Comparisons of Isolated Plasma Membranes from Plant Stems and Rat Liver[1]

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Abstract

A fraction enriched in plasma membrane was prepared from plant stems by low shear homogenization and differential and sucrose density gradient centrifugation. The plasma membrane fragments were identified by electron microscopy after differential staining with a mixture of phosphotungstic acid and chromic acid which specifically and characteristically stained the plant cell plasma membrane. Chemical and enzymatic analyses comparing plasma membrane-rich cell fractions from rat liver and onion stems showed the characteristics of the surface membrane of the plant to be different from that of its mammalian counterpart. Whereas rat liver plasma membranes were characterized by high levels of the enzymes 5′-nucleotidase and Mg++ adenosine triphosphatase and a lipid content high in sphingomyelin, these plasma membrane markers for the rat could not be demonstrated in the plant preparations.

The physiological response of any plant or animal tissue to an external stimulus involves the transmission of the stimulus across the barrier between the cell's interior and its external milieu. This barrier is the plasma membrane (or plasmalemma) plus any surface coat such as the plant cell wall. Three principal functions are usually attributed to plasma membranes: transport (both uptake and secretion); synthesis and/or assembly of surface coats; and transfer of information between the external environment and the cell's interior. Examples of such processes include uptake of ions, metabolites and growth regulators (8); secretion of enzymes (15); cell wall synthesis and assembly (23, 31); and the hormonal control of plant growth through altered cell wall mechanical properties (6, 16, 18).

Studies of the role of the plasma membrane in influencing plant processes have been largely limited to indirect methods of analysis of whole cells and tissues (4, 10). In general, this appears due to the limitations set by the lack of cell free systems suitable for the analysis of such a complex and delicate structure.

Cell membranes have been isolated from mammalian cells (2, 7, 22) but not from plant cells. Progress has been hampered by two factors: (1) the inability to recognize isolated plasma membrane fragments in cell homogenates; and (2) the lack of techniques that rupture the rigid plant cell walls without destroying the fragile plasma membrane.

This report summarizes results of a study directed toward the isolation, identification, purification and characterization of a plasma membrane-rich cell fraction from plant stems.

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Materials and Methods

Biological material. Green onions (*Allium cepa*) were purchased locally and used on the day of purchase or stored at 4°C. Stem explants were harvested by cutting roots and lignified stem regions from the onion base. A cone of tissue 0.5 to 1 cm diam at the base and 0.5 to 1 cm high was then removed from the central portion of the onion bulb using a scalpel fitted with a narrow blade. Included in the explant were stem, meristematic region and leaf bases. Scale leaves and the green (top) portions of the onion were discarded.

Livers were obtained from male rats (200 to 250 g) of the Wistar or Holtzman strains fed Purina Laboratory Chow or fasted 24 hr prior to sacrifice.

Preparation of cell fractions. Approximately 5 g of stem explants from 30 to 50 onions were collected and weighed. Homogenates were prepared using a Polytron 20ST homogenizer (Kinematica, Incerne, Switzerland) operated at slowest speed for about 60 sec (19) or a loose fitting, all-glass homogenizer of the Potter Elvehjem type. The homogenates were then squeezed through a single layer of miracloth (Chicoppe Mills, New York) to remove cell walls and tissue fragments and to break additional cells. Centrifugations were for 30 min using the SW-39 L rotor for the Spinco Model L ultracentrifuge operated at 4°C.

A nuclei fraction, containing occasional wall fragments, proplastids and mitochondria was obtained by low speed centrifugation (3,000 rpm for 10 min). A fraction enriched in proplastids was obtained at 5,000 rpm and a fraction enriched in mitochondria at 10,000 rpm. The fraction sedimenting between 10- and 20,000 rpm contained dictyosomes of the Golgi apparatus and a variety of smooth membrane vesicles suspected of being a mixture of plasma membrane and tonoplast (vacuole membrane) fragments. Everything sedimenting between 20- 30,000 rpm was included in the microsome fraction and consisted largely of fragments of rough endoplasmic reticulum. The 30,000 rpm supernatant is referred to as the soluble fraction of the cytoplasm.

The following homogenization media were employed:

(1) 0.5 M sucrose containing 10 mM sodium phosphate, pH 6.8 and 1% (w/v) dextran (Sigma average mol. wt. 225,000) (20);

(2) 0.5 M sucrose containing 37.5 mM Tris-maleate, pH 6.5; 1% dextran and 5 mM $MgCl_2$ (19);

(3) Medium II minus $MgCl_2$ (for estimation of phosphatidic acid phosphatase which was inhibited by Mg^{++});

(4) 1 mM sodium bicarbonate (22).

Media (1), (2) and (3) gave smiliar results with plant preparations. Medium (4) was used in the rat liver preparations.

Further fractionation of the 10- to 20,000 rpm fraction from plant stems was obtained by centrifugation in a layered sucrose gradient yield-

ing 5 bands (21). The uppermost band contained lipid droplets and was discarded. The lowest bands contained mitochondria and endoplasmic reticulum. The intermediate bands yielded dictyosomes and smooth membranes free of dictyosomes.

Plasma membrane fractions from rat liver were obtained by a modification (7) of Neville's procedure (22). Golgi apparatus, endoplasmic reticulum and other cell fractions from rat liver were obtained as described previously (3, 14, 19).

Chemical assays. Protein was determined by the Lowry procedure (13) or by the biuret method. Inorganic phosphorus was determined by the method of Fiske and Subbarow (9). Polar lipids were separated by two dimensional thin layer chromatography (12) and analyzed for phosphorus by the procedure of Rouser *et al.* (27) as modified by Parsons and Patton (24). Identity of the separated lipids was established by co-chromatography with authentic reference compounds (Applied Science Laboratories, State College, Penn.). Total sterols were measured by the method of Jorgenson and Dam (11).

Morphological assays (electron microscopy). Portions of isolated pellets were fixed in 6% buffered glutaraldehyde (0.1 M potassium phosphate, pH 7.2) for 18 to 20 hr with or without post fixation for 1 to 24 hr in 1% osmium tetroxide (in 0.1 M sodium phosphate, pH 7.2). Specimens were dehydrated through an acetone series; embedded in vestopal and sections were stained by one of the following procedures:

(1) (with osmium post fixation)—Sections were stained with aqueous uranyl acetate and/or lead citrate (25).

(2) (with osmium post fixation)—Sections were treated with 1% periodic acid for 30 min followed by 5 washes of 10 min each with distilled water. The sections were then treated with a mixture of 1% phosphotungstic acid plus 10% chromic acid in water (pH less than 1) for 2 to 5 min (PTA-CA procedure) (2). Finally, the sections were washed to remove excess stain and mounted on copper grids.

(3) (no osmium)—Sections were treated directly with the mixture of 1% phosphotungstic acid plus 10% chromic acid in water for 2 to 5 min; washed free of excess stain and mounted on copper grids (26).

Enzyme assays. Enzyme assays used 0.1 to 0.8 mg protein in a final volume of 1 to 3 ml. The following enzymatic activities were determined according to the procedures referenced: 5'-nucleotidase (7); Mg^{++}-ATPase (7); Na^{+}-K^{+}-Mg^{++}-ATPase (7); phosphatidic acid phosphatase (28); and invertase (5). Assays were carried out at the pH optima for the total particulate fraction determined separately for liver and for onions (see Fig. 5, for example) and at near optimum temperatures for each system (37°C for liver and 25°C for onions).

Results

When onion stem homogenates were fractionated, the fraction sedimenting between 20- and 30,000 rpm contained smooth (ribosome-free) membrane vesicles of various sizes, many of which were suspected of

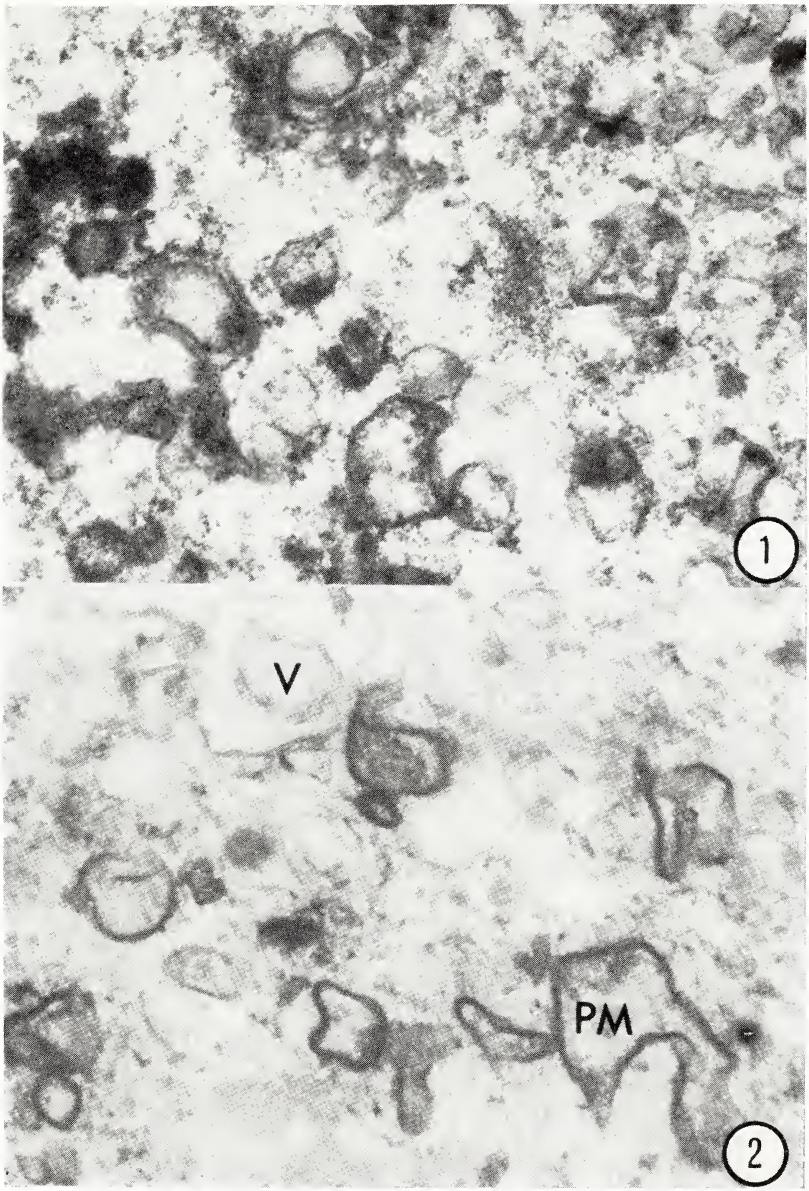

FIGURE 1. *Electron micrograph of an isolated cell fraction containing numerous smooth (ribosome-free) vesicles of onion stem. Obtained by centrifugation between 20- and 30,000 rpm and fixed for 18 hours in 6% buffered glutaraldehyde with post fixation for 24 hours in 1% osmium tetroxide. Section stained with lead citrate and uranyl acetate (Method 1). Isolation Medium 3. X 32,600.*

FIGURE 2. *As in Figure 1 except section staining Method 2 using phosphotungstic acid plus chromic acid which differentiates the darkly staining plasma membrane vesicles (PM) from other smooth vesicles (V) presumed to represent tonoplast fragments. X 32,600.*

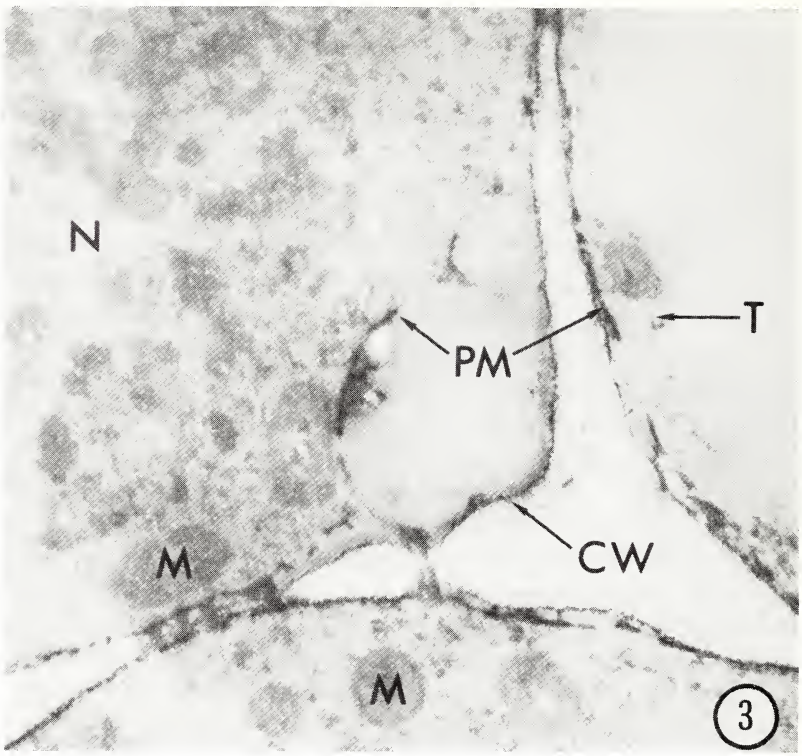

FIGURE 3. *Onion stem tissue fixed in 6% glutaraldehyde for 20 hours and section stained using Method 3. This electron micrograph shows that in the whole cell, the plasma membrane (PM) is the only cell component staining with the phosphotungstic acid-chromic acid mixture except for cell wall which occasionally stains (CW). Nuclei (N), tonoplast (T) and mitochondria (M) are unstained. X 6,400.*

being derived from plasma membrane (Fig. 1). Dictyosomes and mitochondria were also present. However, using ordinary lead and uranyl ion-based staining methods (Method 1), vesicles tended to look alike; for example, those derived from plasma membrane could not be distinguished from those derived from the vacuolar membrane (tonoplast).

To identify plasma membrane-derived vesicles, it was necessary to use a staining procedure specific for plasma membrane. The PTA-chromic acid procedure of Roland (26) was adapted for use with isolated cell fractions (Fig. 2). When applied to whole plant cells, the plasma membrane appeared as a dense, darkly-stained line (Fig. 3). The only other cell component staining with the phosphotungstate-chromic acid procedure (Method 2 or Method 3) was the cell wall. Nuclear membranes, mitochondria, proplastids, Golgi apparatus, endoplasmic reticulum and tonoplast did not stain.

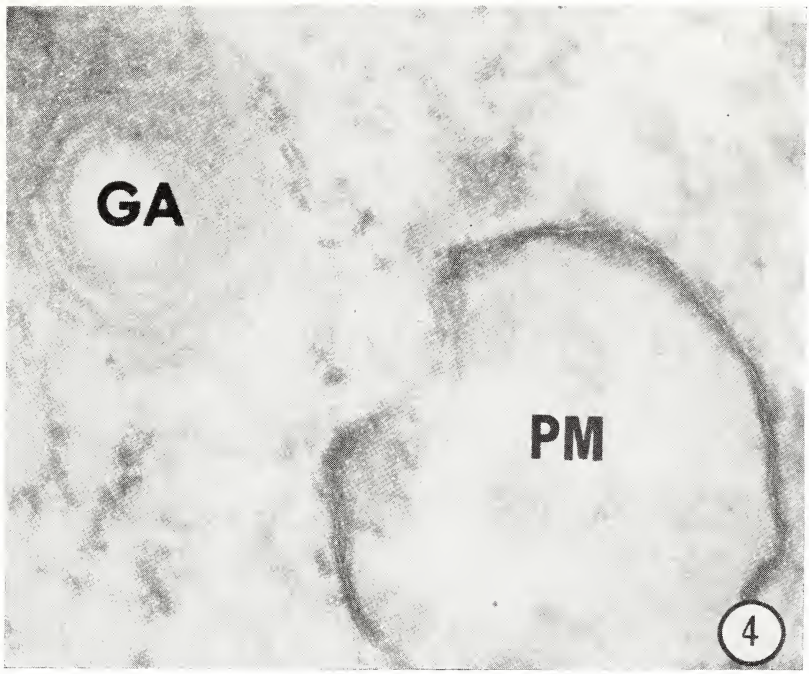

FIGURE 4. *Plasma membrane fragment (PM) at higher magnification showing dark-light-dark trilamellar pattern of the membrane. Isolated dictyosome of the Golgi apparatus (GA) shown for comparison is unstained. Conditions as in Figure 1. X 100,000.*

A similar range of specificity was encountered with the isolated cell fractions. Using staining methods (2) or (3), it was easy to recognize which of the fragments of the pellets were plasma membrane-derived (Fig. 2). Figures 1 and 2 were obtained from sections of the same tissue block. At higher magnifications (Fig. 4), the stained membranes retained the dark-light-dark trilamellar staining pattern characteristic of the plasma membrane when fixed *in situ* and stained by conventional methods. No differences in staining quality could be detected among the cell fractions isolated in each of the three homogenization media used for onion stem.

We estimate our plant fraction to be no more than 50% plasma membrane-derived, but a comparison of some of the properties of the crude preparations with purified plasma membrane fractions from rat liver shows them to be different (Table 1). A striking difference concerns the presence of the so-called marker enzymes for plasma membrane from mammalian sources. These include 5'-nucleotidase, Mg^{++}-adenosine triphosphatase and the Na^{+}-K^{+}-stimulated Mg^{++}-adenosine triphosphatase (2) which are concentrated in rat liver plasma membrane but appear to be absent from the corresponding plant fraction. The results are exemplified by studies of 5'-nucleotidase using AMP as substrate. When examined at pH 7.0, a near optimum pH for the rat liver enzyme,

total homogenate of onion stem had a specific activity of 0.06μmoles inorganic phosphorous/hr/mg protein as compared with 1 to 2 μmoles/hr/mg protein for rat liver homogenates. In contrast, the plant enzymes exhibited a pH optimum at about pH 5.5 with a maximum spe-

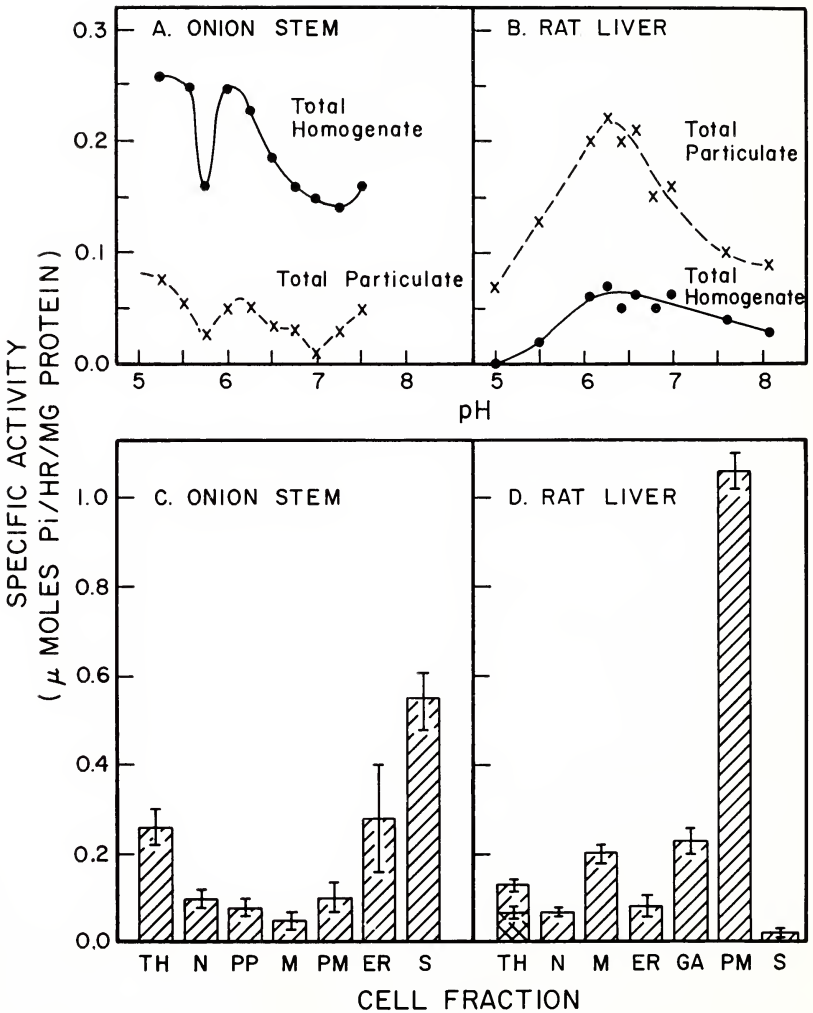

FIGURE 5. *Distribution of phosphatidic acid phosphatase among cell fractions of onion stem and rat liver:*

A. pH relationship of total homogenate and particulate fraction of onion stem.

B. pH relationship of total homogenate and particulate fraction of rat liver.

C. Distribution of activity among cell fractions of onion stem.

D. Distribution of activity among cell fractions of rat liver.

(Key to labeling: TH = total homogenate. N = nuclei-; PP = proplastid-; M = mitochondria-; PM = plasma membrane-; ER = endoplasmic reticulum (microsomes)-; and GA = Golgi apparatus-rich cell fractions. S = soluble fraction of the cytoplasm.)

cific activity at that pH of 0.16 μmoles/hr/mg protein. With both the 5'-nucleotidase and the adenosine triphosphatase, 93% of the activity was in the soluble fraction. The remaining 7% of the combined particulate fractions was removed by washing and no activity was detected in purified plasma membrane fractions.

Phosphatidic acid phosphatase is a fourth enzyme associated with the rat liver plasma membrane (17). This enzyme showed a distribution in the plant similar to that of the 5'-nucleotidase with more than 90% of the activity being soluble, but the particulate fraction was active (Fig. 5A). In contrast, the rat liver phosphatidic acid phosphatase activity was largely particulate (Fig. 5B). The rat liver enzyme showed a single pH optimum at pH 6.25, whereas the plant preparations showed three optima: in the vicinity of pH 5.5; pH 6.0 to 6.25 and pH 8.0 to 8.5. The phosphatidic acid phosphatase activity at pH 6.25 for the plant was more or less equally distributed among the various cell fractions and not concentrated in the plasma membrane fraction as in rat liver (compare Figs. 5C and 5D).

Invertase is one enzyme associated with the cell surface of plant cells (30). Specific activities of various fractions in mμmoles reducing sugar/hr/mg protein were: 0-10,000 rpm fraction = 1.8; 10 to 20,000 rpm fraction (plasma membrane rich) = 0.4; and 20 to 30,000 rpm fraction (microsomes) = 0.

The purity of the fractions precluded a complete phospholipid analysis, but sphingomyelin, a major constituent of the rat liver plasma membrane, was absent from the plant fraction (Table 1). Both fractions were sterol rich although the predominant sterols of plants (stigmasterol and β-sitosterol) are different from those of rat liver (largely cholesterol).

Discussion

Junctional complexes, hexagonal subunit patterns, 100 Å knobs and sialioproteins, all reliable markers for the animal plasma membrane (1, 2, 14), were absent from the plant cell surface. Thus, the first problem was to devise a way to recognize plasma membrane in an isolated cell fraction in the absence of positional relationships to other cell components.

By treating glutaraldehyde-fixed pellets with or without osmium post fixation with a mixture of phosphotungstic acid and chromic acid (PTA-CA procedure), the plant plasma membrane stained specifically and characteristically (26). This staining pattern appears to be applicable to plasma membranes from plants other than onion (26).

Certain enzymes that have proven to be reliable markers for the animal cell membrane appear to be absent from the plant cell surface. Histochemical studies have revealed that certain nucleoside mono-, di- and triphosphatases may be present in the plasma membrane of some plants (29), but even when present they vary from cell to cell type and

TABLE 1. *Comparison of the properties of plasma membrane-rich cell fractions isolated from plant and mammalian sources.*

Constituent	Specific Activity or Amount	
	Rat Liver	Onion Stem
5'-Nucleotidase	50 μmoles/hr/mg protein	not detected
Mg^{++}-adenosine triphosphatase	45 μmoles/hr/mg protein	not detected
Mg^{++}-Na^{+}-K^{+}-adenosine triphosphatase	10 μmoles/hr/mg protein	not detected
Phosphatidic acid phosphatase	1 μmole/hr/mg protein	0.1 μmoles/hr/mg protein
Sphingomyelin	18% of total lipid phosphorous	not detected
Sialic acid	32 mμm/mg protein	not detected
Sterols	5-6% of dry weight	present
Buoyant density	1.16-1.18	est. 1.4*

*Determined for membranes stabilized by the addition of 50 mM glutaraldehyde to the initial homogenization medium.

are often difficult to demonstrate. We found low levels of AMP hydrolyzing activity in the soluble fraction with a pH optimum in the acid range. They probably represent unspecific acid hydrolases. A specific membrane-associated 5'-nucleotidase was not demonstrated. With rat liver plasma membrane, Widnell (32) showed that 5'-nucleotidase is a lipoprotein specifically complexed with sphingomyelin. When the sphingomyelin is removed, enzyme activity is lost. It is of interest that the plant plasma membrane fractions contain neither sphingomyelin nor 5'-nucleotidase.

With phosphatidic acid, the substrate is not only hydrolyzed by the acid hydrolases of the soluble fraction from onion stem but also by an activity of the particulate fraction. This activity has a pH optimum in the range 6.0 to 6.25, similar to that for rat liver. A small fraction of this activity appears to be associated with the plasma membrane-rich fraction, but is present in all cell fractions and cannot be used as a marker enzyme as it is with rat liver.

Our results support the contention of Emmelot and Benedetti (2) that the plasma membrane is a highly differentiated membrane system with its characteristics and composition varying according to species, cell type and perhaps even the metabolic state of the cell. Our comparisons of rat liver vs. onion stem show the plasma membranes to be different in many ways and that species differences are reflected in the characteristics of the surface membrane.

Literature Cited

1. BENEDETTI, E. L., and P. EMMELOT. 1967. Studies on plasma membranes. IV. The ultrastructural localization and content of sialic acid in plasma membranes isolated from rat liver and hepatoma. J. Cell Sci. **2**:499-512.
2. BENEDETTI, E. L., and P. EMMELOT. 1968. Structure and function of plasma membranes isolated from liver, p. 33-120. *In* A. Dalton and F. Haguenau [eds.] The Membranes. Academic Press, New York.
3. CHEETHAM, R. D., D. J. MORRÉ, and W. N. YUNGHANS. 1970. Isolation of a Golgi apparatus-rich fraction from rat liver. II. Enzymatic characterization and comparison with other cell fractions. J. Cell Biol. **44**:492-500.
4. CLOWES, F. A. L., and B. E. JUNIPER. 1968. Plant Cells. Blackwell Scientific Publications, Oxford and Edinburgh. 546 p.
5. EDLEMAN, J., and M. A. HALL. 1965. Enzyme formation in higher-plant tissues. Development of invertase and ascorbate-oxidase activities in mature storage tissue of *Helianthus tuberosus* L. Biochem. J. **95**:403-410.
6. EISINGER, W. R., and D. J. MORRÉ. 1968. The effect of sulfhydral inhibitors on plant cell elongation. Proc. Indiana Acad. Sci. **77**:136-143.
7. EMMELOT, P., C. J. BOS, E. L. BENEDETTI and PH. RÜMKE. 1964. Studies on plasma membranes. I. Chemical composition and enzyme content of plasma membranes isolated from rat liver. Biochim. Biophys. Acta **90**:126-145.
8. EPSTEIN, E. 1960. Spaces, barriers, and ion carriers: Ion absorption by plants. Amer. J. Bot. **47**:393-399.
9. FISKE, C. H., and Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**:375-400.
10. FREY-WYSSLING, A., and K. MÜHLETHALER. 1965. Ultrastructural Plant Cytology. Elsevier Press, Amsterdam, The Netherlands.
11. JORGENSEN, K. H., and H. DAM. 1957. An ultra micromethod for the determination of total cholesterol in bile, based on the Tschugaeff color reaction. Acta Chemica Scand. **11**:1201-1208.
12. KEENAN, T. W., and D. J. MORRÉ. 1970. Phospholipid class and fatty acid composition of Golgi apparatus isolated from rat liver and comparison with other cell fractions. Biochemistry **9**:19-25.
13. LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
14. MIDDLETON, A. E., R. CHEETHAM, D. GERBER and D. J. MORRÉ. 1969. Adenosine mono-, di- and trinucleotidase activities of rat liver cytomembranes. Proc. Indiana Acad. Sci. **78**:183-188.
15. MORRÉ, D. J. Cell wall dissolution and enzyme secretion during leaf abscission. Plant Physiol. **43**:1545-1559.
16. MORRÉ, D. J., and W. R. EISINGER. 1968. Cell wall extensibility: Its control by auxin and relationship to cell elongation, p. 625-645. *In* F. Wightman and G. Setterfield [eds.] Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa, Canada.
17. MORRÉ, D. J., T. W. KEENAN and H. H. MOLLENHAUER. In press. Golgi apparatus function in membrane transformations and product compartmentalization: Studies with cell fractions from rat liver. Proc. 1st Intern. Sym. Cell Biol. Cytopharm., Venice, Italy, 1969.
18. MORRÉ, D. J., and J. L. KEY. 1967. Auxins; pp. 575-593. *In* F. H. Wilt and N. K. Wessels [eds.] Methods in Developmental Biology. Thomas Y. Crowell Co., New York.
19. MORRÉ, D. J., R. L. HAMILTON, H. H. MOLLENHAUER, R. W. MAHLEY, W. P. CUNNINGHAM, and V. S. LEQUIRE. 1970. Isolation of a Golgi apparatus-rich fraction from rat liver. I. Method and morphology. J. Cell Biol. **44**:484-491.
20. MORRÉ, D. J., and H. H. MOLLENHAUER. 1964. Isolation of the Golgi apparatus from plant cells. J. Cell Biol. **23**:295-305.

21. MORRÉ, D. J., H. H. MOLLENHAUER and J. E. CHAMBERS. 1965. Glutaraldehyde stabilization as an aid to Golgi apparatus isolation. Exp. Cell Res. **38**:672-675.

22. NEVILLE, D. M. 1960. The isolation of a cell membrane fraction from rat liver. J. Biophys. Biochem. Cytol. **8**:413-422.

23. ORDIN, L., and M. A. HALL. 1967. Studies on cellulose synthesis by a cell-free oat coleoptile enzyme system: Inactivation by airborne oxidants. Plant Physiol. **42**:205-212.

24. PARSONS, J. G., and S. PATTON. 1967. Two dimensional thin-layer chromatography of polar lipids from milk and mammary tissue. J. Lipid Res. **8**:696-699.

25. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208-212.

26. ROLAND, J. C. 1969. Mise en évidence sur coupes ultrafines de formations polysaccharidiques directement associées au plasmalemme. C. R. Acad. Sc. Paris **269D**:939-942.

27. ROUSER, G., A. N. SIAKOTOS and S. FLEISCHER. 1966. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorous analysis of spots. Lipids **1**:85-86.

28. SMITH, S. W., S. B. WEISS and E. P. KENNEDY. 1957. The enzymatic dephosphorylation of phosphatidic acids. J. Biol. Chem. **228**:915-922.

29. POUX, N. 1967. Localisation d'activités enzymatiques dans les cellules du méristème radiculaire de *Cucumis sativus* L. I. Activités phosphatasiques neutres dans les cellules du protoderme. J. Microscopie **6**:1043-1058.

30. STRAUS, J. 1962. Invertase in cell walls of plant tissue cultures. Plant Physiol. **37**:342-348.

31. VILLEMEZ, C. L., J. M. MCNAB and P. ALBERSHEIM. 1968. Formation of plant cell wall polysaccharides. Nature **218**:878-880.

32. WIDNELL, C. C., and J. C. UNKELESS. 1968. Partial purification of a lipo-protein with 5'nucleotidase activity from membranes of rat liver cells. Proc. Nat. Acad. Sci. **61**:1050-1057.