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An Electron Microscope Comparison of Plasma Membrane Vesicles from Meristematic and Mature Soybean Root Tissue

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ABSTRACT

Plasma membrane vesicles were isolated from homogenates of meristematic and mature soybean root tissue by differential sucrose gradient centrifugation. Vesicles were positively identified by the phosphotungstic acid-chromic acid procedure (PACP). The two preparations were comparable in size class distribution, mitochondrial contamination, and per cent plasma membrane vesicles present. Purity levels were estimated to be greater than 75%. The specificity of PACP was observed for a variety of cell types from both regions. Some variability in PACP staining was offset by careful modulation of the stain protocol and was found to be independent of developmental stage in subcellular fractions. Patchy or discontinuous staining, observed in both intact tissue and in subcellular fractions from both regions, was found to be a function of stain time.

A K-stimulated adenosine triphosphatase was partially characterized in plasma membrane from meristematic and mature root tissue (20). Enzyme studies indicated that ATPase activity per unit membrane protein was greater in the meristematic region, and later studies (21) revealed a declining gradient of ATPase activity between those two extremes. It has not yet been determined whether the difference is due to differences in activity or in concentration. Since the enzyme has been characterized as an *in vitro* component of isolated plasma membrane vesicles, the observable difference clearly reflects intrinsic alterations in the plasma membrane itself. Hence, during cellular development the plasma membrane itself apparently undergoes a specific differentiation that most likely parallels its changing functional role in the maturing cell.

Such observations depend on a procedure for obtaining plasma membrane fractions of comparable and relatively high levels of purity. Purification of a plasma membrane fraction from several plant species has been described (3–5, 7, 9, 11, 23). Estimates of purity have ranged from 60 to 70% from barley root (11) to greater than 75% in fractions from oat root (4). Estimates of purity are based primarily on the PACP,[1] which is reputed to stain specifically plasma membrane both in the intact cell and in subcellular fractions containing membrane vesicles (15, 16). Although the general specificity of acidic PTA staining has been demonstrated in a variety of plants (4–7, 9, 15–17, 23), the degree of staining has been shown to be variable (13, 19), leading to potential misrepresentation of purity levels in subcellular fractions.

It has been suggested (11) that the variability may simply be controlled by staining intact cells along with subcellular fractions and adjusting the destain, stain, and rinse times accordingly. It has also been suggested (3) that the variability may be an inherent property of changing membrane ultrastructure and composition during differentiation and therefore related directly to the heterogeneity of cell populations obtained from tissue homogenates of intact plant organs. It has also been shown (13) that PACP positively stains ribosomes, lipid bodies, and prolamellar bodies. Although those cell components are not of a size or density to allow them to contaminate plasma membrane fractions, the possibility remains that they might complex with various membrane fractions during the isolation procedure. The observable patchiness of PACP positively stained vesicles has been alluded to in this regard (13).

In this paper plasma membrane vesicles obtained from meristematic and mature tissue of soybean root are compared with respect to levels of purity and vesicular diameter. The specificity of PACP staining is compared in both intact cells and in the purified fractions. A comparison of this sort, while providing necessary information on the comparability and degree of purity of mature and meristematic plasma membrane vesicles from soybean root, offers an additional opportunity for assessing the variability of PACP positive staining as a function of factors related to intrinsic cellular variability.

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) and mature tissue (section 1.5–4 cm behind meristematic zone). The region of elongation (section between meristematic and mature zones) was not used. All tissue was excised into ice-cold aerated deionized H_2O.

Isolation of Plasma Membrane Vesicles. Plasma membrane vesicles were prepared essentially by the method of Hodges and co-workers (2, 4, 8) as described previously (22). Twenty to 75 g of mature or 2 to 4 g of meristematic root tissue was homogenized for 3 to 4 min with a mortar and pestle, without added abrasive, in 4 volumes of grinding medium (25 mM Tris-Hepes [pH 7.2], 3 mM EDTA, 25 mM DTT, 250 mM sucrose) and filtered through Miracloth. The crude homogenate was centrifuged for 15 min at 15,000*g*. Membrane vesicles were pelleted from the resulting supernatant by centrifugation at 80,000*g* for 35 min (Spinco SW 27 rotor). Membrane vesicles were resuspended in fresh grinding medium and given a second centrifugation at 80,000*g* for 35 min (Spinco T65 rotor) for further purification. The final pellet was resuspended in 1 ml buffer (1 mM Tris-Hepes [pH 7.2], 1 mM $MgSO_4$, 20% sucrose [w/w]). The resuspended membrane vesicle preparation was layered on a discontinuous sucrose gradient consisting of 8 ml 34% sucrose (containing 0.55 mM $MgSO_4$ and 0.55 mM Tris-Hepes [pH 7.2]), layered over 28 ml 45% sucrose in the same buffer system, and centrifuged for 2 h at 80,000*g* (Spinco SW 27 rotor). Plasma membrane vesicles were recovered from the

[1] Abbreviations: PACP: phosphotungstic acid-chromic acid procedure; PTA: phosphotungstic acid.

interface between the two sucrose layers. All steps were carried out at 0 to 4 C.

Electron Microscopy. Tissue segments were taken from the root tip and from a section 3.0 cm behind the meristematic region. They were fixed at room temperature in 2.5% glutaraldehyde in 0.1 M Na-phosphate buffer (pH 7.3). Segments were postfixed in 1.5% OSO_4, dehydrated in a graded acetone series, and embedded in Spurr's epoxy resin (18).

Membrane fractions recovered from the sucrose gradient were resuspended in glutaraldehyde and left for 15 min at 0 to 2 C. Fractions were pelleted at 80,000*g* for 45 min, washed in cold buffer, postfixed in cold 1.5% OSO_4, and dehydrated in acetone at room temperature. Pellets were then divided into multiple slices and infiltrated with Spurr's over a 48-h period.

Sections were stained with uranyl acetate-lead citrate (14) or with periodic acid-chromic acid-phosphotungstic acid. Sections from intact cells were destained for 30 to 60 min in periodic acid. Sections were rinsed for 20 min in distilled H_2O and then stained in 1% phosphotungstic acid in 10% chromic acid for 5 to 12 min. That was followed by a rinse in distilled H_2O for 50 min. All steps were carried out at room temperature (22 C). Sections from membrane fractions were treated similarly except that periodic acid treatment was for 45 to 60 min and phosphotungstic acid-chromic acid staining was for 5 to 7 min. Sections were viewed with a JEOL-100S electron microscope.

The amount of plasma membranes in proportion to the total number of membranes present in subcellular fractions was determined by comparing sections stained with uranyl acetate-lead citrate with serial sections stained with PACP. The ratio of PACP-stained vesicles to total membrane vesicles was expressed as a percentage. A second estimate was made by the method of Loud (10). A transparent overlay of parallel lines was placed on enlarged prints of PACP-stained sections. Line membrane intersects were quantitated for each component. The ratio of the number of intersects of vesicles positively stained by PACP to the total number of intersects was expressed as a percentage of plasma membrane vesicles present.

RESULTS AND DISCUSSION

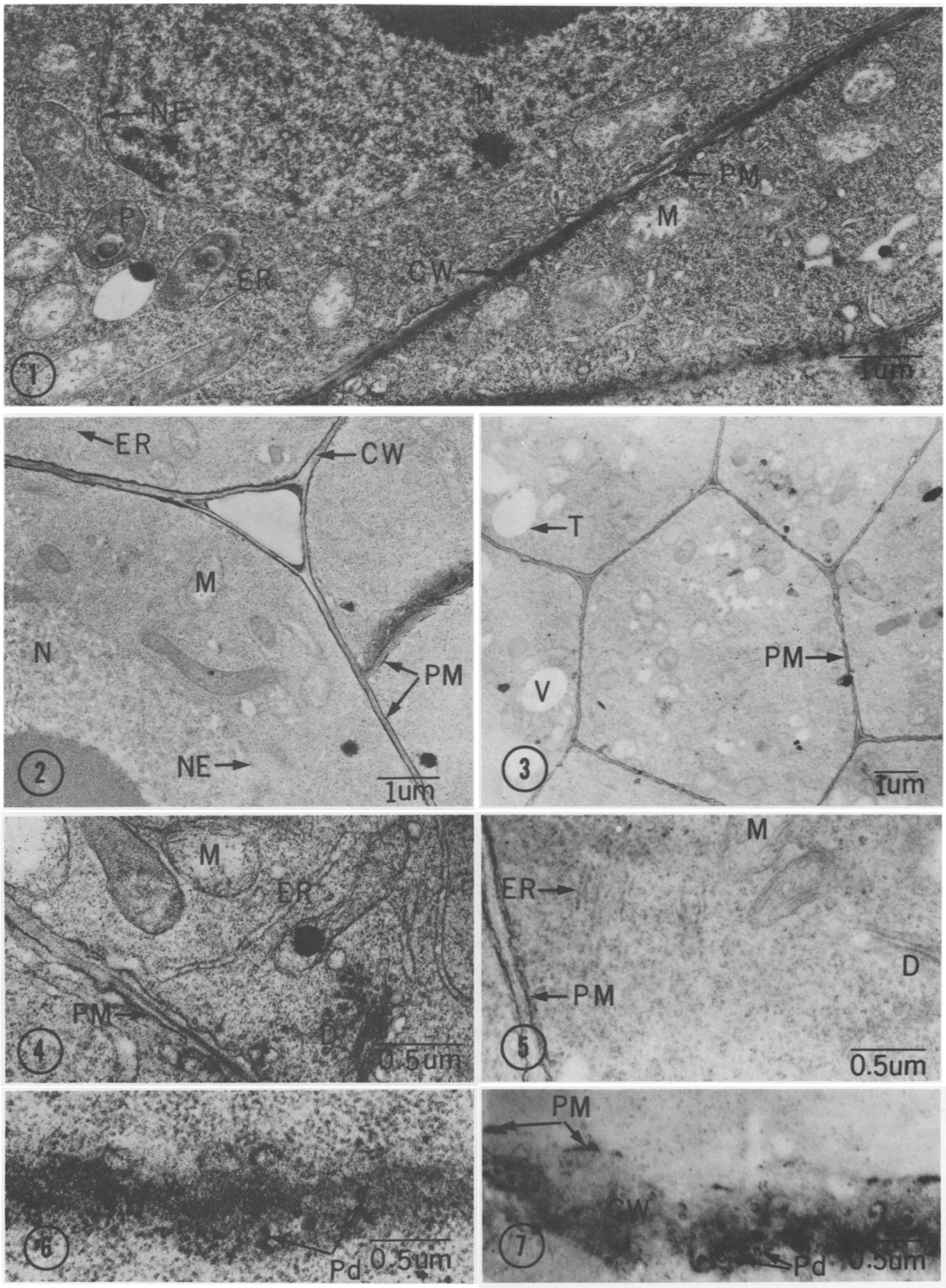
PACP Staining of Plasma Membrane in Intact Cells. The PACP was found to be highly specific for the plasma membrane in a variety of cell types from both mature and meristematic regions of the soybean root. Thin sections from meristematic cells stained with the conventional uranyl acetate-lead citrate (Figs. 1 and 4) showed characteristic staining of all cellular membranes and organelles. In contrast, only the plasma membrane was stained in thin sections treated with PACP (Figs. 2, 3, and 5). In no case was PACP staining observed in membranes other than the plasma membrane. In meristematic cells oblique sections of the cell wall were stained with uranyl acetate-lead citrate (Fig. 6) and PACP (Fig. 7). PACP stained discrete fragments of plasma membrane which were impossible to detect with the conventional uranyl acetate-lead citrate. The delineating membrane of plasmodesmata was also clearly stained. Results were similar in mature tissue (Figs. 8–11). Certain adjustments were necessary in the stain protocol. For cortical cells (Fig. 9), stain times had to be increased for staining of the plasma membrane to be uniform and complete. A similar situation has been observed in oat root (4). For stelar tissue (Fig. 11), longer stain times were unnecessary but destain times with periodic acid had to be increased for effective destaining of the prominent tonoplast in endodermal cells. In endodermal cells and occasionally in cells adjacent to the endodermis, osmiophilic vacuolar condensates, believed to be tannins (24) and found to be associated with the tonoplast inner surface, were resistant to periodic acid treatment within the range of normal destain times. In all cases, adequate destaining was achieved by increasing the time in periodic acid. PACP thus varied with the cell type and location of the cell within the root, but the variability was within a discrete range and did not detract from its intended role as a useful indicator.

PACP Staining of Plasma Membrane in Subcellular Fractions. Although it was possible to offset staining variability in intact cells by careful modulation of the stain procedure, that was not possible in examining subcellular fractions. The suggestion (11) that intact cells be stained at the time of vesicle staining to ensure fidelity obviously does not fully accommodate the difficulties described here unless the intact preparation represents a full cross-section of the population of cells from which the homogenate was derived. Even then, there must be some question as to the validity of the approach since the plasma membrane resides in a distinctly different milieu. To ensure a lower limit of purity for the two subcellular fractions, a protocol was chosen from the upper range of destain times prerequisite for effective destaining in endodermal cells, and from the lower range of stain times which effectively stained the plasma membrane in cells of the meristem.

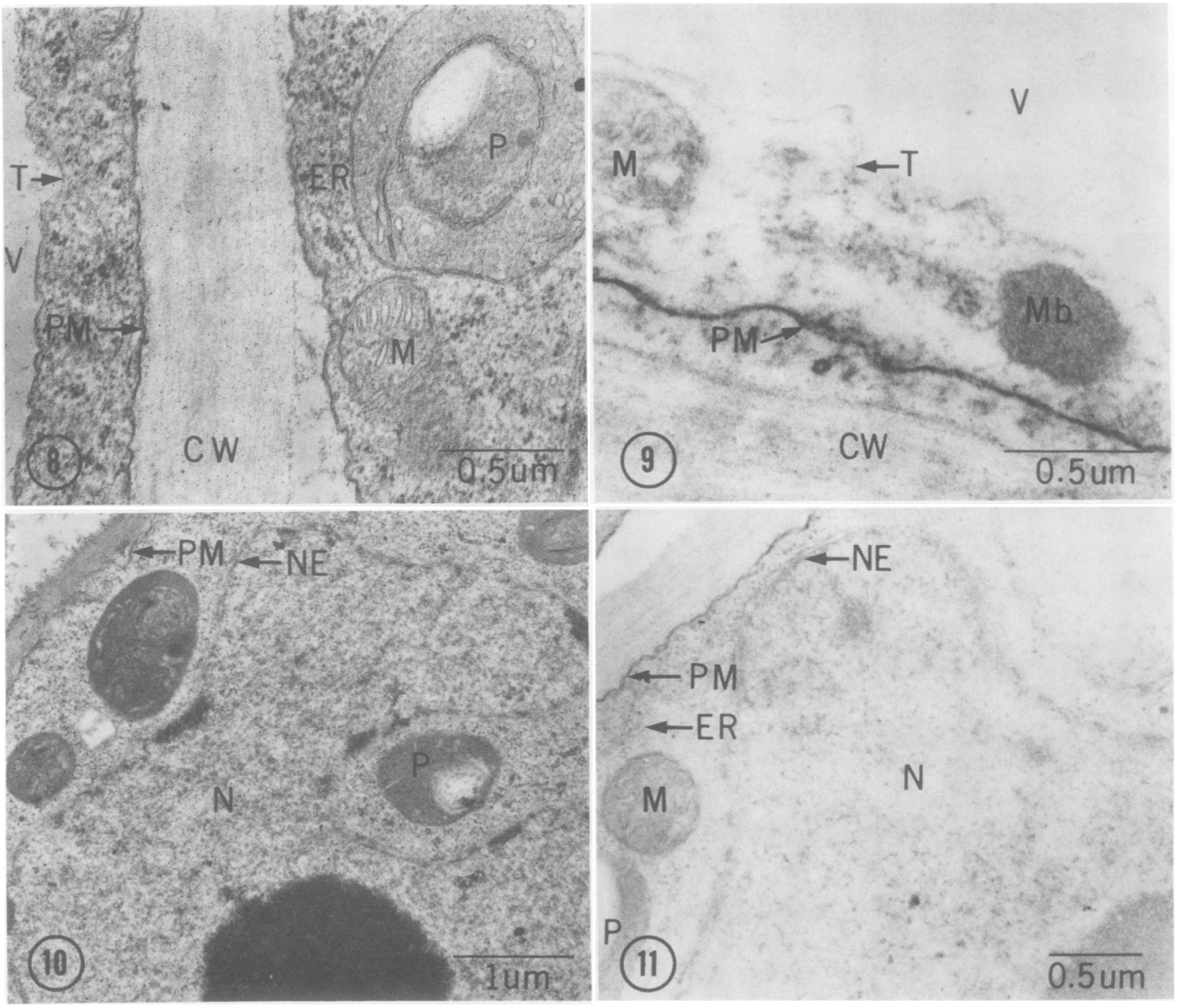
Subcellular fractions from the two regions were stained with uranyl acetate-lead citrate and PACP (Figs. 12–15). Despite certain differences in the relative integrity of the two preparations (discussed below), short PACP stain times effectively stained vesicles from both fractions (Figs. 14 and 15) indicating a possible greater homology with respect to PACP than is sometimes seen in the intact cell. Granularity or the discontinuous staining characteristic of PACP was found to be present in fractions from both developmental stages, indicating that the variability was not a function of an intrinsic biochemical or ultrastructural differentiation. Since ribosomal density is many times as great in meristematic cells as in mature tissue it appears unlikely that granularity is a function of ribonucleo-protein-membrane complexes. In fact, discontinuous staining of the plasma membrane was observed also in the intact cell, increasing with decrease in stain time. Agreement has not been reached on the type of interaction and affinity of acidic PTA for organic substrate. It has been identified primarily with the formation of a complex with highly polymerized carbohydrates (1, 12). Other studies, however, have implicated an ionic PTA-protein complex (25). In either case, the stain has been shown to be additive; stain intensity increases with stain time, with a reciprocal decrease in granularity (Figs. 16 and 17). It appears likely that patchiness of staining reflects a failure to saturate available substrate sites within the short stain times required to ensure specificity for plasma membrane.

Characterization of Mature and Meristematic Plasma Membrane Vesicles. Purified vesicular preparations from mature and meristematic homogenates, respectively, contained an average of 300 and 100 μg protein. An extremely small pellet was obtained from the meristematic region. Meristematic vesicles within the preparations were less stable, as evidenced by a greater incidence of breakage and deformation than in mature preparations, in which the large numbers of vesicles present retained good morphology.

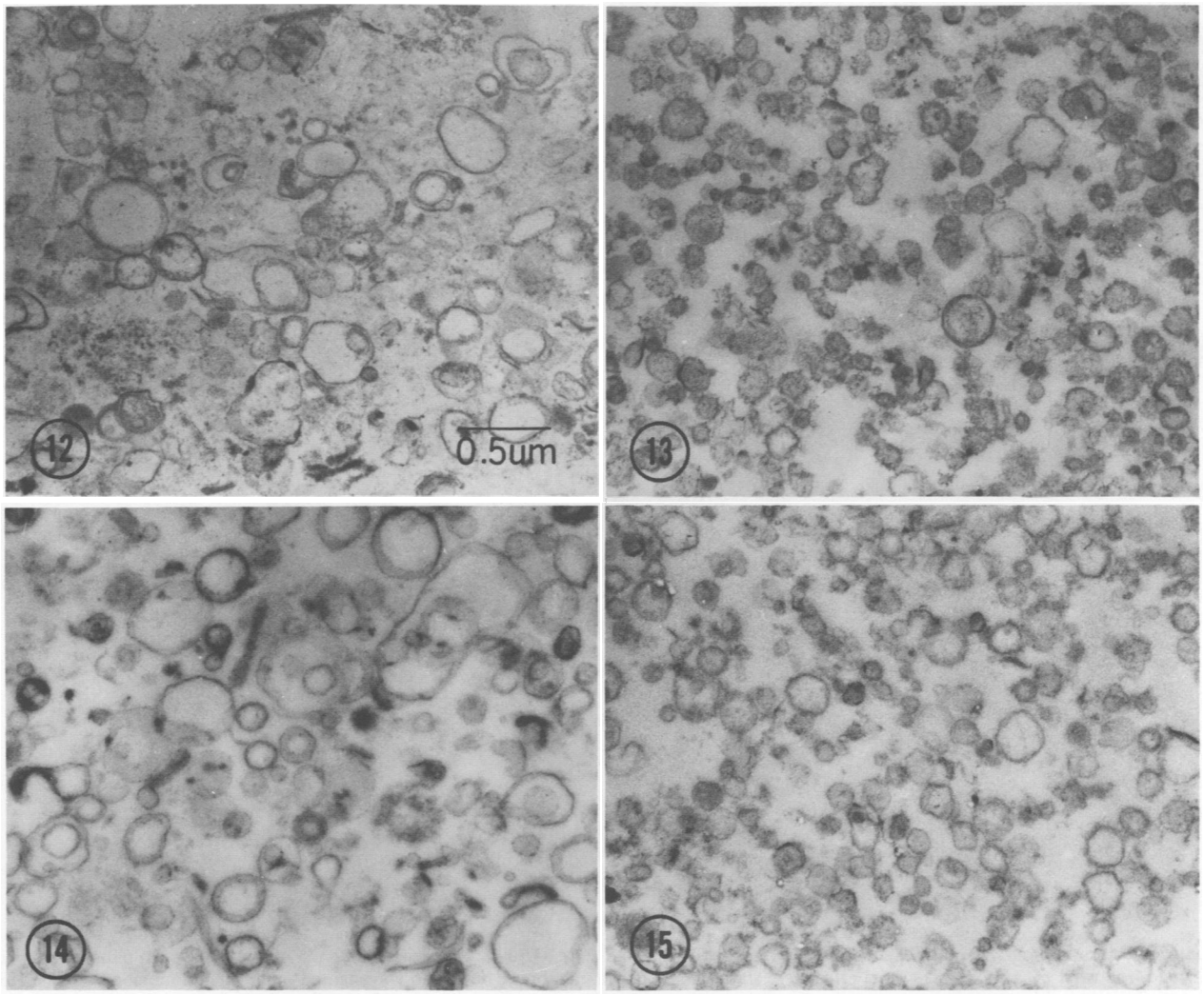
Sections stained with uranyl acetate-lead citrate were compared with sections stained with PACP (Figs. 12–15). In both mature and meristematic fractions it was estimated that more than 75% of the vesicles present were of plasma membrane origin. Similar estimates were obtained by the method of Loud (10). Mitochondrial contamination was less than 2%. Most vesicles were between 0.02 and 0.3 μm in diameter (Fig. 18). The meristematic fraction contained a higher proportion of small vesicles, whereas mature preparations contained a greater percentage of large vesicles, including those with diameters greater than 0.6 μm. An average vesicle diameter of 0.2 μm was computed for the combined populations, a figure which agrees well with the diameters reported for plasma membrane vesicles obtained from whole-root homogenates of other plant species (4, 11). In general, the diameter distribution for vesicles from the two regions was quite similar despite great differences in size between cells from the same two



FIGS. 1–7. Electron micrographs of portions of cells from the meristematic region of soybean root. 1: Meristematic cell stained with uranyl acetate-lead citrate (× 15,400). 2: Meristematic cell stained with PACP (× 11,500). 3: Cells from meristematic region stained with PACP (× 7,800). 4–5: Higher magnifications of meristematic cells stained, respectively, with uranyl acetate-lead citrate and PACP (× 31,100 and 27,460). 6: Cell wall in oblique section from meristematic cell stained with uranyl acetate-lead citrate (× 30,000). 7: Cell wall in oblique section from meristematic cell stained with PACP (× 30,000). CW: cell wall; PM: plasma membrane; N: nucleus; ER: endoplasmic reticulum; D: dictyosome; M: mitochondrion; P: plastid; L: lipid; NE: nuclear envelope; V: vacuole; T: tonoplast; Pd: plasmodesmata.



FIGS. 8–11. Electron micrographs of portions of cells from mature region of soybean root. 8: Cortical cell stained with uranyl acetate-lead citrate (× 37,000). 9: Cortical cell stained with PACP (× 50,100). 10: Stelar parenchyma cell stained with uranyl acetate-lead citrate (× 17,900). 11: Stelar parenchyma cell stained with PACP (× 27,400). CW: cell wall; PM: plasma membrane; V: vacuole; T: tonoplast, M: mitochondrion; P: plastid; ER: endoplasmic reticulum; N: nucleus; NE: nuclear envelope; Mb: microbody.



FIGS. 12–15. Electron micrographs of purified plasma membrane fractions obtained from meristematic and mature regions of soybean root by differential sucrose density centrifugation. 12: Purified plasma membrane fraction from mature region stained with uranyl acetate-lead citrate (× 26,600). 13: Purified plasma membrane fraction from meristematic region stained with uranyl acetate-lead citrate. 14: Purified plasma membrane fraction from mature region stained with PACP. 15: Purified plasma membrane fraction from meristematic region stained with PACP (× 26,600).

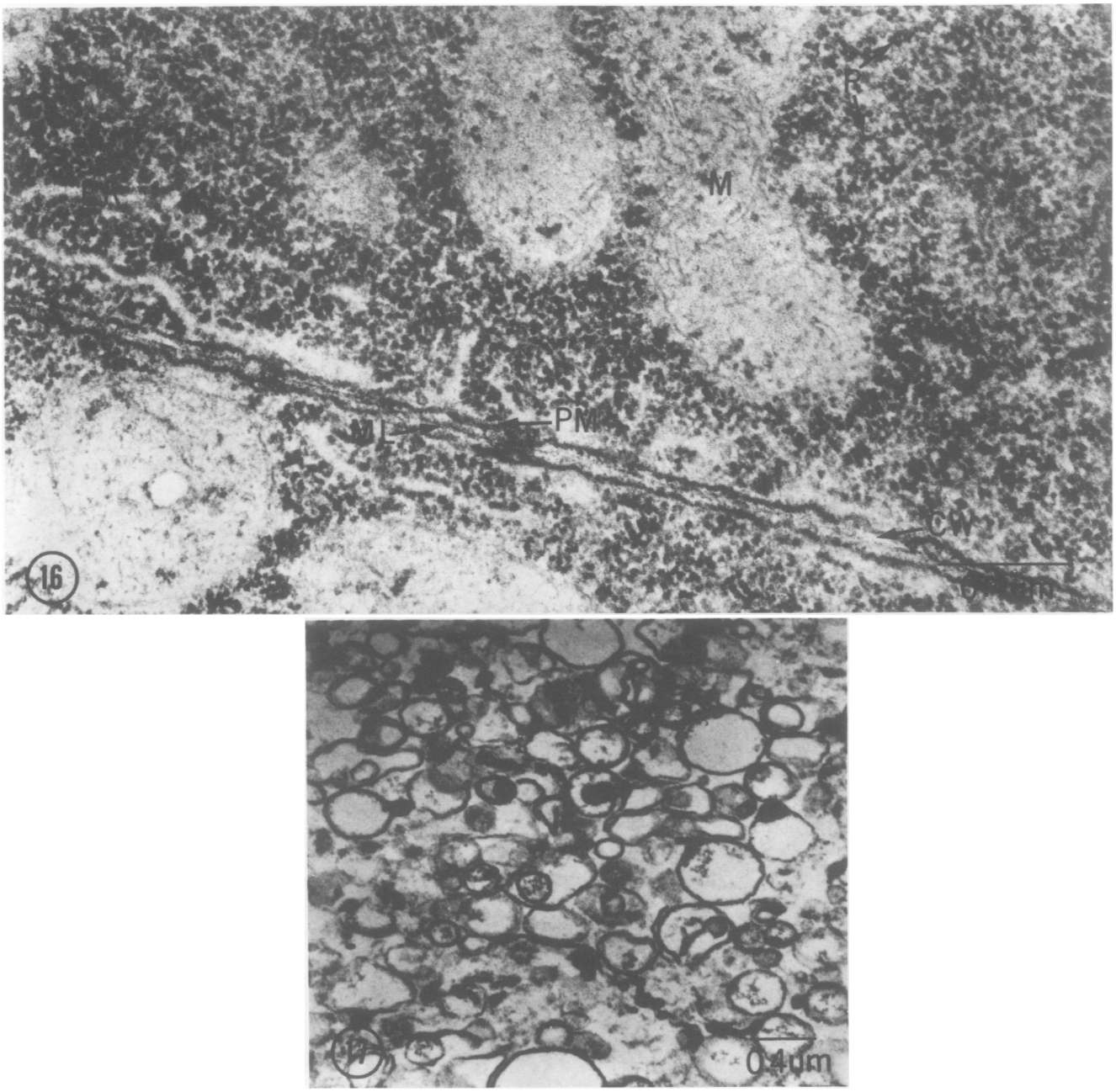


FIG. 16. Electron micrograph of meristematic cell overstained with PACP (× 53,300). A long stain time (25 min at 22 C) with phosphotungstic acid-chromic acid, followed by a short rinse time, effectively stained the plasma membrane but in no way stained other membrane components. However, intensity of ribosomal staining was greatly increased over intensity as seen in other micrographs (*cf.* Figs. 5, 9, and 11) obtained with shorter stain times (5–12 min at 22 C) and long rinse times. CW: cell wall; ML: middle lamella; PM: plasma membrane; ER: endoplasmic reticulum; M: mitochondrion, R: ribosomes.

FIG. 17. Electron micrograph of purified plasma membrane fraction from mature region overstained with PACP (× 26,700). Despite increase in stain intensity with reciprocal decrease in granularity of positively stained plasma membrane vesicle, the possible loss of specificity and increase in background are negative features which accompany long stain times (*cf.* Fig. 14).

regions. The average cell length of a cortical cell from a region 3 to 3.5 cm behind the apex was 12.5 times the length of the same cell in the vicinity of the apex. The diameter was nearly four times as great (21).

CONCLUSION

The special affinity of acidic phosphotungstic acid for the plasma membrane in cells of higher plants has been described in several cases (11, 15, 16, 23). That characteristic of PACP has been demonstrated graphically in this report by its ability to recognize plasma membrane fragments and the delimiting membrane of plasmodesmata in oblique sections of meristematic cells of the soybean root (Fig. 7). In one report (19) in which staining was not found to be absolute, some staining of tonoplast and ER was noted. It is of interest that even in that case the plasma membrane was stained uniformly, in contrast to a partial or transient staining of other membranes. Since there is little disagreement that acidic PTA effectively stains the plasma membrane, the question is whether partial staining of other membranes represents a true loss of specificity or in fact reflects some specific technical error. The loss of PACP specificity observed occasionally in cells of barley

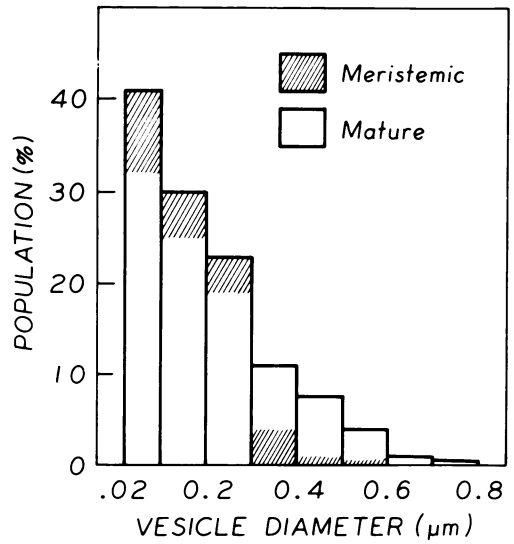


FIG. 18. Diameter distribution of meristematic and mature plasma membrane vesicles.

root (11) was attributed either to incomplete rinsing of the phosphotungstic-chromic acid or to an incomplete periodic acid treatment. In cells of soybean root, specificity was lost with incomplete periodic treatment. If nonspecific PACP staining indeed occurs in some cells but not others or in some plants and not others, such variability may in fact reflect differences in membrane composition or ultrastructure. Since PACP was found to be a reliable indicator for the plasma membrane in soybean root in a variety of cell types from different stages in the developing root, it appears unlikely that changes in membrane structure are in any way refractory to the formation of a specific phosphotungstic acid-plasma membrane complex within this plant.

Isolated plasma membrane vesicles were obtained from homogenates of meristematic and mature root tissue by differential sucrose density centrifugation. This procedure, used with varied success with roots from several plant species (4, 6, 11), effectively yielded subcellular fractions which were enriched in K-stimulated ATPase activity (20). The activity was nearly three times as high in the fraction from the meristematic region, yet sterol to phospholipid ratios indicated a similar chemical composition for the two preparations (20). With the aid of the PACP the fractions were found to be comparable in vesicle size and level of purity. It seems likely that differences in enzyme activity reflect real differences in the plasma membrane complement within each preparation and are not the result of gross levels of differential contamination.

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