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Orientation and Integrity of Plasma Membrane Vesicles Obtained from Carrot Protoplasts

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ABSTRACT

Two fractions enriched in plasma membrane derived from suspension-cultured carrot (*Daucus carota* L.) cells were examined to determine if they differed from each other either in physical nature or in orientation. Parameters studied included the protein composition of purified membranes derived from trypsinized and nontrypsinized protoplasts as well as from trypsinized purified plasma membranes, the effect of inhibitors and membrane perturbants on ATPase activity, the binding of [*acetyl*-^{14}C]concanavalin A to purified membrane fractions, and the competitive removal of [*acetyl*-^{14}C]concanavalin A from purified membranes derived from [*acetyl*-^{14}C]concanavalin A-labeled protoplasts. One fraction (at density of 1.102 grams per cubic centimeter on Renografin gradients) appears to be a mixed population of 'tightly' sealed vesicles with the majority being rightside-out vesicles of plasma membrane, and the other fraction (density 1.128 grams per cubic centimeter) apparently is a population of predominantly 'leaky' vesicles and/or nonvesicular fragments of plasma membrane, a large portion of which appear to be 'leaky' inside-out vesicles. In addition, it is shown that plasma membrane-enriched fractions can be distinguished from cellular endomembranes on the basis of protein and glycoprotein composition.

Membrane preparations of high purity and known physical orientation (sidedness) have been extremely valuable in the elucidation of membrane properties in various non-plant systems (10, 14, 31). Knowledge of the purity and orientation of plasma membrane vesicles is critical for proper interpretation of transport and other biochemical activities of the plasma membrane such as cell wall synthesis. Relatively pure plant plasma membrane preparations have been obtained from a number of sources (2, 12, 13, 21, 25). In only one instance (2) was the protein composition of the plasma membrane fraction (as determined by electrophoretic analysis) clearly distinguishable from a mitochondria-enriched fraction, but even this plasma membrane-enriched fraction did not appear to be significantly different from other fractions enriched in Golgi, ER, and possibly tonoplast. This was attributed to the plasma membrane being a significant contaminant in the other fractions. Although a preparation may be primarily plasma membrane as measured by PACP[3] staining (29), the protein composition as determined by electrophoretic analysis may not truly represent plasma membrane components. Endomembranes, which may constitute only a low percentage of the total membrane in a fraction, might make a significant contribution to a total protein analysis. Such contamination might even be sufficient to obscure plasma membrane-specific proteins.

Several recent vesicular preparations of unproven origin are thought to contain sealed vesicles of plasma membrane (27, 33), but their orientation is not known. Although the orientation of vesicles is thought to be inside-out, this has not been rigorously tested. Assumptions of sidedness are valid only if the origin of the vesicles involved in uptake is unambiguously ascertained.

Methods utilizing Renografin gradient centrifugation have been developed to obtain relatively pure plasma membrane from gently disrupted protoplasts derived from suspension cultured carrot cells (3). Plasma membrane obtained in these studies distributed in one peak (on continuous gradients) that could be separated into two fractions (on discontinuous gradients). We now show that these two fractions contain vesicles with significantly different properties with respect to physical orientation.

Membrane fragments resulting from homogenization could be expected to fall into one of the following three classes: (a) sealed vesicles (inside-out); (b) sealed vesicles (rightside-out); and (c) unsealed ('leaky') vesicles or fragments. To distinguish between the above possibilities in these two plasma membrane-enriched fractions, we studied Con A binding, the effects of inhibitors and membrane disruptive agents on ATPase activity, and the protein composition of plasma membrane-enriched fractions (electrophoretic analysis).

It is necessary to define the degree of sealedness used in the present experimental system. We have utilized the term 'tightly' sealed vesicles to designate those that are impermeable to ATP or are capable of sustaining a potential gradient. Sealed vesicles are those that meet the minimum requirement of exclusion of macromolecules (Con A, trypsin, or dextran). 'Leaky' vesicles (or nonsealed fragments) are those that do not meet the above requirements.

MATERIALS AND METHODS

Protoplasts were isolated (3) from suspension-cultured carrot cells in early to mid log phase of growth (3 d after transfer) following a 2.25-h digestion in 2% (w/v) Driselase and 0.4 molal sorbitol. Generally, 0.4 ml packed volume of protoplasts was homogenized (3) in 4.0 ml 8% Renografin in THM. The 1000g supernatant was loaded directly onto discontinuous gradients consisting of the following volumes and concentrations (v/v) of Renografin in THM: 0.5 ml of 38%, 3.5 ml of 27%, 3.0 ml of 23%, 4.0 ml of 18%, 2.0 ml of 10%. Gradients were centrifuged at 25,000 rpm for 2.5 h in a SW28.1 rotor (87,000g at r_{ave}).

Dextran Gradients. Methods were adapted from Sze (33) with the following modifications. Pelleted membranes were resuspended in 8% (v/v) Renografin in THM and layered over 7.5 ml

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[3] Abbreviations: PACP, phosphotungstic acid-chromic acid procedure; THM, Tris homogenizing medium (contains 50 mM Tris, 10 mM KCl, 1.0 mM EDTA, 0.1 mM MgCl, pH 7.5 at 4°C); Con A, concanavalin A; α-MG, 1-*o*-methyl-α-D-glucopyranoside.

10% (w/w) dextran (mol wt = 79,000) in 8% Renografin in THM. The gradients were centrifuged for 2 h at 22,000 rpm in a SW25.2 rotor (60,000*g* at r_{ave}).

Enzyme Assays. ATPase and IDPase assays (3) were performed on pelleted, washed membranes. ATPase assays generally contained 10 to 30 μg protein as determined by the Lowry method (19). Pi was measured according to Taussky and Shorr (34) except that when Triton X-100 was present the reaction was terminated with 5% SDS in 5% TCA. Cyt *c* oxidase and NADH:Cyt *c* reductase were assayed spectrophotometrically (28).

For ATPase inhibition, vanadate, NaN_3, or oligomycin was added simultaneously with the substrate. Vanadate was supplied as vanadium pentoxide, a less potent source of $H_2VO_4^-$ than ortho- or meta-vanadate (30).

Where noted, ATPase activity was stimulated with a 1-min trypsin incubation at 1.25 mg trypsin/mg membrane protein, pH 7.5 (Tris-HCl) and 27°C. Protolysis was terminated with a 5-fold excess of soybean trypsin inhibitor. The solution was then sonicated on ice for 15 s at 85% output in a Heat System-Ultrasonic sonicator and immediately assayed for ATPase activity. An unsonicated control contained trypsin inhibitor and trypsin.

For Triton X-100 treatments, 60 μg protein was incubated in 0.4 ml 0.05% Triton X-100 in 30 mM Tris-Mes (pH 6.5), and 0.1 mM DTT for 30 min on ice (vortex-mixed periodically). Following centrifugation for 45 min at 105,000*g*, the pellet was resuspended (in the above buffer minus Triton) and assayed for ATPase activity.

Con A Binding Assays. For competition studies, [^{14}C]Con A was prepared (see 3) with 36.8 mCi/mmol Con A (utilizing a low specific activity acetic anhydride preparation) and was stored in the lyophilized state at −20°C until use. Protoplast surfaces were labeled as previously described (3), but to a level where approximately 23% of the binding sites were occupied as estimated by Scatchard analysis (4). Washed membranes obtained from Con A-labeled protoplasts were incubated in 0.1 M α-MG, 5 mM EDTA, with or without 0.1% Triton X-100 (samples containing Triton were also sonicated) in 50 mM Tris-HCl (pH 7.2). Membranes were collected on Whatman GF/C glass fiber filter discs after agitation for 1 h at 27°C and the filters were washed four times with 5.0 ml ice-cold 50 mM Tris-HCl (pH 7.5).

For labeling purified membranes, [^{14}C]Con A (prepared as in Ref. 3) was suspended in 5 mM Tris-HCl, 0.1 mM $MnCl_2$ (pH 7.5) at 4°C to a concentration of 1.0 mg/ml. This suspension was filtered through Whatman GF/C glass fiber filters to remove aggregates and was diluted in the same buffer to 106,000 cpm/25 μl. The specific activity of the filtered Con A was 134 mCi/mmol Con A.

The reaction mixture contained approximately 40 μg membrane protein, 50 mM Tris-HCl (pH 6.8 at 27°C), either with or without 0.1 M α-MG in a volume of 1.0 ml. To this we added 106,000 cpm of [^{14}C]Con A, and the mixture was agitated for 1 h at 27°C. The reaction was terminated by transfer to GF/C glass fibers filter discs and washed as described previously. Binding was linear to at least 40 μg membrane protein for fraction C, which had the higher specific binding of the two fractions tested.

Tryptic Digests of Protoplasts and Purified Membranes. To 1.0 ml packed volume of protoplasts, 1.0 ml of a solution containing 1 mg/ml trypsin, 0.34 M mannitol, 50 mM Tris-HCl (pH 6.5 at 38°C) was added. Digestion was carried out at 38°C, resuspending the protoplasts every 30 min. Digestions were terminated by the addition of 2.0 ml 0.5 mg/ml soybean trypsin inhibitor in 0.45 M mannitol. The protoplasts were then pelleted and washed twice with 3.5 ml ice-cold 0.45 M mannitol, homogenized, and centrifuged on Renografin gradients. Fractions were retrieved from gradients and prepared for electrophoresis as described below. The viability of protoplasts (*i.e.* integrity of plasma membrane) was monitored by incubating protoplasts 5 min in 0.5% Evan's Blue in 0.4 M mannitol.

For tryptic digests of purified membranes, fraction C or fraction D was incubated with 125 μg/ml trypsin, in 25 mM Tris-HCl (pH 6.5) at 38°C, at a trypsin to membrane protein ratio (w/w) of 1.25. The digestion was terminated with a 3-fold excess of soybean trypsin inhibitor. The membranes were diluted with ice-cold Tris-HCl (pH 7.5), pelleted, and prepared for electrophoresis.

Vacuole Preparation. Vacuoles were isolated from protoplasts using Kringstad's (16) Ficoll step-gradient method. To prepare vacuole membranes for electrophoresis, vacuoles were diluted approximately 10-fold with Tris-HCl (pH 7.5), sonicated, and centrifuged at 106,000*g* for 2 h. The pellet was washed twice with 5% TCA to remove Ficoll.

Electrophoresis. Washed membranes were extracted with 90% methanol (to remove all traces of Renografin) and the protein pellet obtained was resuspended in a standard sample buffer (17) containing 1.0 M urea. Samples were boiled 2 to 4 min and insoluble material was removed by centrifugation prior to gel loading. Polyacrylamide gel electrophoresis was run (17) with the following modifications. Acrylamide gradients of 7.5 to 15%, accompanied by a 5 to 17.5% sucrose gradient, were set up in either 12- or 20-cm-long slabs (0.5 or 1 mm thick, respectively). The current applied was 17.5 mamp and electrophoresis was continued for 1 h after the tracking dye ran off the gel (15 min for 12 cm gels). Gels were fixed and stained using standard methods with Coomassie brilliant blue R-250 for protein visualization or with a coupled Con A-peroxidase method for glycoprotein (24).

Scintillation Counting. Whatman GF/C glass fiber filter discs were counted in 5.0 ml of Aquasol (counting efficiency, 82%).

RESULTS

Identification of Gradient Fractions. The marker enzyme distribution on discontinuous Renografin gradients confirmed that obtained previously (3) (data not shown). Fractions A (P = 1.042 g/cm^3) and B (P = 1.068 g/cm^3) are enriched in ER as shown by NADH:Cyt *c* reductase activity. Fraction C (P = 1.102 g/cm^3) contains Golgi membranes based upon latent IDPase activity and plasma membrane based upon Con A binding and ATPase activity. Fraction D (P = 1.128 g/cm^3) also is enriched in plasma membrane but contains little Golgi material and relatively little mitochondrial contamination based upon Cyt *c* oxidase activity. Fraction E (P = 1.146 g/cm^3) contains predominantly mitochondria and a small proportion of plasma membrane.

Inhibitor Studies: ATPase Activity. The plasma membrane ATPase of fraction D exhibits properties similar to those described for other plasma membrane ATPases, including a pH optimum less than 7.0 (3) and KCl-stimulated activity (data not shown). Vandadate, an inhibitor of the plasma membrane-localized ATPase in plants (8), likely acts on the same side of the plasma membrane as the site of ATP hydrolysis (7). Because we can measure only ATPase activity that is localized on the exposed surface of the membranes, the chemical inhibition of plasma membrane ATPase by vandate should not vary according to the orientation or intactness of the vesicles. Vandate significantly inhibits K^+,Mg^{2+}-ATPase activity (pH 6.5) in fractions C and D (Table I). NaN_3, a specific inhibitor of mitochondrial ATPase in *Neurospora* (5) and higher plants (9, 11), and oligomycin, a specific inhibitor of mitochondrial ATPases, have little effect on ATPase activity in fractions C and D, indicating an insignificant amount of mitochondrial ATPase activity at pH 6.5 in those fractions, despite the small but significant amounts of mitochondrial contamination in this fraction (3). These inhibitor studies show that when interpreting ATPase stimulation at pH 6.5, we

Table I. *Effects of Inhibitors and Membrane Disruptants on ATPase Activity*

The specific activities (± SD) of the controls are: fraction C, 15.8 ± 0.9 and fraction D, 21.6 ± 1.5 μmol Pi released/mg protein·h. Activity in the presence of oligomycin is expressed as percentage of 1% ethanol activity. All other values (± SD) are expressed as percentage of the control rates.

Gradient Fraction	ATPase Activity						
	No Treatment	50 μM Vanadate	10 mM Na Azide	10 μg/ml Oligomycin	Trypsin	0.05% Triton X-100	1% Ethanol
	% of control						
C	100	15 ± 8	93 ± 5	87 ± 2	195 ± 65	241 ± 37	97 ± 5
D	100	14 ± 7	90 ± 4	82 ± 5	108 ± 24	133 ± 22	94 ± 4

can be reasonably certain that increase in activity is plasma membrane ATPase activity, not mitochondrial ATPase activity.

Activation of ATPase. Triton X-100 at concentrations ranging from 0.02 to 0.2% (w/v) has been used to expose inner surfaces of sealed vesicles. In microsomal membranes from maize (27), as well as in other systems, the stimulation of enzyme activity in the presence of detergent is attributed to the increased accessibility of the substrate to enzyme. Alternatively, Sze (33) attributes the stimulation of a K^+-ATPase by Triton X-100 to the dissipation of an electrochemical (K^+ or H^+) potential, resulting in the release of a feedback inhibition of the ATPase since the stimulation can be induced to a similar extent by application of either detergent or specific ionophores. The latter assumption would imply that our examination of physical orientation by detergent activation of ATPase activity can distinguish with certainty only between tightly sealed vesicles and unsealed vesicles or fragments. We have utilized two methods of permeabilization to stimulate the ATPase activity in these plasma membrane-enriched fractions. Trypsinization followed by sonication resulted in marked stimulation of ATPase in fraction C but not in D. This activation by trypsin and sonication is not necessarily due to a specific activation of the ATPase by trypsin as sonication results in an additional stimulation over that of trypsin alone (data not shown). The mitochondrial ATPase from corn leaf has been shown to be activated by mild trypsin treatment (26). If this were the case in the present study, fraction D would be expected to show a greater stimulation than fraction C following the tryptic digest because fraction D contains more mitochondrial membranes. It is unlikely that the stimulation of ATPase activity in fraction C is due to an activation of a mitochondrial ATPase. Treatment with Triton X-100 also stimulates activity in fraction C to a much greater extent than it does in fraction D. Triton activates ATPase activity in plasma membrane-enriched fractions of oat roots without changing the apparent pH optima, suggesting that the same ATPases are responsible for the activated activity (6), but we cannot be certain that there are no additional ATPases activated in our membrane-enriched fractions without further characterization of the Triton-activated activity. These data, together with that of inhibitors, suggest that fraction C contains tightly sealed vesicles of plasma membrane, while fraction D contains mostly membranes that are not tightly sealed. The low amount of ATPase stimulation in fraction D is consistent with the presence of only a small number of tightly sealed vesicles. The distribution following centrifugation of fractions C and D on 10% dextran gradients, as described by Sze (33), provides further support for this conclusion. In this system, sealed microsomal vesicles remain on top of a dextran layer while leaky vesicles, nonvesicular membranes, and mitochondria pellet. A great proportion of fraction C protein (see Table II) was retained at the interface, whereas in fraction D the greatest proportion of protein was found in the pellet. The lower-than-expected amount of [^{14}C]Con A associated with the pellet from fraction D is probably due to the competition effect of dextran (a polymer of B-1, 6-linked glucose residues) that would occur as the membranes passed through the 10% dextran step. This explanation is supported by the fact that lesser amounts of the radioactivity added to the gradient are recovered in fraction D than in fraction C, indicating that competition may have removed greater amounts of Con A from fraction D than fraction C. Although we have not eliminated the possibility that the pelleted protein from fraction D represents the mitochondrial contamination that occurs in this fraction, it is unlikely to be a significant factor because the electrophoretic patterns of proteins in the dextran pellet and interface fractions from fraction D were essentially identical (data not shown).

Table II. *Behavior of Fractions C and D on 10% Dextran Step Gradients*

All values represent the average of three experiments (±SD).

Fraction Loaded	Fraction Recovered[a]	Total Protein[b]	Total Radioactivity[c]
		%	
C	Interface	75 ± 12	76 ± 16
	Pellet	25 ± 12	24 ± 16
D	Interface	30 ± 3	58 ± 14
	Pellet	70 ± 3	42 ± 14

[a] 5.0 ml including the interface was collected.
[b] Percentage of total protein recovered from interface and pellet.
[c] Percentage of total radioactivity recovered from interface and pellet.

Analysis of Protein Composition of Membranes. If the two plasma membrane-enriched fractions differ from each other only in their physical orientation, their protein composition should be identical. Electrophoretic analysis of the protein composition of gradient fractions indicates that fractions A, B, and E differ from each other as well as from C and D, indicating that they represent distinctly different membrane populations (Fig. 1). The electrophoretic patterns of the two plasma membrane-enriched fractions (C and D) are similar and their composition is markedly different from that of the mitochondria-enriched fraction (E) and the ER-enriched fraction (B). Several protein components distinguish plasma membrane-enriched fractions from all other fractions. The plasma membrane-enriched fractions bear no resemblance to membranes obtained from a tonoplast-enriched preparation (T). Protein components unique to Golgi do not constitute a significant portion of fraction C, as its pattern is similar to that obtained in D. However, proteins in Golgi should contain components similar to those found in plasma membrane as Golgi vesicles are likely about to fuse with and become plasma membrane. Similar gels stained for glycoprotein (Fig. 2) indicate that the glycoprotein (those containing glucosyl and mannosyl residues) compositions of fractions C and D are relatively similar and clearly distinguishable from fractions A and B. Although most components in fraction C are also found in D, the relative amounts of some of these glycoproteins do vary. Two high mol wt glycoproteins (bands 1 and 2) are present in successively lesser amounts in the ER (B), Golgi plus plasma membrane (C), and plasma membrane (D)-enriched fractions. Bands 1 and 2 are

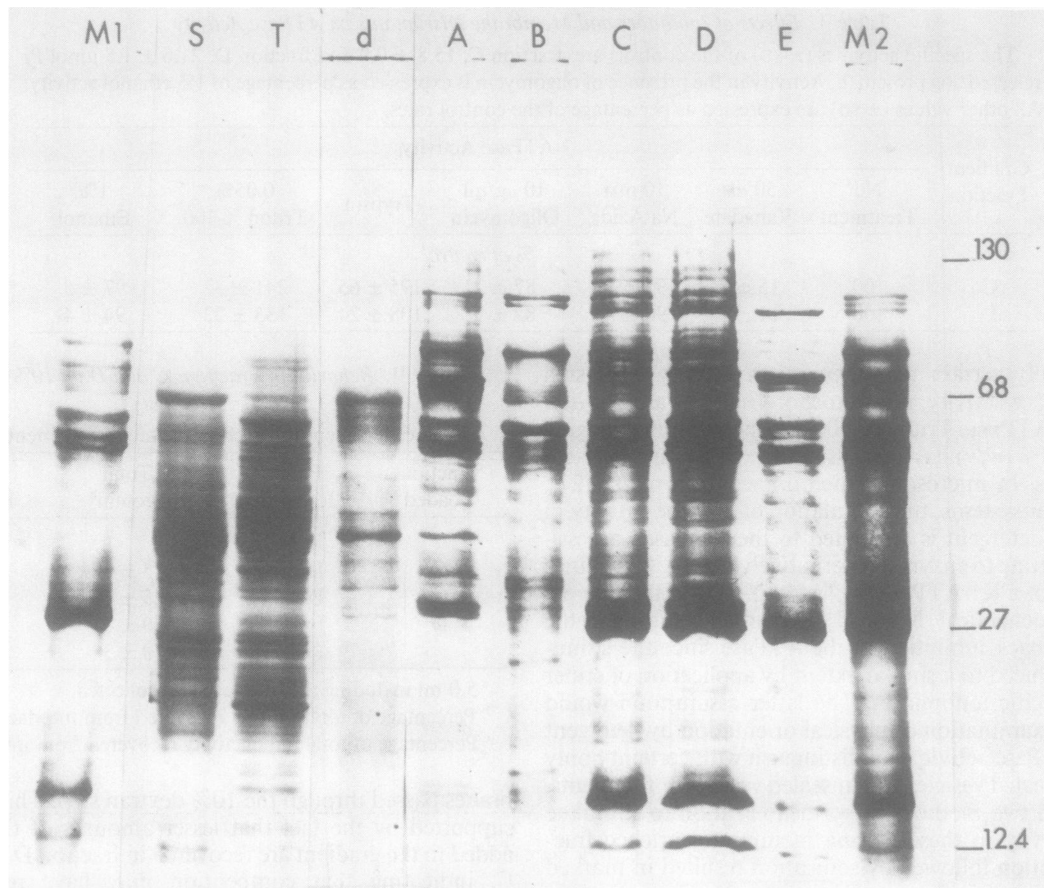

FIG. 1. SDS-polyacrylamide gel (20 cm) of enriched membrane fractions stained for protein. Lanes A–E are of Renografin gradient fractions A–E; lane T is of a tonoplast-enriched fraction (see "Materials and Methods"); lane S is a 30,000*g* supernatant derived from protoplasts; and lanes M1, M2 are from 1,000–30,000*g* (microsomal) pellets. Lane d is of the protoplast releasing enzyme mixture (driselase). Lanes were loaded with the following amounts of protein: lanes A and C, 70 μg; B and E, 40 μg; D, 100 μg; M1 and M2, 60, 80 μg; T, S, and d, 40, 90, and 40 μg. Fractions A–E, M1, and M2 were run on one gel; fractions T, S, and d on another. Molecular mass of protein standards are expressed in kD along the ordinate for B-galactosidase, BSA, Con A, and Cyt *c*.

present as only minor components in fraction D. In addition, there is a group of four abundant glycoproteins (see arrows) that occur in the ER fraction (B) and Golgi plus plasma membrane fraction (C) that are not present in the most pure plasma membrane fraction (D). Band 3 (a rather diffuse high mol wt glycoprotein[s]) and band 4 are found only in the plasma membrane-enriched fractions C and D. In terms of both quality and quantity of mannosylated and glucosylated proteins, fraction C appears to represent an intermediate state between the ER-enriched fraction (B) and the most highly enriched plasma membrane enriched fraction (D). The glycoprotein array of fraction E is similar to that of plasma membrane-enriched fractions and is very low relative to protein content. The glycoprotein content likely results from minor plasma membrane contamination in this fraction (the small amount present is not perceptible in Fig. 2).

Limited proteolysis has been used to determine the distribution of proteins in isolated red blood cell membranes (32) and this technique is applicable to the study of sidedness of higher plant plasma membrane vesicles. The electrophoretic patterns of proteins of fractions C and D derived from protoplasts that had been proteolytically digested with trypsin were examined (Fig. 3, panel A). The patterns represent the protein remaining membrane-bound following a tryptic digest. The great similarity of the protein patterns is consistent with membranes in both fractions C and D being derived from the plasma membrane. A test for the integrity of the plasma membrane utilizing Evan's blue dye indicated that 95% of the protoplasts excluded the dye after 2 h in the presence of trypsin, suggesting that trypsin is not entering the protoplasts and that only the proteins exposed on the extracytoplasmic surface of the plasma membrane of the intact protoplast are accessible to tryptic digest. Isolated gradient fractions (C and D) were also trypsinized after purification (on Renografin gradients) and a digestion pattern (Fig. 3, panel B) very similar to that of the trypsinized intact protoplasts was obtained; however, fraction D protein appeared significantly less susceptible to proteolysis. These data suggest that although the majority of the plasma membrane fragments in these two fractions have their extracytoplasmic surface exposed, fraction D is partly composed of plasma membranes that do not have their extracytoplasmic surface exposed.

No proteins are detectable localized on the cytoplasmic side of the plasma membrane (*i.e.* no bonds are lost during the *in vitro* digestion that were not lost during the *in vivo* digestion). There are several possible explanations. Trypsin specifically hydrolyzes only peptide bonds adjacent to lysine or arginine residues. Such sites may not be accessible in cytoplasmic proteins. Proteins exposed only on the cytoplasmic surface may make up only a small fraction of the total membrane protein and hence be undetectable. Finally, cytoplasmic surface proteins may simply not be resolved on one-dimensional gels. Golgi membranes should be protected from tryptic digestion *in vivo* but not *in*

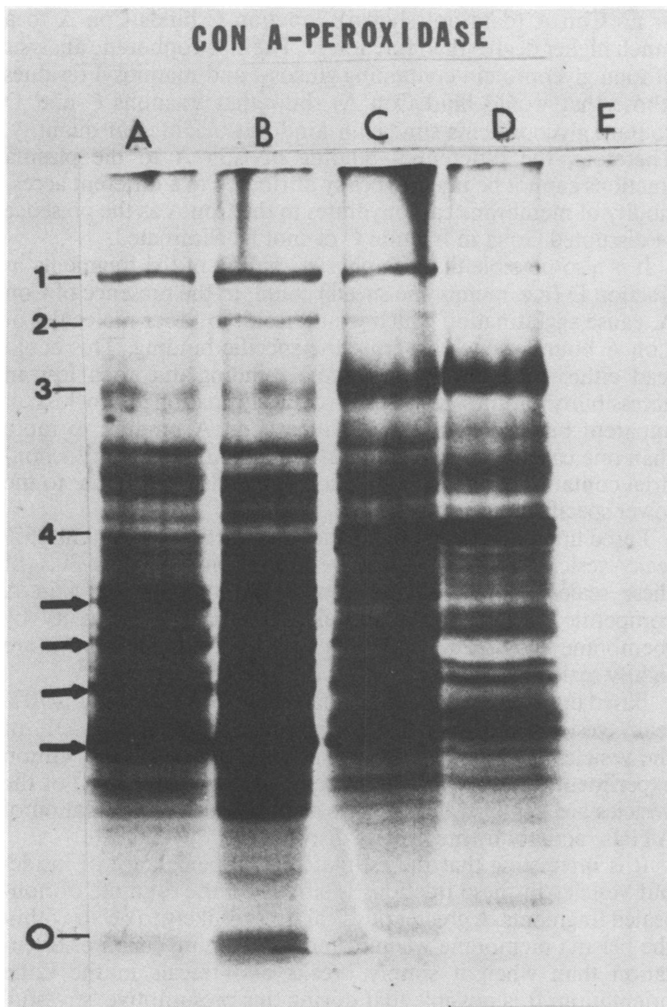

FIG. 2. SDS-polyacrylamide gel (20 cm) of Renografin gradient fractions stained for glucosylated and mannosylated proteins with a coupled Con A-Peroxidase method. All lanes loaded with the same amount of protein as in Figure 1. The position to which ovalbumin (mol wt = 43,000) migrated is designated by 'O.'

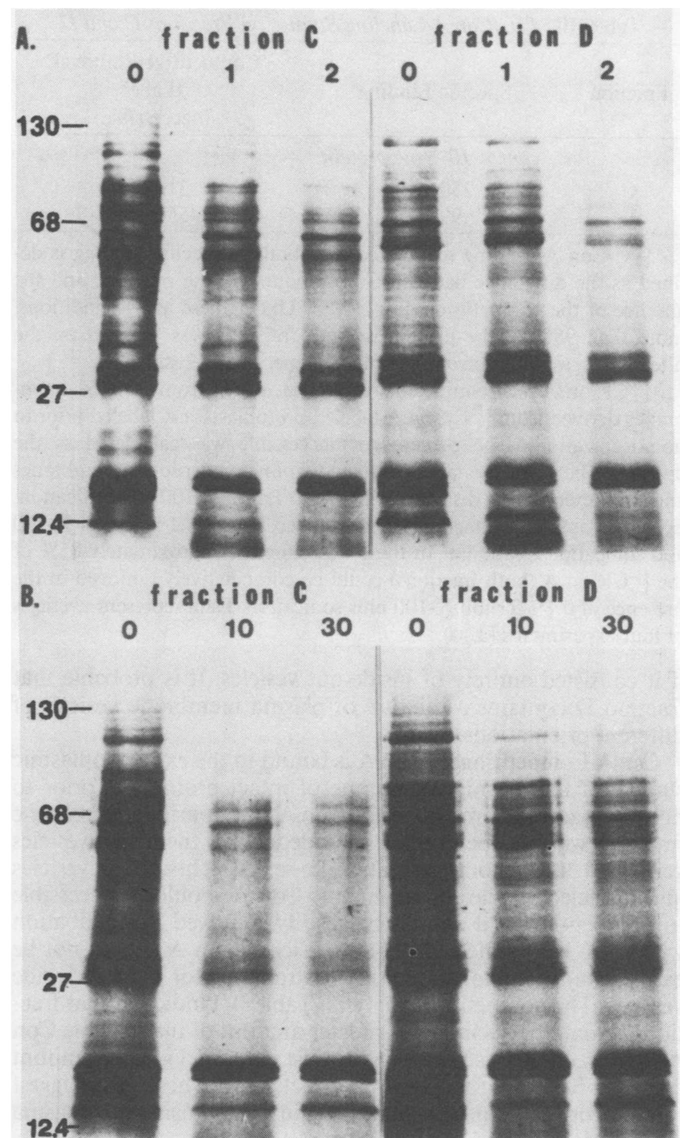

FIG. 3. SDS-polyacrylamide gels (12 cm) of fractions C and D subsequent to tryptic digestion. Panel A, Intact protoplasts digested with trypsin for 0, 1, and 2 h. Fractions C and D were then isolated (see "Materials and Methods"). All lanes were loaded with 30 μg protein (determined just prior to electrophoresis). Panel B, Purified membrane fragments digested with trypsin for 0, 10, and 30 min. The equivalent of 45 μg protein (estimated prior to tryptic digest) was loaded in each lane. The zero time control for both experiments had both trypsin and soybean trypsin inhibitor present. Trypsin and trypsin inhibitor are not detectible on these gels. Molecular mass standards are as in Figure 1.

vitro. If protein components of the Golgi are similar to those of plasma membrane, it is possible that the apparent enhanced sensitivity of fraction C proteins to *in vitro* proteolysis could result from Golgi membrane proteins. The possibility that proteins of mitochondrial origin in fraction D contribute to the apparent 'protected' protein in this fraction can be eliminated as several of the protected proteins in vesicle preparations are readily accessible when intact protoplasts are proteolytically digested (Fig. 3A). These must therefore represent proteins normally exposed on the extracytoplasmic surface of plasma membrane.

Con A Binding. Carbohydrate moieties have been shown to be located asymmetrically on the extracytoplasmic surface of red blood cells (23). Several investigators suggest that this is likely to be true for soybean (35) and spinach (10) and may be a general characteristic of the plasma membrane. In mammalian systems, the plasma membrane is the major site of Con A binding, with endomembranes binding little or no Con A (15). Con A-binding membranes isolated from soybean (1) by relatively harsh techniques include both ER and plasma membrane (Golgi and nuclear membranes were not examined).

In a purified plasma membrane fraction containing minimally disrupted endomembranes, Con A binding should be limited primarily to the plasma membrane because carbohydrate moieties are probably located on the luminal surface of endomembranes (20, 22). Sealed vesicles of plasma membrane of opposite orientations should be distinguishable based on their specific binding of Con A. As shown in Table III, fraction C binds Con A at greater specific activity than fraction D. This suggests that fraction C must contain rightside-out vesicles of plasma membrane. However, electrophoretic analysis of Con A-binding glycoproteins (Fig. 2), indicates that fraction C contains a much greater amount of Con A-binding glycoproteins (on a per total protein basis) than does fraction D. Thus, the possibility of disrupted Golgi contributing to the higher specific binding in fraction C cannot be ruled out. As carbohydrate moieties are found primarily on the extracytoplasmic surface of the plasma membrane, fraction D binds more Con A than would be expected

Table III. *[^{14}C]Con A Labeling Studies on Fractions C and D*

Fraction	Specific Binding[a]	Competitive Removal[b] (Label Inaccessible)
	cpm × 10^{-3}/mg protein	%
C	250 ± 56	31 ± 13
D	99 ± 54	55 ± 11

[a] [^{14}C]Con A binding to isolated membranes. Specific binding is defined as the difference between Con A bound in the presence and the absence of the competitor 0.1 M α-MG. Under these assay conditions, more than 95% of the protein in both fractions was retained on the filters. Data represent averages of three experiments (±SD).

[b] [^{14}C]Con A was competed with 0.1 M α-MG from isolated membranes derived from [^{14}C]Con A-labeled protoplasts (*i.e.* labeled prior to homogenization). The percentage inaccessible was calculated as the difference between the label competed from nondisrupted membranes and from membranes disrupted with 0.1% Triton X-100 and sonication, expressed as a percentage of label removed by the 0.1% Triton X-100 and sonication treatment. In these experiments, approximately 85% of the [^{14}C]Con A (both fractions) could be competetively removed in the presence of 0.1% Triton X-100 plus sonication. Data represent averages of four experiments (±SD).

if it consisted entirely of inside-out vesicles. It is probable that fraction D contains a mixture of plasma membrane vesicles of different orientations.

Con A Competition. If Con A is bound to the extracytoplasmic surface of the plasma membrane of intact protoplasts prior to homogenization, removal of the Con A from purified membrane fractions would depend upon the sidedness of the sealed vesicles generated during homogenization. In sealed rightside-out vesicles and unsealed vesicles or fragments, Con A should be accessible to competition and should be readily removed by incubation with α-MG. In sealed inside-out vesicles, Con A should not be easily removed due to physical entrapment of Con A inside vesicles. The competition analysis (Table III) indicates that fraction D contains a somewhat greater amount of inaccessible Con A than fraction C, so fraction D must contain a greater amount of sealed inside-out vesicles than fraction C. These data suggest that fractions C and D both contain some inside-out plasma membrane vesicles.

DISCUSSION

Electrophoretic analysis indicates that fractions C and D are virtually indistinguishable based on their protein composition and have similar glycoprotein composition as well. It is also apparent (Fig. 1) that proteins of tonoplast, cytoplasm (soluble components), and protoplast-releasing enzymes (driselase) are not likely to be localized in these fractions. The tryptic digests of protoplasts confirm that proteins accessible on the outer surface of plasma membrane are found in the plasma membrane-enriched fractions. When isolated membranes are subjected to tryptic digest, fraction D shows a proportion of protein that is resistant to proteolytic attack. Inasmuch as most of these proteins are susceptible to protolysis when present in plasma membrane of intact protoplasts, they must be located on the extracytoplasmic surface of the plasma membrane. Thus, a small but significant amount of membranes in fraction D must be in the form of inside-out vesicles.

We have calculated that 3.7×10^{14} molecules of Con A are bound per μg plasma membrane protein (assuming a mol wt for tetramer = 110,000) in fraction D, which is on the same order of magnitude as that found in soybean root plasma membrane (1). We have also confirmed the work (1) showing that ER binds approximately 2× greater amounts of Con A than highly purified plasma membrane (fraction D) and that mitochondria bind little or no Con A (data not shown). Fraction C binds Con A to a much higher degree than fraction D. The electrophoretic analysis of total glycoprotein containing glucosyl and mannosyl residues (those that would bind Con A) show that fractions C and D contain glycoproteins similar in kind but of different quantity. Therefore, the differential binding of Con A to the plasma fractions cannot be unequivocally attributed to a different accessibility of membrane carbohydrates to the Con A as the presence of disrupted Golgi in fraction C cannot be eliminated.

It is also possible that the physical nature of the fragments in fraction D (*e.g.* membrane sheets) could, in the presence of Con A, cause agglutination which would result in fewer molecules of Con A bound, hence lowering the specific binding. This could lead either to a decrease in actual binding due to a loss in accessibility of sites as a result of agglutination or to loss of apparent binding due to multivalent Con A binding to more than one carbohydrate moiety. The small amount of mitochondrial contamination in this fraction could also contribute to the lower specific binding of Con A.

Based upon dextran gradient analysis, fraction C contains 25% leaky vesicles and 75% sealed vesicles. Approximately 30% of these sealed vesicles must be inside-out based upon Con A competition experiments. Stimulation of ATPase activity by membrane disruptants indicates that approximately 50% are tightly sealed vesicles.

Based upon dextran gradient analysis, fraction D contains 70% leaky vesicles and 30% sealed vesicles. Approximately 55% of the vesicles must be inside-out based upon Con A competition experiments. Only a small number (approximately 10%) of the vesicles are tightly sealed vesicles as measured by stimulation of ATPase activity by membrane disruptants.

It is interesting that the estimate of the percentage of inside-out vesicles in these fractions is similar to the estimate of non-sealed fragments. A greater physical stress is likely to occur within the plasma membrane when it undergoes an inversion of orientation than when it simply breaks and reseals in the same orientation. It is possible that during this presumptive 'stressful' inversion the resultant inside-out vesicles might sustain damage and become leaky. Thus, a large proportion of membrane fragments that do not fall into the category of sealed vesicles might indeed be leaky inside-out vesicles. This hypothesis is in apparent contradiction to the assumptions of other workers who have assumed that they were working with tightly sealed inside-out plasma membrane. It should be noted that in red blood cells, production of sealed inside-out vesicles is favored by homogenization in a medium that is of low ionic strength and lacking in divalent cations (31). In the experiments described here, the protoplasts were broken in a medium that does not meet these criteria, although other workers (27, 33) have homogenized whole cells under conditions approaching those described for red blood cells. It remains to be shown whether these conditions are also favorable to the production of sealed inside-out vesicles from plant cells.

In addition to the characterization of the physical nature of two plasma membrane-enriched fractions, we have shown that these fractions are pure enough that plasma membrane-specific proteins and glycoproteins can be distinguished on polyacrylamide gels. The patterns of mannosylated and glucosylated proteins in ER (fraction B), Golgi plus plasma membrane (fraction C), and plasma membrane (fraction D) are striking. This system provides an excellent opportunity to study the processing of components (glycoproteins in particular) whose ultimate destination is the plasma membrane or exterior to the plasma membrane, an avenue of research previously not feasible in plants.

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