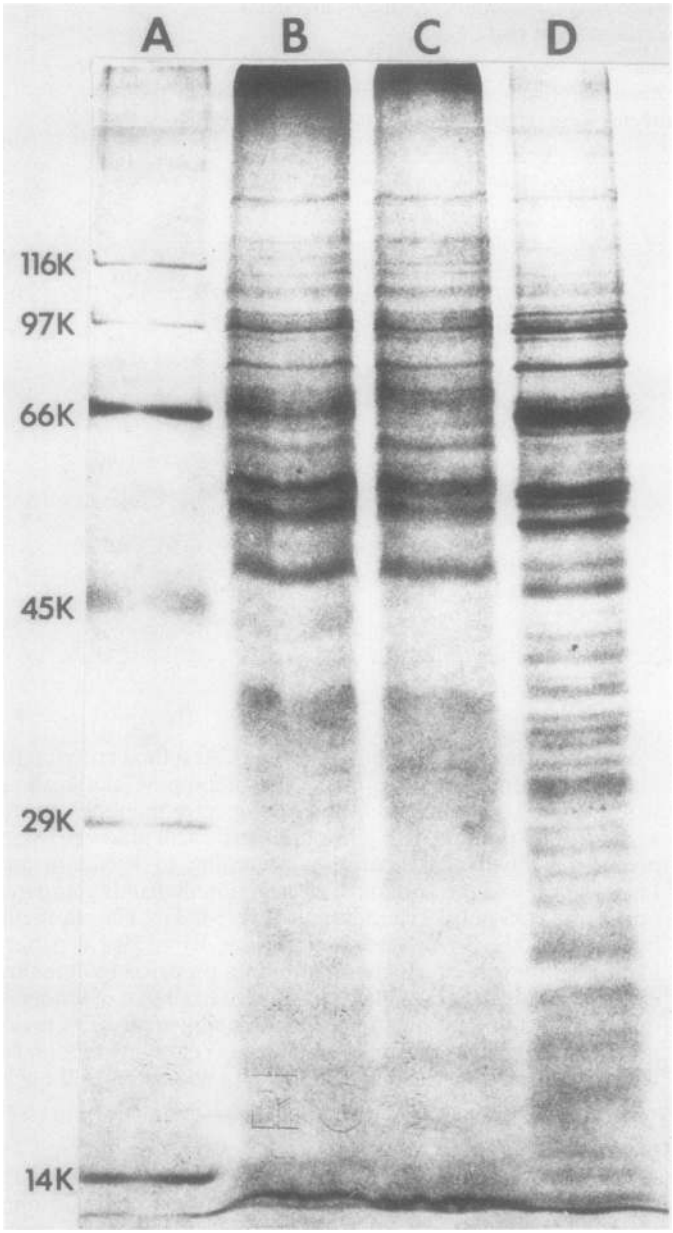

FIG. 9. SDS-polyacrylamide (12.5%) gels of plasma membranes from soybean hypocotyls prepared by two phase polymer partition according to Kjellbom and Larsson (13) (lane B) compared to plasma membranes prepared by free-flow electrophoresis (pooled fraction E) (lane C). Lane D is tonoplast (pooled fraction A) and lane A contains the mol wt standards. These analyses were carried out on three different membrane preparations with similar results.

were fixed with glutaraldehyde and osmium tetroxide simultaneously (3), a technique that enhanced the dark-light-dark pattern of plasma membrane and the differences in membrane thickness between different classes of cytomembranes. As detailed by Powell *et al.* (25) for a fungus, *Gilbertella persicaria*, these differences are retained during tissue homogenization and can be used as a reliable index for distinguishing tonoplast from plasma membrane and of both tonoplast and plasma membrane from all other internal membranes with the exception of thylakoids and certain elements of the Golgi apparatus.

Under the conditions of the simultaneous fixation procedure, plasma membranes were about 10 nm thick, tonoplast was 7 to 9 nm thick, and other internal membranes were 5 to 7 nm thick. Various factors which affect the accuracy of such measurements were evaluated in an earlier publication (19). The membrane measurements, taken together with the ability of phosphotungstic acid at low pH to stain selectively the plasma membrane (27), established the least electronegative shoulder, the one to the right of the main separation peak, to be derived of plasma membrane vesicles free of tonoplast contamination. The other membrane shoulder, the most electronegative fractions to the left of the main separation, appeared to consist of tonoplast vesicles free (less than 1%) of plasma membrane contamination.

To verify a plasma membrane origin of the vesicles to the right of the main separation, NPA binding was used. This criterion was correlated previously with PTA reactivity (15, 22) for plant fractions and could serve as an independent biochemical criterion for plasma membrane identification. Also correlated with PTA-staining of isolated membrane vesicles was a glucan synthetase with a high K_m for UDP-glucose (33) termed glucan synthetase II (26). This enzyme activity resided primarily with the PTA-staining vesicles of plasma membrane origin in our studies. UDP-glucose-sterol glucosyl transferase, while concentrated in the plasma membrane, appears to be present as well in the tonoplast and cannot be regarded as an absolute marker on this basis.

The plasma membrane fraction was judged to be about 90% vesicles of plasma membrane origin as evidenced from the number of vesicles staining with phosphotungstic acid at low pH, the number of vesicles with membranes 10 nm thick that showed clearly the dark-light-dark pattern of membrane staining, and from the virtual absence of marker enzyme activities indicative of contamination by mitochondria, ER, Golgi apparatus, or plastids. Contamination from tonoplast was discounted on the basis of morphological criteria, the absence of nitrate inhibition of a Mg^{2+} ATPase, and the fact that the plasma membranes and the putative tonoplast membranes were so widely separated in the electrophoretic separation. Since 20% or more of the plasma membrane markers and 40% of the PTA-stained membranes were found associated with fraction C, the possibility that we have purified only a subfraction of the total plasma membrane, as with procedures based on step gradients (6, 10), remains.

For the moment, identification of tonoplast was based on the positive morphological criteria set forth above and derived from positional relationships *in situ* together with the presence of a nitrate-inhibited, Cl^--stimulated, Mg^{2+} ATPase. There was little or no plasma membrane contamination as evidenced from no PTA staining at low pH, no NPA binding, and very low glucan synthetase II activity. Contamination of tonoplast by other membranes also appeared to be minimal based on marker enzyme analyses. The fractions were low or absent in succinate INT-reductase and NADPH-cytochrome reductase. These two activities correlated with the content of mitochondria and ER of soybean membranes, respectively (14). Also low was contamination by plastid membranes based on content of monogalactosyldiacyldiglyceride synthetase and β-carotene content. Both content of β-carotene and mono- and digalactosyldiacyldiglyceride correlated with content of plastid membranes in soybean as identified by permanganate reactivity and quantitated by morphometry (12).

As criteria for Golgi apparatus, two markers were used. The first, latent IDPase was described first by Ray (26) for Golgi apparatus fractions of pea. This activity was present in Golgi apparatus of soybean as verified by direct measurement and by cytochemistry (21) and correlated with dictyosome content of sucrose gradient isolated fractions (6, 20). The second marker was fucosyltransferase using endogenous acceptors, a Golgi apparatus marker in animal cells. Based on the presumptive biosynthesis of fucose-containing polymers by Golgi apparatus in plants as identified by direct incorporation of radioactive fucose by electron microscope autoradiography into Golgi apparatus

(*e.g.* 23), the activity should be present in Golgi apparatus of plants as well. Indeed, the two activities coincided reasonably well. Because the Golgi apparatus of soybean unstack extensively during the membrane isolation prior to free-flow electrophoresis, unequivocal morphological identification of Golgi apparatus membranes in the free-flow electrophoresis was not achieved. In any event, neither the plasma membrane nor the tonoplast fraction appeared to be cross-contaminated by Golgi apparatus fragments.

K^+-stimulated vanadate-inhibited Mg^{2+} ATPase (8) has been used widely as a marker for plasma membrane but as pointed out by Hodges and Mills (9), there are K^+-stimulated ATPases associated with other membranes of plant cells and the specificity of vanadate for the plasma membrane ATPase may not be absolute (16). Despite problems with an exclusive assignment of this activity to the plasma membrane, it appears to be absent from tonoplast and at least present in plasma membrane. In contrast, NO_3^--inhibited, Cl^- Mg^{2+} ATPase (17, 34, 35) is used almost routinely now as a marker for the tonoplast (24, 31, 32). Our data support such an assumption although an association of this activity with other fractions in addition to tonoplast cannot be ruled out.

While the putative tonoplast and plasma membrane fractions were clearly different based on the various markers as well as overall protein patterns from PAGE analysis, the highly purified plasma membranes prepared either by free-flow electrophoresis or by two-phase partition methods appeared similar. While there was no obvious advantage to free-flow electrophoresis over two-phase procedures for plasma membrane isolation, the two-phase methods do not resolve tonoplast from internal membranes. In addition, the free-flow separations are facile, rapid, and preparative. With continuous operation possible, membranes from 20 g of hypocotyls can be processed in less than 1 h.

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