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Characterization of Soybean Plasma Membrane during Development

FREE STEROL COMPOSITION AND CONCANAVALIN A BINDING STUDIES

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ABSTRACT

Plasma membrane preparations from soybean root and hypocotyl contained the following free sterols: cholesterol, campesterol, stigmasterol, and sitosterol. The cholesterol level was relatively low in root plasma membrane (less than 0.5%) but was 1.4 to 2.4% in hypocotyl membrane. The relative levels of the three other sterols fluctuated with cellular development and tissue source. Campesterol level decreased with the development of both root and hypocotyl membrane. With development, stigmasterol increased greatly in root membrane but remained constant in hypocotyl membrane, and sitosterol, the major free sterol component of all membrane preparations, decreased in root membrane but increased slightly in hypocotyl membrane.

Electron microscope studies indicated that all root plasma membrane preparations were equivalent in terms of relative purity. Hypocotyl membrane preparations contained significantly greater levels of contaminating membrane components.

Root plasma membrane fractions were between 70 and 80% pure as determined by staining with the phosphotungstic acid-chromic acid procedure (PACP). Staining was most definitive for vesicles present in complete cross-section. Electron micrographs showed that vesicles treated with concanavalin A (Con A)-ferritin were extensively labeled at the outer surface indicating the presence of mannosyl and/or glucosyl residues at the vesicle surface. Densities of ferritin were highest on vesicles present in oblique section. PACP and Con A-ferritin were thus complementary with respect to topological specificity.

The percentage of Con A-ferritin-labeled and/or PACP-stained vesicles in plasma membrane root preparations was greater than 80%. Con A did not bind in purified tonoplast preparations, and binding was reduced in regions of low PACP reactivity in a root membrane fraction containing a lowered proportion of plasma membrane. Con A specificity for the plasma membrane in subcellular membrane preparations is discussed.

It has been suggested that sterols in higher plants might act directly as hormones or as precursors to steroid hormones or that they may be involved in the structural arrangement of the membrane (16). This paper concerns the sterol content of plasma membranes of soybean root and hypocotyl and the changes in the relative proportions of membrane sterols that accompany development. It also provides further evidence of the relative purity of the root plasma membrane preparations, and presents new evidence for the presence of mannosyl and glucosyl residues on the plasma membrane vesicle surface.

Changes in sterol concentration and composition have often been related to development in higher plants. Davis and Poneleit (7) reported that free stigmasterol content decreased whereas sitosterol increased during the linear phase of development in *Zea mays* kernels. Ingram *et al.* (21) described changes in the distribution of sterols during germination and early stages of growth in cruciferae. Nearly all species studied showed a decline in campesterol level with increasing seedling age. Sitosterol was more variable. It increased, decreased, or remained constant in different species. Stigmasterol, usually a major sterol in higher plants, was a minor component in all cruciferae species studied. Kemp *et al.* (23) reported that the stigmasterol content of the root of *Zea mays* increased during growth with a concomitant decrease in the proportion of sitosterol. Geuns (9) reported that young tissues of mung bean contained more sterols than older tissues and were characterized by a high sitosterol to stigmasterol ratio. This ratio gradually decreased as the tissue aged. Grunwald (15) reported that the absolute level of stigmasterol in tobacco increased with leaf maturity, whereas sitosterol, campesterol, and cholesterol did not change. Thus, the relative proportion of stigmasterol increased whereas that of the other three sterols decreased.

Sterol levels have been compared also in etiolated and green leaves. Bush *et al.* (6) reported that the ratio of free sitosterol to stigmasterol decreased from 2:1 to 1:1 during the greening of etiolated barley leaves. Campesterol and cholesterol content were the same in both tissues. Thus, excepting filling maize kernels and some cruciferae species, the relative level of sitosterol generally decreased with development, whereas stigmasterol increased.

In all of the above cases, only total tissue sterols were considered. Although these results probably reflect membrane sterol composition, it is also possible that they were governed by the presence of nonmetabolic sterol pools (15). If sterols are an essential structural component of membranes, membrane sterol composition deserves evaluation. Information is relatively scant on the plasma membrane sterol composition of higher plants. Microsomal fractions (which include plasma membrane) of maize shoots (24) and bean leaves (3) contained campesterol, stigmasterol, and sitosterol in similar proportions. The major sterol components were stigmasterol (27–33%) and sitosterol (53–60%). Campesterol ranged from 8 to 12%. Cholesterol ranged from 1% in maize shoot (24) and green bean leaf microsome fractions (3) to 6% in the microsome fraction isolated from etiolated bean leaves (3). Grunwald (12) reported that the microsome fraction isolated from tobacco leaves contained sitosterol and stigmasterol in relatively equal proportions (38 and 40%, respectively). The cholesterol level was 8%. Hartmann *et al.* (17) later reported that an enriched fraction of plasma membrane from maize coleoptiles contained relative concentrations of the four major sterols as follows: campesterol, 19%; stigmasterol, 56%; sitosterol, 24%; and cholesterol, 1%. The sitosterol to stigmasterol ratio was the opposite of that reported for the maize shoot microsome preparation of Kemp and Mercer (24). Thus, there appears to be no clear-cut definition of plasma membrane sterol composition; species variation appears to be

considerable. It must be emphasized that enriched plasma membrane preparations have been studied only in maize coleoptiles (17). In all other cases the preparations used (designated as microsomal) were not enriched for plasma membrane.

The study reported here was undertaken to clarify the sterol composition of the plasma membrane of higher plants. Only free sterols were considered, because the hydroxyl group at the C_3 position (3β-hydroxyl) must be free for the sterol molecule to interact in a molecular association with phospholipids and other components of the membrane (14, 16). Furthermore, Hartmann *et al.* (17) reported that sterol esters constituted less than 1% of the sterol composition of enriched fractions of maize coleoptile plasma membrane.

In addition, because it is essential to know to what extent biochemical analyses of plasma membrane enriched fractions reflect the presence of contaminating membranes, the possibility was also examined that surface-oriented oligosaccharide receptor sites associated with plasma membrane glycoproteins or glycolipids might represent an additional identifying characteristic of plasma membrane in subcellular fractions. The presence of surface carbohydrates associated with the plasma membrane of higher plants has been inferred from lectin-binding studies with protoplasts from several plant species (4, 5, 33, 35). It is known from lectin-binding studies with isolated membranes from animal cells, that membrane-bound oligosaccharides, when present, are asymmetrically disposed on the extracytoplasmic membrane surface of the plasma membrane and the various endomembrane components (22, 27). Their presence and disposition on isolated membranes from higher plants has not been studied.

The identification and quantitative analysis of Con A[1] receptor sites on plasma membrane from plant cells in different stages of development in itself deserves further study (Berkowitz and Travis, in preparation). The present work is concerned with establishing evidence for the general presence of these sites on plasma membranes of differing developmental age. The use of Con A-ferritin labeling of plasma membrane vesicles in conjunction with PACP staining was also examined as a possible method of obtaining a more precise estimate of the purity of plasma membrane preparations.

MATERIALS AND METHODS

Plant Tissue. Soybean seedlings (*Glycine max* L. var. Wells) were germinated in darkness in plastic dishpans containing moist Vermiculite at 30 C. Four-day-old roots, excised below the region of lateral root development, were separated into meristematic tissue (terminal 3–4 mm), elongating tissue (1-cm section behind the meristematic zone), and mature tissue (section 1.5–4 cm behind meristematic zone). The hypocotyl was divided into the hook region and a region 1–4 cm below the hook. The region between the hook and mature zone was not used. All tissue was excised into ice-cold aerated deionized H_2O.

Isolation of Plasma Membrane Vesicles. Plasma membrane vesicles were prepared by differential and sucrose density gradient centrifugation as described previously (31).

Sterol Analyses. Membrane sterols were determined by a modification of the procedure of Donaldson and Beevers (8). Briefly, plasma membrane vesicles were recovered from sucrose gradients, diluted with resuspension buffer (1 mM Tris-Hepes [pH 7.2], 1 mM $MgSO_4$, 20% sucrose [w/w]), pelleted at 80,000*g* for 35 min (Spinco T-65 rotor) and then resuspended in 0.5 ml of resuspension buffer. Lipids were extracted with 20 volumes of chloroform-methanol (2:1, v/v) and stored overnight at −20 C. Extracts were washed twice with 0.2 volumes of 0.1% $MgCl_2$ (w/v), evaporated to dryness under N_2, and redissolved in 50 μl chloroform-methanol (9:1, v/v). Total unesterified sterols were recovered by TLC. TLC plates (Redi-Coat G, Supelco, Inc.) were prewashed overnight in chloroform-methanol (2:1, v/v), dried at room temperature for 30 min, and activated for 1 h at 110 C. Duplicate 100-μl samples were applied to TLC plates in horizontal bands about 2–3 cm long. Cholesterol was used as a standard. Plates were developed in benzene-diethyl ether-ethanol-acetic acid (50:40:2:0.02, v/v). Spots corresponding to free sterols, identified by I_2 vapor (R_F 0.35), were removed and eluted with 4 ml of chloroform. Samples were again evaporated to dryness under N_2 and redissolved in 25–50 μl of ethyl acetate.

Sterol composition was determined by GC. One to 3 μl of the prepared sample, containing about 1 μg cholestane as an internal standard, was injected into a Varian model 3700 gas chromatograph equipped with a flame ionization detector and an automatic data analysis system. The 1-m column, 3% OV 101 on Gas chrom-Q, was maintained at 250 C. Injector and detector temperatures were 260 C. Retention times relative to cholestane were: cholesterol, 1.75; campesterol, 2.26; stigmasterol, 2.44; and sitosterol, 2.79. Campesterol and stigmasterol were not completely resolved under these conditions. Resolution was made nearly complete by increasing the column length to 2 m, but that did not increase the efficiency of the automatic data analysis system in determining the relative percentages of the major sterol components. Hence, to minimize retention time, the 1-m column was used routinely. All sterol data are presented as relative per cent of the four major components.

Electron Microscopy. Enriched fractions of plasma membrane recovered from sucrose density gradients were diluted with 0.1 M phosphate buffer (pH 7.3) and centrifuged at 80,000*g* for 30 min (Spinco T-65 rotor). The pellets were washed twice to remove sucrose, a weak inhibitor of Con A binding. After each wash, resuspended aggregates were pelleted at 50,000*g* for 20 min (Sorvall SS 34 rotor). Membrane vesicles were fixed in 2% buffered glutaraldehyde for 12 h at 0 to 2 C, washed in cold buffer, and post-fixed in buffered 1% OsO_4 for 1.5 h. Samples were dehydrated through a graded acetone series and embedded in Epon. Sections were stained with uranyl acetate-lead citrate (28) or PACP (30). The PACP-staining protocol and methods of quantitation have been described (2).

Some membrane preparations were incubated with Con A-ferritin (Calbiochem) before fixation in glutaraldehyde. In all

Table I. *Free Sterol Composition of Soybean Root Plasma Membrane Preparations*

The values are means ± SD for three experiments, each with three replications.

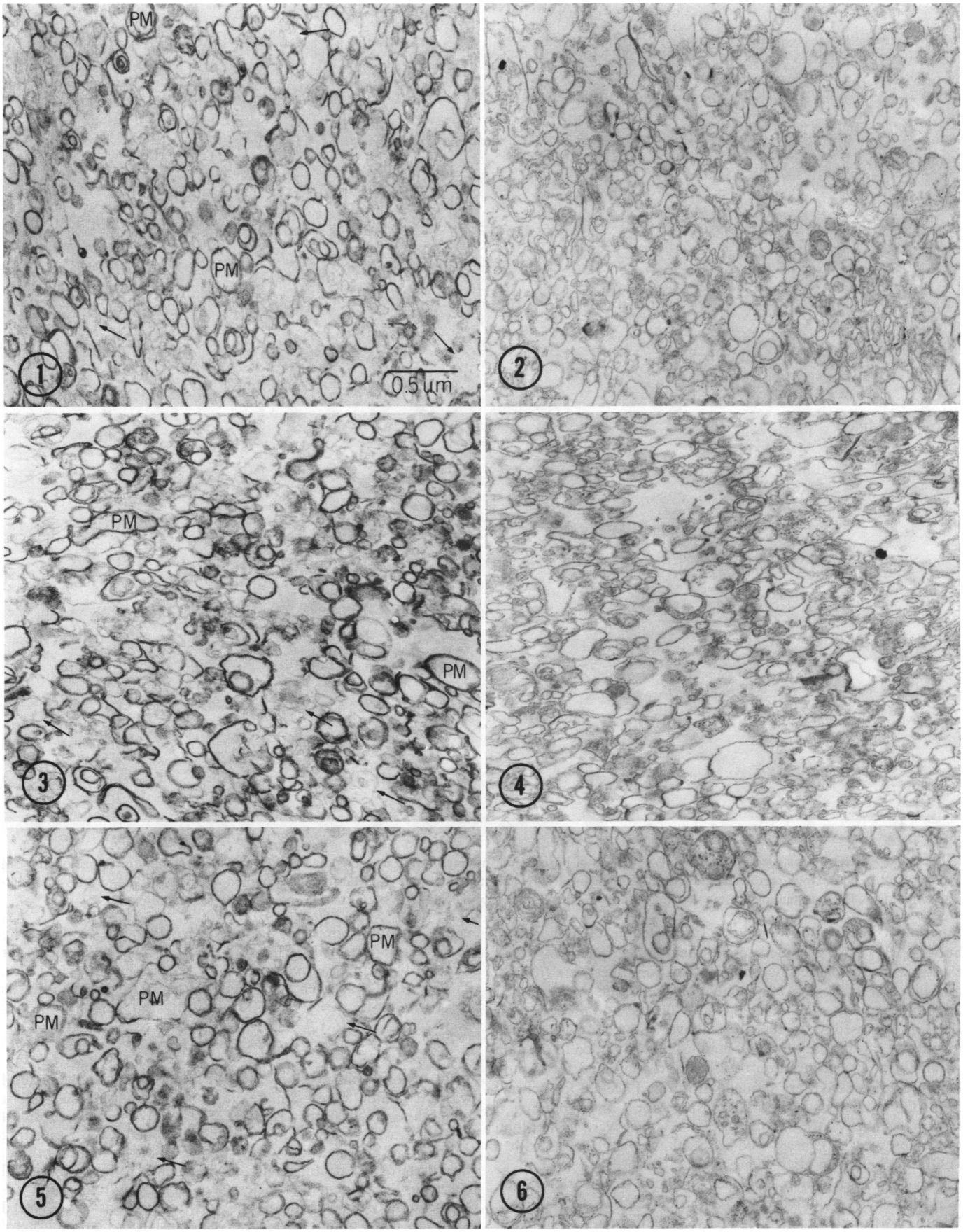
Section	Relative Per cent			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Meristematic	0.3 ± 0.07	15.0 ± 0.25	10.4 ± 0.80	74.4 ± 0.91
Elongating	0.05 ± 0.06	11.9 ± 0.25	12.3 ± 1.5	75.7 ± 1.50
Mature	0.2 ± 0.07	10.6 ± 0.35	31.1 ± 0.53	58.1 ± 0.50

Table II. *Free Sterol Composition of Soybean Hypocotyl Plasma Membrane Preparations*

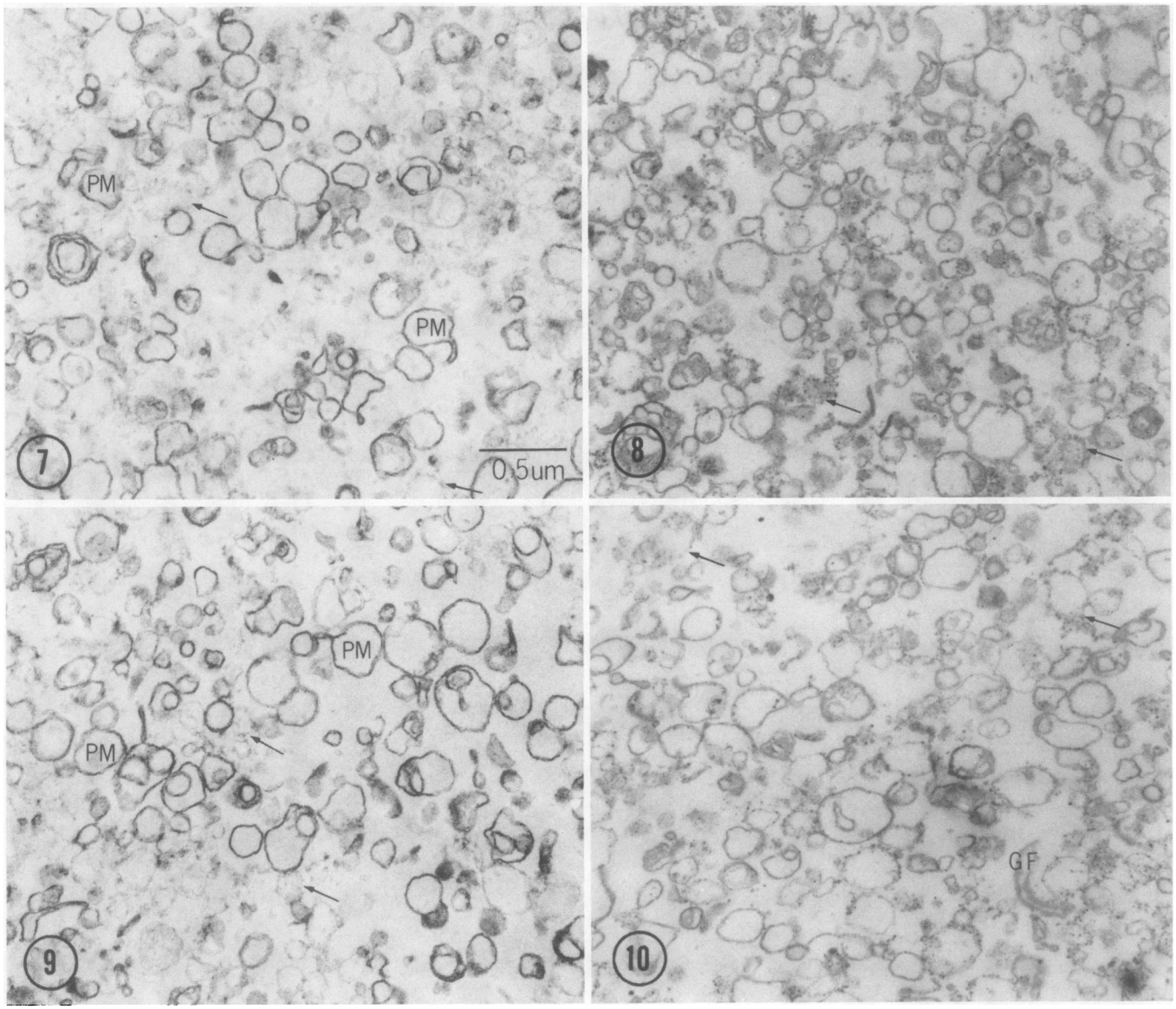
The values are means ± SD for three experiments, each with three replications.

Section	Relative Per cent			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Hook	2.4 ± 0.07	12.0 ± 0.60	24.0 ± 1.09	61.4 ± 1.31
Mature	1.4 ± 0.17	9.6 ± 0.31	23.5 ± 0.17	65.5 ± 0.55

[1] Abbreviations: Con A: concanavalin A; PACP: phosphotungstic acid-chromic acid procedure.



FIGS. 1–6. Electron micrographs of sections of pellets of purified plasma membrane fractions obtained from meristematic, elongating, and mature root tissue (× 27,000). Membrane vesicles from meristematic region stained with PACP (1) and uranyl acetate-lead citrate (2). Membrane vesicles from elongating region stained with PACP (3) and uranyl acetate-lead citrate (4). Membrane vesicles from mature region stained with PACP (5) and uranyl acetate-lead citrate (6). PM: plasma membrane vesicles. Arrows: unidentified vesicles.



FIGS. 7–10. Electron micrographs of sections of pellets of purified plasma membrane fractions obtained from hook and mature regions of soybean hypocotyl (× 27,000). Hook region stained with PACP (7) and uranyl acetate-lead citrate (8). Mature region stained with PACP (9) and uranyl acetate-lead citrate (10). PM: plasma membrane vesicles; GF: golgi fragment; arrows: unstained vesicles with associated electron-dense particles.

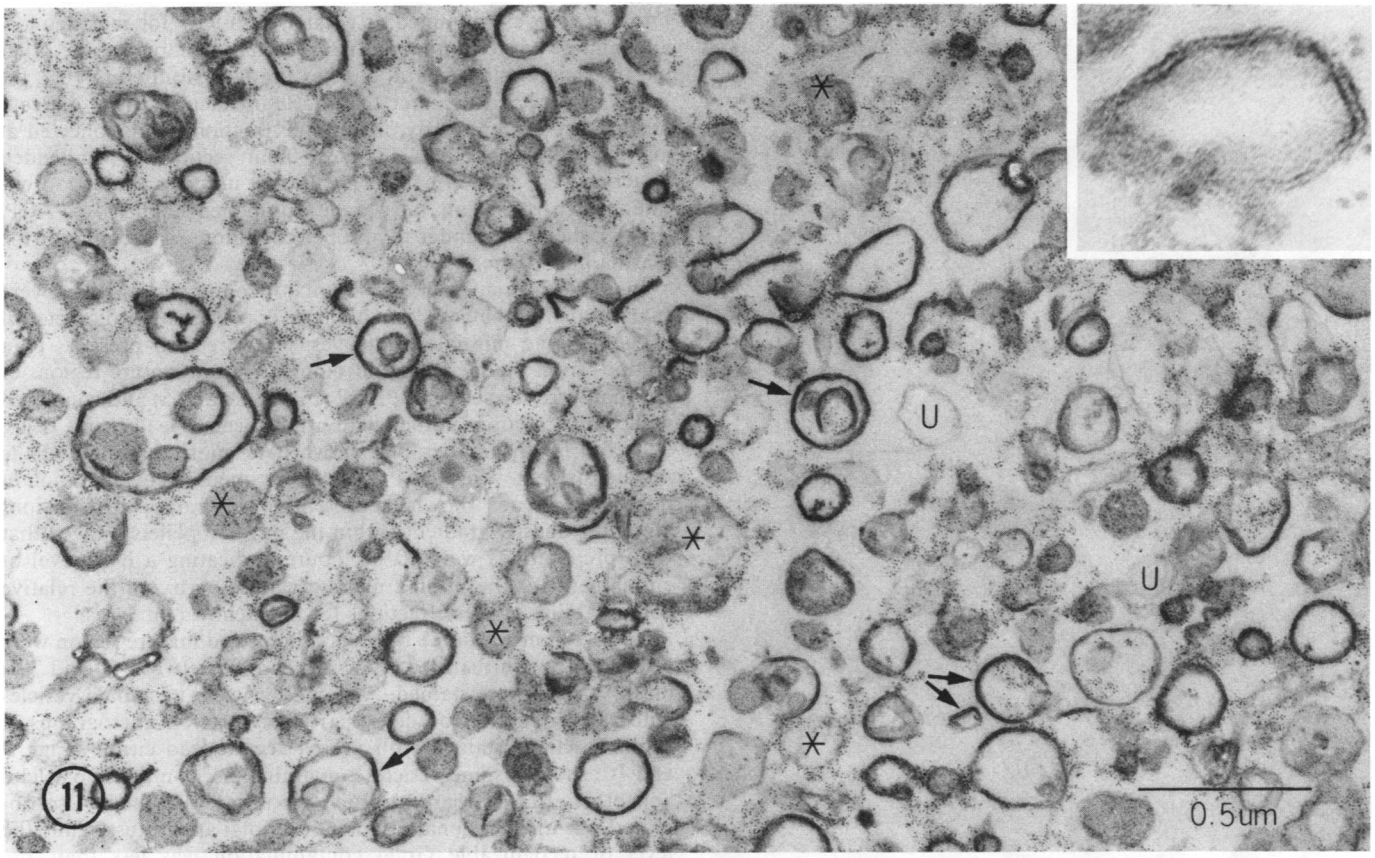
experiments the ferritin compound, suspended in 0.1 M phosphate buffer (pH 7.3), was added to the membrane protein in 5-fold excess (w/w) to ensure saturation. Incubation times were from 30 min to 2.5 h at 22 C. After incubation the pellets were washed three times to remove unreacted Con A-ferritin and processed for electron microscopy as described above. Controls were treated identically except that α-methyl mannoside was added to the Con A-ferritin at a concentration of 0.1 M and incubated for 45 min before use. Sections were stained with uranyl acetate-lead citrate or PACP. Vesicles were scored positive for Con A binding if ferritin densities on the outer surface of a vesicle were greater than those observed in control studies. Sections were viewed with a JEOL-100S electron microscope.

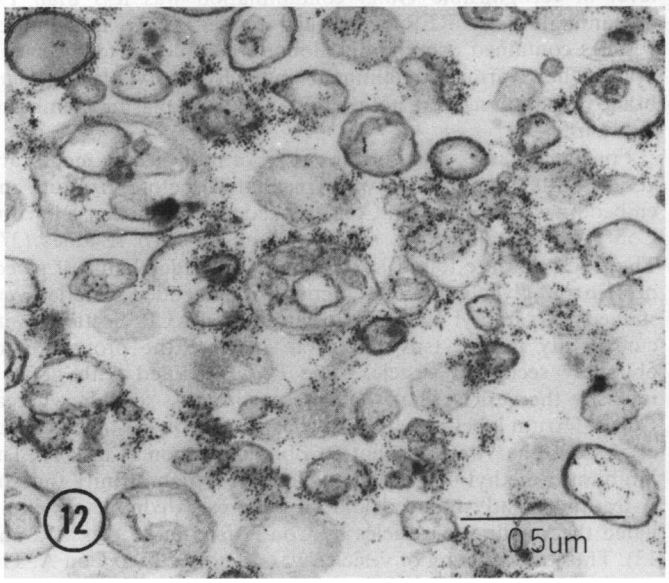
Tonoplast Preparation. Vacuoles, prepared from barley leaves, were generously supplied by R. Granstedt and R. C. Huffaker, Plant Growth Laboratory, University of California, Davis (methodology to be published elsewhere). The vacuole suspension, in 0.3 M mannitol, was centrifuged at 80,000*g* (Spinco T-65 rotor) for 45 min to remove residual chloroplast and protoplast contaminants. Vacuoles remaining in the supernatant were collected by centrifugation at 50,000*g* (Sorvall SS 34 rotor) for 30 min after dilution with an equal volume of 0.1 M Na-phosphate (pH 7.3). This fraction was then processed for lectin-binding studies as described above.

RESULTS

Free Sterol Composition of Root Plasma Membrane. Sitosterol was the major sterol component in all soybean root plasma membrane preparations (Table I). The relative level of sitosterol was greatest in vesicles isolated from meristematic and elongating regions, and markedly lower in vesicles from the mature region. Campesterol also declined with development. However, the initial decrease was evident in vesicles from elongating tissue. The decrease in relative percentage of these two sterols was compensated for by a concomitant increase in stigmasterol. The cholesterol level in root plasma membrane preparations was less than 0.5%.

Free Sterol Composition of Hypocotyl Plasma Membrane. Sterol composition patterns were less clearly defined in hypocotyl





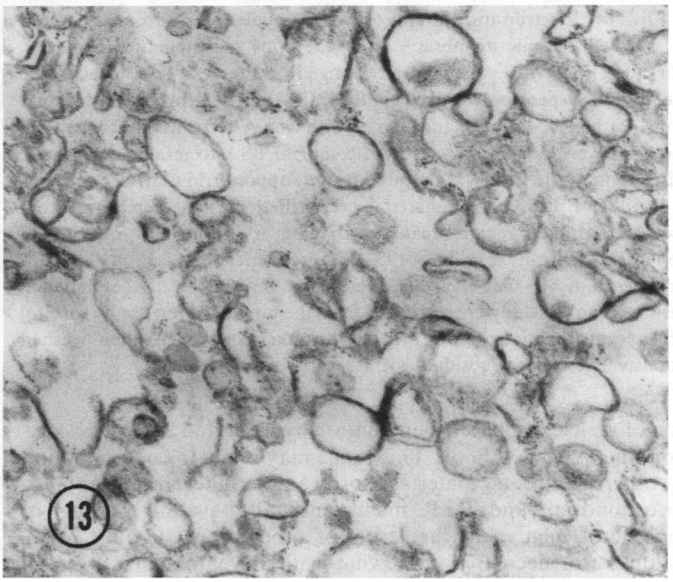
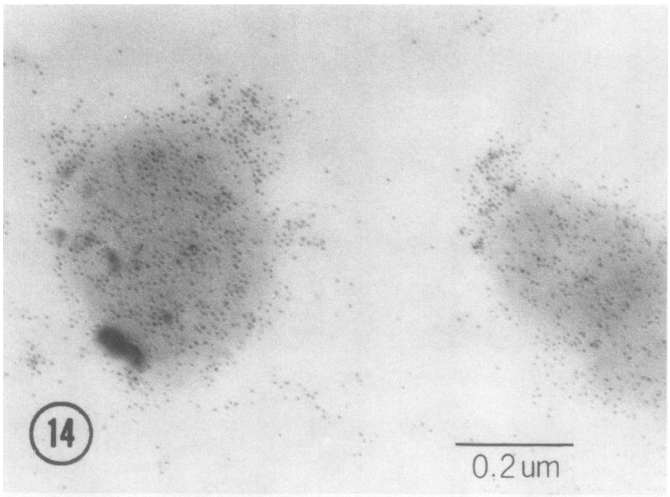


FIG. 11. Electron micrograph of section of pellet of Con A-treated purified root plasma membrane from mature region (× 46,700). The section, cut slightly thicker to increase the proportion of obliquely sectioned vesicles, was stained with PACP. In cross-section the triple-layered structure of the vesicle membrane (arrows) can be seen in many places. Relatively little or no Con A-ferritin is present in those regions. A greater density of ferritin particles is associated with portions of the vesicles sectioned obliquely (*). U, unidentified vesicles. Inset: higher magnification (× 260,000) of vesicle membrane showing trilaminar appearance. Some ferritin particles are present where vesicle is in oblique section.

FIGS. 12–13. Thin-section electron micrographs of pellets of purified plasma membrane from root mature region incubated with Con A-ferritin in the absence (12) and presence (13) of the Con A inhibitor α-methyl mannoside (× 43,750). Sections were stained with uranyl acetate-lead citrate.

vesicle preparations. The reason may be the greater difficulty in obtaining relatively pure plasma membrane vesicles from hypocotyl tissue. Electron microscope studies discussed below suggest that the preparations probably contained a significantly greater level of contaminating membrane material.

Sitosterol was the major sterol component of the enriched fraction of hypocotyl plasma membrane (Table II). One major difference between root and hypocotyl plasma membrane in sterol composition was the greater level of cholesterol in hypocotyl membrane. Cholesterol ranged from 1.4% of the sterol composi-



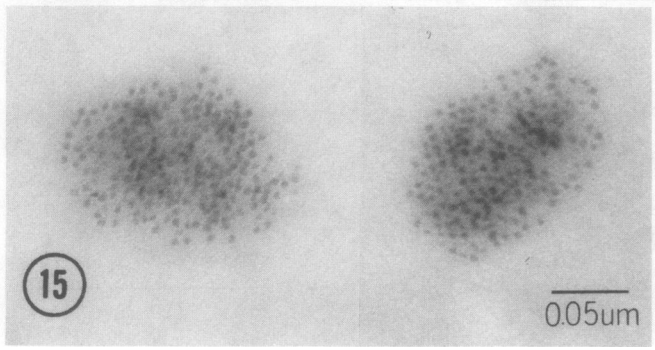


FIG. 14. Electron micrograph of Con A-labeled fresh droplet material of purified plasma membrane from the root meristematic region (× 82,600). The ferritin particles are arranged on the vesicular surface. The membrane suspension was fixed in 0.5% OsO_4 for 1 h, placed on formvar grids, washed extensively, and stained with Con A-ferritin. Before staining, the grids were floated on a drop of 5 mg/ml BSA to reduce nonspecific adsorption of the Con A compound to the support grid (23).

FIG. 15. Fresh droplet material of purified plasma membrane from root meristematic region treated with Con A in the test tube before application to formvar grids (× 195,000). Frequent superposition of ferritin particles indicate that they are present on both sides of the flattened vesicles. The vesicles were separated from the unreacted ferritin by sorbitol densitycentrifugation before grid application. Grids were then washed extensively to remove residual sorbitol.

tion of vesicles prepared from mature hypocotyl to 2.4% of vesicles from the hypocotyl hook. This range may relate to contamination by other membrane material. For example, nuclear, chloroplast, mitochondrial, and RER membranes all have high levels of cholesterol relative to the levels of other plant sterols (3, 12). Campesterol decreased somewhat with development (12.2% in hook, *versus* 9.6% in mature hypocotyl), as it did in the developing root. Stigmasterol remained constant in hypocotyl tissue with development, and sitosterol increased slightly. The latter pattern was the reverse of that during root development. Thus, while it is possible to relate specific sterol ratios to certain developmental stages in the root, the trends do not hold for the hypocotyl.

Electron Microscope Study of Vesicle Preparations. In assessing a given characteristic of plasma membrane vesicles isolated from different tissues or from similar tissue but in various stages of development, one must consider the potential problem of contamination by other cellular membrane fractions. This topic was addressed in an earlier communication from this laboratory (2). In that report the degree of purity of soybean root plasma membrane preparations was assessed by electron microscopy. Purity level was estimated at greater than 75% for meristematic and mature vesicle preparations. Estimates of purity for other plasma membrane preparations have ranged from 60 to 70%, for barley root (22), to greater than 75%, in fractions from oat root (16). In the current study, membrane fractions were collected at an average density of 1.17 g/cc. Thin sections of embedded material were stained with conventional uranyl acetate-lead citrate, which stains all membrane components, and by PACP, which, when used under the proper conditions, is specific for the plasma membrane (2, 30).

In all electron micrographs the major components present were smooth membrane vesicles with an average vesicular diameter of 0.2 μm. Some multivesiculate vesicles were also present. Thin sections from the meristematic, elongating and mature regions of the root were stained with PACP (Figs. 1, 3, and 5) and compared with similar sections stained with uranyl acetate-lead citrate (Figs. 2, 4, and 6). In micrographs shown here, more than 85% of the vesicles present were PACP positively stained. An examination of micrographs (not shown) representing several distinct regions along the density gradient within the purified pellets showed that total preparations were 70–80% pure, indicating a high level of enrichment for the plasma membrane and verifying the relative comparability of these fractions for biochemical analyses.

Thin sections from the hypocotyl hook and mature region were also stained with the PACP (Figs. 7 and 8). Between 60 and 70% of all vesicles were stained. Also present were unstained vesicles with associated electron dense particles. Electron micrographs of similar sections stained with uranyl acetate-lead citrate (Figs. 9 and 10) showed some vesicles containing electron dense particles and others that appeared to have ribosomes attached. Also observed was an occasional fragment of Golgi apparatus. While the level of recognizable Golgi contamination was less than 1%, contamination by RER was much higher. Some unidentified vesicles contained osmiophilic globules complexed to the membrane. These various contaminants in the hypocotyl plasma membrane preparations were not present in the root plasma membrane fractions.

For further assessment of the purity of root plasma membrane vesicles, the preparations were incubated, before fixation and embedding, with the lectin Con A linked to electron dense ferritin. Con A specifically binds mannosyl and glucosyl residues (27). These residues are found in the oligosaccharide chains of glycoprotein and glycolipids. Thin sections of embedded material were stained with uranyl acetate-lead citrate or PACP. Sections were cut slightly thicker (silver-gold) to increase the proportion of obliquely sectioned vesicles. The PACP stained with greatest intensity the vesicles that were present in cross-section, whereas Con A-ferritin labeling was most conspicuous on the outer surface of vesicles that were present in oblique section (Fig. 11). The inhibitor α-methyl mannoside, which has a high affinity for Con A-ferritin, greatly reduced and in some cases completely eliminated the labeling of vesicles by Con A-ferritin (*cf.* Figs. 12 and 13). Thus, the labeling of vesicles was due directly to Con A. The percentage of lectin-labeled and/or PACP-stained vesicles was greater than 80%.

A full surface view of membrane vesicles was obtained by placing a droplet of membrane suspension directly on a grid. When the collapsed vesicles were stained with Con A-ferritin the electron dense ferritin particles were arranged on the exposed vesicular surface (Fig. 14). Results were similar when vesicles were incubated with Con A-ferritin before grid application (Fig. 15). In this case, however, ferritin particles were present on both sides of the flattened vesicles.

Also treated with Con A-ferritin/PACP were vesicles present in the first gradient step comprising the membranes that are denser than 20% sucrose (1.08 g/cc) but less dense than 34% (1.15 g/cc). Enzyme marker studies (19) indicate that fraction to contain

significant amounts of plasma membrane. The proportion of plasma membrane present is diluted, however, by other membranes, particularly in the low density range. Electron micrographs of sections from four different regions of that pellet (Figs. 16–19) showed that the number of vesicles with Con A-ferritin bound decreased with a decrease in the number of PACP positively stained vesicles present. (In some cases Con A-ferritin-reacted vesicles were extensively agglutinated.)

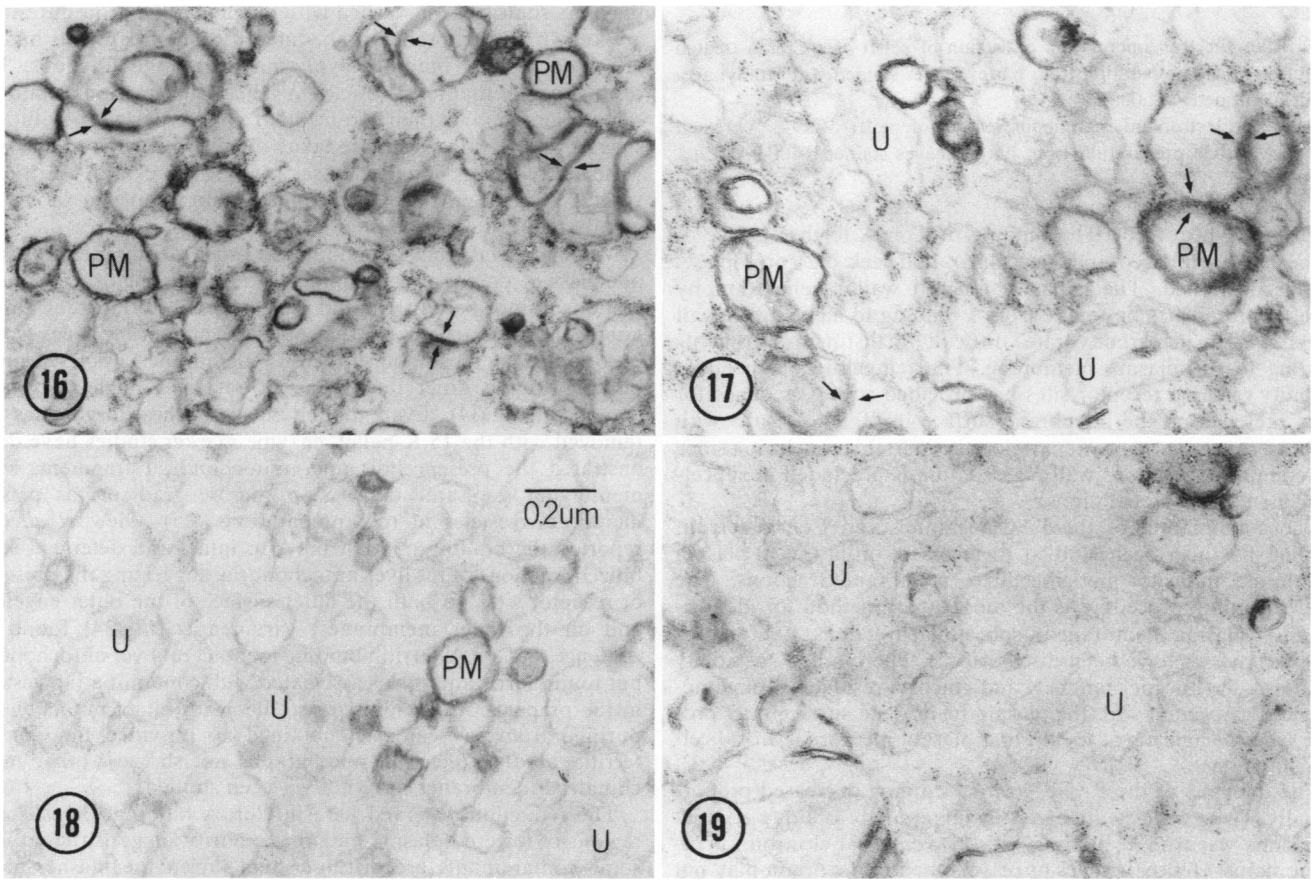
Tonoplast vesicles prepared by lysis of intact barley leaf vacuoles were treated similarly to define further the specificity of Con A in subcellular fractions. Vesicles identified in sections stained with uranyl acetate-lead citrate (Fig. 20) were extremely large, with a diameter range of 0.45–3.6 μm. Membranes were unstained by PACP (Fig. 21). No measurable Con A binding was detected in these experiments.

DISCUSSION

The variation in sterol composition with the varying stages of development of tissues of higher plants, especially in microsome and plasma membrane preparations, precludes a complete assessment of the physiological significance of the data presented here. Sterols have been implicated in the control of membrane permeability (11, 13, 14, 18). In each of those studies, exogenously applied sterols modified the permeability of plant cells. Cholesterol and campesterol were most effective at decreasing permeability. The effects of stigmasterol and sitosterol were less well defined. Although these sterols had no effect on membrane permeability in excised barley roots (14) they were effective in preventing leakage in a red beet system (11). If sterols are involved in membrane permeability, then variation in sterol composition of soybean root plasma membrane may relate to permeability differences and possibly functional differences in the plasma membrane associated with development.

The increase in the relative level of stigmasterol and concomitant decrease in sitosterol in the maturing root suggests that a developmentally regulated conversion of sitosterol to stigmasterol may occur. Both are C_{29} sterols differing only in one double bond at C_{22}-C_{23} position. The pathway of synthesis of sitosterol and stigmasterol has not been clearly resolved. Grunwald (16) recently reviewed literature relative to sterol biosynthesis. He noted either that sitosterol may serve as a precursor for stigmasterol or that both sterols may arise from a common intermediate. The former possibility was suggested by experiments in which labeled sitosterol was converted to stigmasterol *in situ*. Early reports were generally unconvincing because of the relatively low level of conversion, but Grunwald (16) later observed conversion levels as high as 10%. A $C_{22\text{-}23}$ dehydrogenase was suggested to mediate the conversion. If that enzyme does exist in soybean root, the increase in stigmasterol in the maturing root might reflect a developmentally associated increase in enzyme activity. Alternatively, the shift in relative proportions of those sterols may represent turnover or exclusion of sitosterol and synthesis and/or incorporation of stigmasterol during membrane development. Those possibilities will be pursued in future studies.

The extensive binding of Con A-ferritin to vesicles in membrane preparations enriched for K^+-stimulated ATPase activity (31) and containing a large percentage of PACP positively stained vesicles indicates that receptor sites for Con A are present on the plasma membrane surface of cell types from both meristematic and mature



FIGS. 16–19. Sections of a pellet obtained from Con A-ferritin treated vesicles which were present at the 34% interface of the sucrose gradient (× 46,000). Sections were stained with PACP. PM: plasma membrane. Arrows: closely appressed membranes of vesicles agglutinated by Con A. U, unidentified vesicles (unstained by PACP, unlabeled by Con A-ferritin).

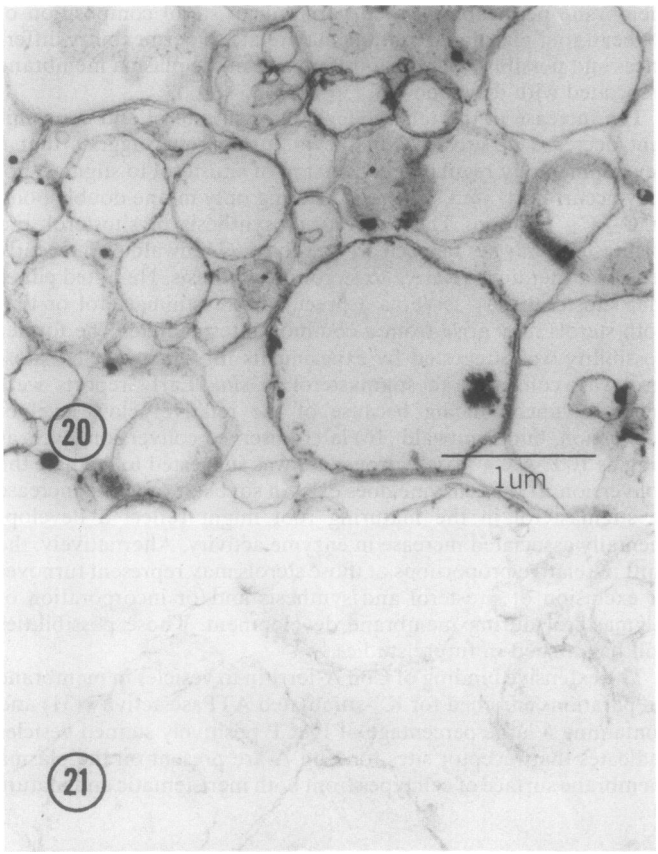


FIG. 20. Electron micrograph of section of pellet of a Con A-treated tonoplast vesicle preparation from barley leaves stained with uranyl acetate-lead citrate (× 22,000).

FIG. 21. Electron micrograph of section of pellet of Con A-treated tonoplast vesicle preparation from barley leaves stained with PACP (× 22,000).

tissue of soybean root. The binding of Con A to the surface of soybean (33), tobacco and vine (4, 5), and leek (32) protoplasts has been studied. The capacity for cell wall regeneration by isolated protoplasts has made Con A binding to nascent cell wall material, rich in carbohydrate, difficult to distinguish from its binding to the plasma membrane. The temperature-dependent mobility of these receptor sites has provided strong evidence for their presence on the membrane surface itself. The results with soybean root membrane preparations support this conclusion since the complication of cell wall regeneration is precluded in subcellular membrane preparations.

The combination of the PACP staining and Con A-ferritin binding has also been studied to assess its utility as a plasma membrane indicator in subcellular membrane fractions. The PACP staining procedure is the most direct method for identification of plasma membrane in subcellular fractions. Its strengths and weaknesses have been discussed (2). The binding specificity of Con A-ferritin for mannosyl and glucosyl residues of oligosaccharides associated with the plasma membrane surface may provide a further means of identifying plasma membrane in subcellular fractions.

The intensity of the PACP positive staining decreased proportionally when vesicles appeared in increasingly oblique section. The same was true in staining with conventional electron microscope stains. Hence, vesicles of plasma membrane origin may not be identified as such. This situation can be clarified by "double-staining" with Con A-ferritin and PACP. Con A-ferritin was most definitive when vesicles appeared in oblique section, providing a larger surface view of the plasma membrane. Electronmicrographs of membrane vesicle suspensions placed directly on grids and stained with Con A-ferritin (Fig. 14) or labeled *in vitro* prior to grid application (Fig. 15) confirmed that binding was extensive and was most evident for vesicles in total surface view. Conversely, vesicles, or portions of vesicles, sectioned in perfect cross-section were characterized by the presence of triple-layered membrane structures (Fig. 11, inset) and thus showed a minimal surface view, with concomitant decrease in Con A-ferritin labeling. The two staining procedures are thus complementary with respect to topological specificity and will allow a more definitive identification of the plasma membrane in subcellular fractions if Con A plasma membrane specificity can be demonstrated unequivocally.

The affinity of Con A for the plasma membrane, like that of PACP (26), appears to be related to an inherent characteristic of the carbohydrate complement of that membrane. For Con A, that apparently relates to the presence of surface-oriented mannosyl and glucosyl residues. The success with Con A in identifying plasma membrane vesicles depends on a lack of those carbohydrates in other endomembrane components. Alternatively, if present, they should be in relatively low concentration or in a position that keeps them inaccessible to the lectin. In studies with membrane fractions from rat liver cells in which Con A-ferritin (34) and [^{3}H]Con A (22) were used it was shown that Con A-binding sites for various membrane components were exclusively located on the extracytoplasmic surface. The amount of [^{3}H]Con A bound/μg protein by plasma membrane fractions was greater than 2-fold that in Golgi fractions and from 8- to 30-fold greater than that by other membrane components. Membrane fractions other than plasma membrane had a limited ability to bind Con A unless the preparations were subjected to several cycles of freezing and thawing, sonicated, or washed with distilled H_2O, procedures that are known to disrupt membrane integrity. The treatments had no effect on Con A binding to plasma membrane fractions. The results suggest that most vesicles in subcellular fractions retain the same orientation as their intracellular counterparts. They further suggest that when vesicles are taken directly off the gradients and washed free of residual sucrose, binding of Con A-ferritin to vesicles other than those of plasma membrane origin should be greatly reduced or nonexistent.

Mitochondrial, plastid, and nuclear inner membranes represent one class of potential contaminants for which the above considerations do not pertain. Should these organelles fragment during homogenization it is likely that cisternal membrane faces would be oriented at the outer surface of membrane vesicles. Con A binding to the inner membrane of rat liver nuclei has been demonstrated (34). Even though most of these organelles are removed with the 15 K pellet, enzyme marker studies have demonstrated the presence of inner mitochondrial fragments sedimenting at the same density on sucrose gradients as plasma membrane vesicles in root preparations (25). Glew *et al.* (10) reported the binding of [^{3}H]Con A to intact and detergent-solubilized fractions of rat liver mitochondria, suggesting the presence of receptor sites on both the outer surface of the outer envelope and on the inner membranes. Virtanen *et al.* (34) found no evidence for Con A-ferritin binding to intact rat liver mitochondria but found ferritin particles associated with contaminating vesicles in the preparation. Preliminary studies with intact mitochondria purified from soybean root obtained no provided for Con A-ferritin binding (electron micrographs not shown). Inner mitochondrial membranes have not yet been studied.

The reduction observed here in Con A binding to vesicles in regions of lowered plasma membrane purity suggests that at least some contaminants are distinguishable from the plasma membrane on the basis of that characteristic. The isolation of highly purified vacuolar membranes containing little or no plasma membrane (membrane profiles were unstained by PACP) has enabled

identification of one membrane component that lacks affinity for Con A at the outer surface.

Several lines of evidence, including enzyme marker enrichment studies (19, 20, 25, 31), PACP staining (2, 26, 29, 30), and [^{14}C]UDPG surface-labeling (1), have made it possible to assert that specific membrane fractions of characteristic density are enriched for plasma membrane. Our results have indicated a high capacity for Con A binding by those same fractions. The results also indicate that under specified conditions the capacity to bind Con A may reside primarily with the plasma membrane portion of the total membrane fraction.

The "double-staining" procedure described suggests that the level of enrichment for plasma membrane in these root preparations may be higher than 80%, the upper range of estimate on the basis of PACP staining alone. Thus, chemical (sterol data presented here) and enzyme analyses reported earlier (29) reflect developmental changes of the root plasma membrane.

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