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Hunt Institute was dedicated in 1961 as the Rachel McMasters Miller Hunt Botanical Library, an international center for bibliographical research and service in the interests of botany and horticulture, as well as a center for the study of all aspects of the history of the plant sciences. By 1971 the Library's activities had so diversified that the name was changed to Hunt Institute for Botanical Documentation. Growth in collections and research projects led to the establishment of four programmatic departments: Archives, Art, Bibliography and the Library.

SCIENCE

①

Author **MORRE, D. J.; BRACKER, C. E.; VANDENWOUDS, W. J.**

Title **Pinocytosis in Plants? -- or Exocytosis?**

Comments: 1) According to Wheeler & Hanchey, the fact that pinocytotic vesicles are dilated is an indication that these vesicles originated from the plasmalemma. If this is the case then the contents of the vesicles (whether they contain uranyl granules or not) would be of secondary importance.

2) If uranyl ions move into the cytoplasm in the outer secretory cells and if the packets in figs C & D are moving outward, why do those that bind have about the same crystal content as the secretory products outside the protoplast?

3) Newcomb (in 1969 Ann. Rev. Pl. Physiol. 20: 254) reports the "average value of microtubules as "about 240 A". But they add: "because of technical and biological variations, ordinarily no great significance can be assigned to reported differences in diameter." If this is accepted, can the discrepancies between your measurements and Wheeler & Hanchey's (not very large in any case) be critical?

(Continue on additional sheet if necessary)

Overall Evaluation

- ☐ Excellent, merits rapid publication
- ☒ Publish if space is available
- ☐ Belongs in a specialty journal
- ☐ Should not be published anywhere

If you recommend publication in Science, please check one or more of the following:

- ☐ Opens a new and significant area of research
- ☐ In an established field, rates in the upper tenth with respect to significance
- ☒ Has broad appeal to non-specialists
- ☒ Is important to specialists in three or more disciplines, namely

physiology

cytology

pathology

Confidential Comments:

My impression is that the general tone of the authors' criticism is harsh. Considering the controvertial nature of the subject, this hardly seems warranted. In any case, if the paper is published, Wheeler & Hanchey should certainly be given equal space for rebuttal.

LOUISIANA STATE UNIVERSITY
AND AGRICULTURAL AND MECHANICAL COLLEGE

BATON ROUGE · LOUISIANA · 70803

College of Arts and Sciences — Agricultural Experiment Station

DEPARTMENT OF BOTANY AND PLANT PATHOLOGY

DEPARTMENT OF PLANT PATHOLOGY

Feb. 8, 1971.

Dear Harry,

Enclosed is a xerox copy of a paper sent to me by Science for review. The authors question a number of points in your Jan. 1971 paper, which of course they are entitled to do, but the whole tone of their comments seems to me unnecessarily harsh. i.e. "This article merely adds to the confusion " (p. 5) and "..... the consequences of propagating such unsubstantiated concepts are obvious."

No doubt you will make a rebuttal at the proper time and place, but for the purpose of this review, if you would care to make a few succinct statements, I would like to incorporate them into my criticism of the Morra' et al. letter.

Sincerely,

Bernie

Feb. 10, 1971

Dear Bernie:

The three main points:

1) Morré says that in the outer secretory cells uranyl ions move into the cytoplasm, are bound in secretory vesicles, and that the packets we see inside (see Fig. C + D) are moving out. We say if so, why do only a few secretory vesicles bind uranyl and why do those that do bind always have about the same number of crystals as secretory products outside the protoplast?

2) Morré says that in Fig. F, G, J. + K the plasma membrane is of normal width. We say it is not and that the fact that only the plasmalemma and the membranes around pinocytotic vesicles are dilated provides evidence that the vesicles arose from the plasmalemma and are moving into the protoplast rather than vice-versa. His objection that these vesicles do not contain uranyl crystals is irrelevant - if the vesicles arose from the plasmalemma, then they are pinocytotic regardless of their content.

3. He says we ignore evidence against pinocytosis - we say there is no such evidence.

Best wishes
Harry

SCIENCE

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Author MORRE, D. J.; BRACKER, C. E.; VANDERWOUDE, W. J.

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physiologycytologypathology

Confidential Comments:

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JAN 22 1971

For: SCIENCE (Technical Comment)

PINOCYTOSIS IN PLANTS? -- OR EXOCYTOSIS?

At best, an electron micrograph represents a frozen moment in the eternal flux of life. Thus, it is necessary to employ reliable markers and objective criteria if dynamic processes are to be interpreted from these static images of biological systems. Wheeler and Hanchey's article claiming support of pinocytosis in plant roots (8 January, p. 68) not only attempts to derive dynamic interpretations from static images in the absence of correlative information but at the same time fails in its avowed purpose of presenting evidence in support of pinocytosis. We call attention to considerations that challenge Wheeler and Hanchey's interpretations concerning pinocytosis in plant roots and offer an alternative explanation of their results.

The experiments in question consisted of placing oat roots in toxic solutions of uranyl acetate, followed by "desorption" in the absence of a counter ion, transfer to fixative solution, and preparation for electron microscopy. Subcellular location of uranyl crystals in electron micrographs and the conformation of the plasma membrane in chemically fixed cells were the only criteria applied for recognizing pinocytotic activity. We contend that the results would be better interpreted within the existing framework of ion absorption and exocytosis.

Most of the electron micrographs presented were derived from cells of the outer rootcap. In outer rootcap cells, the Golgi apparatus is known to produce large secretory vesicles (1) containing acidic polysaccharides (2). The direction of migration of these vesicles is

outward, i.e., they are clearly exocytotic and not endocytotic vesicles, as established by detailed physiological (3) and autoradiographic (4) studies. The anionic polymers in the cell wall and within these secretory vesicles (2) not only bind UO_2^{2+} readily, but would explain why uranyl crystals were localized primarily within the cell wall and the contents of these secretory vesicles in the Wheeler and Hanchey study. This is especially evident in their Figures 1A and B where masses of secretory product in an extraprotoplasmic location (i.e., already discharged from the protoplast and no longer surrounded by a limiting membrane) show uranyl binding equal to or greater than that of the secretory vesicles. The authors state that "most crystals were confined to the wall and structures in direct contact with it." However, "some packets of crystals were within the protoplast." To conclude pinocytosis from this evidence, assumes the impermeability of the plasma membrane and vesicle membrane to uranyl ions. This assumption is not warranted (5). Uranyl ions are taken up by plant roots and could cross the plasma membrane through normal, non-pinocytotic cation transport mechanisms (5). They would then be free to diffuse through the cytoplasm. Membranes surrounding secretory vesicles of the Golgi apparatus are plasma membrane-like and capable of fusing with the plasma membrane (6). On the basis of the plasma membrane-like character of the vesicle membranes, the same cation transport mechanisms would be expected to be associated with membranes of secretory vesicles. Thus, there is no reason to believe that uranyl ions cannot enter secretory vesicles of the Golgi apparatus and bind to the anionic polymers of the secretory product in the same manner as if the secretory product were external to the protoplast.

That uranyl ions appear in vacuoles of roots exposed to uranyl acetate for 10-20 hr is not unexpected, since the vacuole is a well established repository for diffusing cations in plant cells. Why discrete uranyl crystals appeared in the vacuole is less readily explained. Yet, in cells of the rootcap interior where conspicuous polysaccharide-containing vesicles were absent, uranyl crystals were restricted to cell walls and vacuoles, and the authors provided no evidence for pinocytotic activity. In cells of the rootcap exterior where conspicuous polysaccharide-containing vesicles were present, the authors provided no evidence for the occurrence of crystals in vacuoles. Even assuming that an occasional secretory vesicle did re-enter the cytoplasm briefly from the protoplast surface after initial contact with the plasma membrane, this does not constitute a demonstration of pinocytotic activity since the flow of materials is still outward.

What the article fails to prove or even consider adequately is the direction of migration of the secretory vesicles which bind uranyl ions in the outer rootcap cells. The authors assume a priori that migration is from the exterior to the interior. This assumption is totally untenable in view of physiological (3) and autoradiographic (4) studies of outer rootcap cells which show vesicle migration to be in exactly the opposite direction. In fact, their own results provide an excellent demonstration of exocytosis. In outer rootcap cells subjected to 1mM uranyl acetate for 1 hr and then "desorbed" in the absence of uranyl acetate for 2 hr (Fig. 1D), uranyl crystals were restricted to vesicles adjacent to the cell wall, whereas secretory vesicles in more interior positions were free of crystals. This is exactly the result predicted for exocytotic vesicles and exactly

opposite the result predicted for pinocytotic vesicles. If the vesicles had been migrating in the opposite direction as claimed by the authors, the stained vesicles should have occupied the interior position. That the stained vesicles occupied the exterior position shows the process under study to be exocytosis and not pinocytosis as claimed.

Other evidence cited in support of pinocytosis was the irregular, invaginated profile of the plasma membrane in cells exposed to uranyl acetate. An irregular profile of the plasma membrane is a common plant response to injury (7) including that induced by uranyl acetate (the concentrations of uranyl acetate employed were toxic). Furthermore, it is significant that none of the structures labeled as plasma membrane invaginations or as pinocytotic vesicles in Fig. 1F and G contained uranyl crystals, even though crystals were present in adjacent cell wall regions. Wheeler and Hanchey show invaginations of the plasma membrane but provide no evidence to implicate a function of these invaginations in any type of transport.

The authors use membrane characteristics to support their interpretation of pinocytosis. They emphasize the effects of uranyl ions on the dimensions and conformation of the plasma membrane and "membranes of vesicles apparently derived from it", but no control cells are illustrated. Even the reported membrane dimensions are suspect. In fact, in Fig. 1J inset, comparison of the thickness of a so-called "dilated" plasma membrane profile with two adjacent microtubules in cross section shows the plasma membrane to have dimensions in the 90-100 Å range, not in the 150-200 Å range reported as characteristic of "dilated" plasma membrane profiles in their study. If the correction derived from the microtubule diameters in Fig. 1J is applied to Figs.

1G and K, the membrane thickness in the latter figures is also 90-100 Å and hence in the normal range for plasma membranes, secretory vesicles and tonoplasts. Furthermore, the membranes they report as being dilated are those which normally appear thicker than other cytoplasmic membranes and show the dark-light pattern most clearly (6). What the authors describe as membrane overlap would result from the tilt of the membrane in the section.

Wheeler and Hanchey propose that anionic polymers serve multiple functions in pinocytosis. They suggest that acidic polysaccharides of secretory products might "account for cell wall swelling and invaginations of the plasmalemma" in addition to binding uranyl ions for transport. Yet, their evidence fails to show both binding of uranyl ions to secretory product and "swelling" or invagination to occur simultaneously as would be necessary for this argument to support their interpretations relative to pinocytosis. None of their figures showing "cell wall swelling" or "plasma membrane invaginations" (Figs. 1F, G and J) clearly show uranyl-binding secretory products. Furthermore, the cell wall described as swollen (Fig. 1J) is actually a lomasome-type structure and shows few if any uranyl crystals of any sort.

The great body of evidence against the occurrence of pinocytosis in plant roots (cf. 8) is largely ignored in the report as is the requirement for examining living cells as a requisite to establishing the reality of the process (8). We can only conclude that pinocytosis, if it occurs in plant roots, has not been demonstrated in the Wheeler and Hanchey article. This article merely adds to the confusion already generated by an overabundance of inconclusive experimentation in this important area of plant physiology.

We call attention to this study in order to stem the inevitable result of it having appeared in SCIENCE -- namely widespread acceptance

of pinocytosis in plant roots as a valid concept. If left unchallenged, the consequences of propagating such unsubstantiated concepts are obvious. The concept of pinocytosis is too important to risk non-critical acceptance of Wheeler and Hanchey's interpretations without considering evidence to the contrary and the rational alternatives (9).

D. JAMES MORRE

CHARLES E. BRACKER

WILLIAM J. VANDERWOUDE

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Purdue University
Lafayette, Indiana 47907

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SCIENCE

Author **WHEELER, H.; & HANCEY, P.**

Title **Uranyl Induced Pinocytosis and Membrane Dilation**

Comments:

Although lomasomes are not mentioned, could the irregularly evaginated plasmalemma and/or pinocytotic vesicles shown in the electron micrographs (especially figs. 2 C,D) be similar if not identical structures ? If so, the mechanism of their formation may be elucidated here for the first time.

(Continue on additional sheet if necessary)

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- ☒ In an established field, rates in the upper tenth with respect to significance
- ☒ Has broad appeal to non-specialists
- ☒ Is important to specialists in three or more disciplines, namely

cytology physiology pathology

Confidential Comments: This paper may well serve as a model of excellence in conception, organization and execution. Apart from a few trivial technical details, I would recommend no changes. All criteria outlined measure up except for length, but occasionally an exceptional paper should merit making an exception. The electron micrographs are of very high quality. Use of the proposed frontispiece is of course an editorial prerogative, but I would surely consider this one among the candidates.

B. Lowy

September 9, 1970

Advisor's Name

Date

AUG 26 1970

Uranyl induced pinocytosis and membrane dilation.

Treatment of plant roots with uranyl salts induces pinocytotic activity and dilates the plasmalemma and tonoplast.

Harry Wheeler and Penelope Hanchey

The authors are Professor of Plant Pathology, University of Kentucky, Lexington and Assistant Professor of Botany and Plant Pathology, Colorado State University, Fort Collins.

Several lines of evidence indicate that negatively charged groups on the outer surface of plant cells are capable of firmly binding uranyl ions (UO_2^{++}). In yeast cells exposed to UO_2^{++} , sugar transport is blocked and invertase, an enzyme reported to be located in the outer cell surface, is inhibited (1). In barley roots, UO_2^{++} selectively inhibits the absorption of other cations (2). In oat leaves, uranyl salts greatly reduce the toxic effects of the pathotoxin victorin (3). In view of evidence that the site of action of victorin may be the outer surface of the plasmalemma (4), we studied the ultrastructural effects on oat cells of various combinations of victorin and uranyl salts. The results of this study will be published elsewhere but the effects on roots exposed only to uranyl acetate were so striking that we believe they will be of general interest to those concerned with membrane structure and function and the mechanisms by which materials enter and escape from plant cells.

Although some work was done with other species and tissues (5), we will describe only the effects of UO_2^{++} on cells of the oat root cap. The ultrastructure of the cap region of untreated oat roots has been described (4) and is very similar to that of maize and wheat (6). The outer 3-5 layers of cells of the oat root cap exhibit high secretory activity and, in this area, intercellular spaces are filled with slime. Very convincing evidence has been presented that the slime excreted by other grasses in the outer region of the root cap consists of polysaccharide material produced by Golgi secretory activity (7). Although similar direct evidence for oats is lacking, dictyosomes in outer root cap cells are hypersecretory and secretory vesicles are common in the cytoplasm (4). Occasionally, accumulations of secretory products are found between the cell wall and

plasmalemma in secretory cells of untreated oat roots (Fig. 1A). When treated with uranyl acetate, outer root cap secretory cells undergo changes quite different from those in cells in the root cap interior which normally do not exhibit secretory activity. Because of this, the effects of UO_2^{++} on cells in these two regions will be discussed in separate sections.

Uranyl binding and uptake by secretory root cap cells.

When oat roots were placed in solutions of uranyl acetate, then fixed in phosphate-buffered glutaraldehyde, post-fixed in osmium tetroxide (Ga-OsO_4 fixation) and post-stained with lead citrate (5), very dense, needle-shaped crystals were found in the walls, the intercellular spaces, and secretory products in contact with the walls of outer secretory cells of the root cap (Fig. 1 B and C). The crystals were also present in material fixed only in phosphate-buffered glutaraldehyde without post-fixation or post-staining. In view of this and their very high electron denseness we conclude that they represent some type of uranium complex. They are probably not simply uranyl phosphate crystals since they were not found when aqueous or phosphate-buffered KMnO_4 was used as a fixative (5) or when uranyl acetate was mixed in a variety of concentrations with phosphate buffer and deposited on collo dion coated grids. We assume that areas in which these crystals are found contain sites available to and capable of binding uranyl ions.

The number and distribution of uranyl crystals in secretory cells of the outer root cap varied with the concentration of uranyl solutions used and the length of time the roots were exposed. In one test, roots were exposed to 1 millimolar uranyl acetate for 5, 10, 20 and 30 minutes then removed and rinsed in three changes of distilled water for a total desorption

time of 30 minutes. Although the number of crystals increased as exposure time to uranyl increased, the distribution of crystals was the same for all treatments. Uranyl crystals were sharply localized in the cell walls, intercellular spaces, and in secretory products in direct contact with cell walls. Except for an unusually massive accumulation of secretory products along the cell wall, Figure 1 B is typical of the results of this test. To check the possibility that changes in the localization of uranyl occurred during desorption, the test was repeated with desorption time reduced to 30 seconds. Results were entirely similar except that, in addition to crystals, cell walls and intercellular spaces contained dense, non-crystalline deposits.

More massive depositions of crystals were found in secretory cells of roots exposed for 1 hour to 1 millimolar uranyl. Most of the uranyl crystals were confined to the cell wall and structures in direct contact with it (Fig. 1 C) but occasionally packets of crystals were found, and by serial sections shown to be, within the protoplast (Fig. 1 D)). To test how firmly uranyl was bound, desorption time in distilled water was increased to two hours. Although some reduction in number was obtained, many crystals were still present (Fig. 1 E). The chief result of extended desorption was the presence of many more packets of crystals free in the cytoplasm in the vicinity of the cell wall.

Since exposures of two hours or more to 1 millimolar uranyl caused severe disruption of many exterior root cells, roots were treated for 10 and 20 hours in 0.1 millimolar solutions. After 10 hours, uranyl crystals were present in the cell wall and in secretory products in contact with the wall. Packets of crystals which appeared embedded in secretory products were also common in the cell interior (Fig. 2 A). After 20 hours,

crystals were concentrated in distinct packets inside the protoplast (Fig. 2 B). Serial sections demonstrated that many such packets were surrounded by cytoplasm and not in contact with the cell wall.

In general, results with secretory outer root cap cells indicated rapid initial binding of UO_2^{++} in the cell walls, in secretory products in contact with the walls, and in intercellular slime. With exposures to uranyl of 30 minutes or less, no uranyl crystals were found inside the protoplast. With longer exposures, masses of secretory products which contained uranyl crystals appeared to separate from the wall and migrate into the interior of the protoplast in a pinocytotic fashion (8).

Uranyl binding and uptake by interior root cap cells.

In roots exposed to 1 millimolar uranyl for 30 minutes or less a few uranyl crystals were found in the walls of cells immediately beneath the secretory region of the root cap. When exposure time was increased to 1 hour, many uranyl crystals were present in cell walls in this area (6-8 layers of cells beneath the root surface). The surface contours of these cells were extremely irregular with numerous invaginations of the plasma-lemma (Fig. 2 C and D). Vesicles which appeared to have arisen pinocytotically from invaginations of the plasmalemma were common and some of these were shown by serial sections to be free in the cytoplasm. Otherwise the cells appeared to be normal with no evidence of secretory activity and with no uranyl crystals other than those in the cell walls.

Cells in the root cap interior were highly variable in roots exposed to 1 millimolar uranyl for 2 hours. Most cell walls contained large numbers of uranyl crystals (Fig. 2 E) but some lacked crystals, had dense, noncrystalline deposits, and appeared grossly and irregularly swollen (Fig. 3 A). Both

types of cells contained large numbers of secretory vesicles and hyper-secretory dictyosomes (Fig. 2 E, 3 A). Dense amorphous or membranous structures were often present in vacuoles (Fig. 2 E).

After exposures for 4-6 hours, cell walls in the area just beneath the secretory layers were packed with uranyl and had many wart-like protrusions (Fig. 3 B). Deeper in the interior (10-12 cell layers beneath the surface) cell walls were grossly distorted and irregularly shaped masses which contained uranyl crystals and appeared to have originated from the cell wall were common in the cytoplasm (Fig. 3 C).

More consistent results were obtained with roots exposed to 0.1 millimolar uranyl for 10 and 20 hours. Cell walls in the interior of roots exposed for 10 hours were swollen and contained, in addition to uranyl crystals, a variety of membranous, vesicular, and tubular elements (Fig. 3 D). Uranyl crystals were found in some vacuoles but most vacuoles contained dense, noncrystalline material (Fig. 3 E). Results were similar in roots exposed to 0.1 millimolar uranyl for 20 hours except that fewer crystals were present in cell walls and more were found in vacuoles. Also, the difference in response of secretory outer cells of the root cap and those in the interior was clearly evident. Secretory cells with uranyl crystals confined to secretory products were often adjacent to cells with few or no secretory products and in which uranyl crystals were concentrated in vacuoles (Fig. 3 F).

In cells of the root cap interior, uranyl appears to be bound initially in the cell walls where it induces pinocytotic activity. If exposure to 1 millimolar uranyl is extended beyond 1 hour, secretory activity by the Golgi apparatus is induced and dense material (which may contain uranyl) appears in vacuoles. With prolonged exposures (4-6 hours) to 1 millimolar uranyl, the

cell walls appear to become overloaded and cell wall fragments containing uranyl crystals break off and enter the cytoplasm. Prolonged exposure to 10-fold less uranyl allows the cells to respond and to sequester the uranyl in vacuoles. How the uranyl is transferred from the walls to vacuoles is uncertain but pinocytotic vesicles and Golgi secretory products could serve as vehicles.

Uranyl induced changes in membranes

In cells of higher plants fixed by the methods we have used (5) the plasmalemma appears as a unit structure (two dark lines separated by an electron-lucid area) with an overall width of $70-100 \text{ A}^\circ$ (9). In untreated oat roots, the plasmalemma averages 90 A° in overall width (4). In oat roots treated with 1 millimolar solutions of uranyl acetate for 1 hour, the plasmalemma of cells in the root interior was greatly dilated (Fig. 2 C and D). These dilated membranes ranged in overall width from $150-200 \text{ A}^\circ$ and the clear central area comprised about two-thirds of the total width.

In cells with dilated membranes the plasmalemma contour was highly irregular with many invaginations (Fig. 2 C) and, at high magnifications, an undulate appearance (Fig. 2 D). Vesicles with dilated membranes which appeared to have arisen pinocytotically from invaginations of the plasmalemma were common (Fig. 2 C) and some of these were shown by serial sections to be free in the cytoplasm. In the invaginations of the plasmalemma there were abrupt transitions from a normal tripartite unit structure to one with two dense outer lines, two adjacent clear lines, and a dense center line about twice as wide as the two outer ones. (Fig. 2 D arrow). Similar transitions in unit structure were found in the dilated membranes around vesicles (Fig. 2 D, inset).

Membrane systems other than the plasmalemma and vesicles apparently derived from it showed no changes in roots treated with 1 millimolar uranyl for 1 hour. At the magnification used in Fig. 2 D, the unit structure of other membranes was not resolved. When unit structure was resolved at higher magnification, the overall width was in the normal range and no structural transitions such as those in Fig. 2 D were seen.

The plasmalemma was also dilated in roots exposed to 0.1 millimolar uranyl for 10-20 hours (Fig. 3 D, inset). In addition, it was often asymmetric with the dark line next to the cell wall more densely stained than the one adjacent to the cytoplasm. Clear transitions in unit structure were not found but in many areas two or more overlapping layers appeared to be present (Fig. 3 D arrow). Similar dilation with more pronounced asymmetry and areas of apparent membrane overlap was characteristic of tonoplasts surrounding vacuoles which contained uranyl crystals or dense noncrystalline deposits (Fig. 3 E).

Secretory activity in uranyl-treated roots

Secretory products, at least superficially similar to those in uranyl-treated oat roots, have been described in a variety of plant cells and tissues (10). Massive accumulations of vesicles between the cell wall and plasmalemma, such as those in Figure 1 B (and frontispiece), are readily visible when sections 1.5-2.0 microns thick are viewed with a light microscope. They stain intensely when treated with periodic acid followed by Schiff's reagent (PAS reaction for insoluble carbohydrates). This and their location in the slime-producing outer layers of the root cap indicate that they represent polysaccharide material produced by Golgi secretory activity.

Either increased secretory activity or decreased excretion (or a combination of the two) could account for the very massive accumulation of secretory products in the outer layers of cells of uranyl-treated roots. The fact that after prolonged exposures secretory material containing uranyl crystals ~~is~~ ^{is} common inside the protoplast (Fig. 2 A and B) suggests that in these cells uranyl may interfere with the normal process of slime excretion. In cells of the root cap interior, uranyl clearly induces secretory activity (Fig. 2 E and 3 A) since cells in this region of untreated roots do not contain large numbers of secretory vesicles or hypersecretory dictyosomes. In some cases, secretory vesicles appeared to be in contact with elaborations of the cell wall (Fig. 3 A). In view of evidence that polysaccharides secreted by the Golgi apparatus are involved in the synthesis of cell walls (6, 7, 9), the secretory vesicles in interior cells may contribute to uranyl-induced cell wall swelling and distortion (Fig. 3 A-D).

Discussion and summary

Although we have demonstrated uranyl crystals inside protoplasts, we believe our results support rather than contradict Rothstein's (1) conclusion that uranyl ions as such cannot readily penetrate to the interior of intact, isolated cells. After short exposures to uranyl, crystals were found only in cell walls and in secretory products in direct contact with the walls. Crystals were not present in secretory vesicles free in the cytoplasm or in vacuoles and no electron-dense material was found on any membrane system or organelle inside the protoplast. Even after prolonged exposures, mitochondria and nuclei in close proximity to packets of uranyl crystals appeared normal (Fig. 2 B). These results suggest that few, if any, free uranyl ions move into or through the protoplast. Failure to find dilation of any membrane other than the plasmalemma in roots exposed to uranyl for 1 hour supports the same suggestion.

On the other hand our results do not support the conclusion that the initial binding sites for uranyl ions are restricted to the outer surface of the plasmalemma. Instead, large quantities of uranyl are bound in the cell wall and in secretory products along its surface. The slime secreted by outer root cap cells has been postulated to function as a lubricant to facilitate growth of the root tip through soil (13). The ability of this material to bind and retain large quantities of uranyl suggests that such secretory products may also serve to protect the root from rapid influxes of toxic cations.

The root cap was chosen for this study partly because its ultra-structural features were well known and partly because it represents a mass of cells isolated from the vascular system of the plant. We emphasize that the time-course of events found in the root cap may not apply to other regions of the root. In preliminary work, crystals have been found localized in vacuoles in the root hair region of roots exposed to uranyl for as little as 10 minutes. The fragility of root hairs and their tendency to rupture when placed in hypotonic solutions preclude any conclusion as to how such localization occurred.

Invaginations of the plasmalemma in root cap cells held for an hour in hypotonic uranyl solutions and then transferred to distilled water for 30 minutes (Fig. 2 C and D) present a paradox. The isotonic sucrose concentration for these uranyl-treated root cap cells was similar to that of untreated controls; approximately 0.3 molar at 20° C (4), and a value which indicates an osmotic potential of about -8 bars (14). The large deposits of uranyl crystals in cell walls in Fig. 2 C and D indicate that these walls, like cell walls in general, represent free space readily accessible to water and other small molecules. Even allowing for diffusion gradients, cells held in water for 30 minutes should be fully turgid with the plasmalemma held in close

contact with the cell wall by osmotic pressure. Similar invaginations have been described in many different types of plant cells fixed by a variety of methods (9-12) and are present, but less abundant, in untreated oat roots. If they are not artifacts, they could result from localized accumulations of material between the cell wall and the plasmalemma, which either (a) was capable of exerting several atmospheres of osmotic pressure but incapable of passing freely through the cell wall or (b) was a hydrophilic gel capable of physically forcing the membrane away from the wall by swelling during hydration.

✓ Evidence that materials enter cells of higher plants by pinocytosis has been, with the exception of work with isolated protoplasts (11), circumstantial rather than direct and conclusive (12). Although many electron micrographs which suggest that pinocytosis does occur in plant cells have been published (8-12), lack of suitable markers by which the process could be followed has precluded firm conclusions. The crystals and the unique changes in membrane structure found in uranyl treated roots provide such markers. Because of the toxicity of uranyl ions, our results do not establish pinocytosis as a normal mechanism of intake of material by plants. However, the clearest indications of pinocytotic activity were found in roots exposed to 1 millimolar uranyl for 1 hour (Fig. C and D) or to 0.1 millimolar for 10-20 hours (Fig. 2 A and B). Roots were not killed by either of these treatments. They grew at 30-50% of the normal rate when transferred to distilled water.

In animal cells, salts and proteins induce pinocytosis (15), and this process is considered by some to be the main mechanism of active uptake of soluble macromolecular substances (16). In plants, Sutcliffe (17) has postulated that pinocytosis may play an important role in the active uptake of ions. A report that pinocytotic vesicles are centers of high adenosine

triphosphatase activity and that such vesicles increase in number in maize roots exposed to weak salt solutions (18) has provided some support for Sutcliffe's hypothesis.

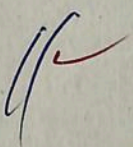
Uranyl acetate induces both pinocytotic and secretory activity in root cap cells. The best chemically characterized secretory products of plants are acidic polysaccharides or polysaccharide-protein complexes of high viscosity (10). Such anionic polymers would not only provide binding sites for UO₂⁺⁺ and other cations but also could account for irregular swelling of cell walls and invaginations of the plasmalemma. Released pinocytotically into the cytoplasm, they could serve as macromolecular carriers to transport bound uranyl to vacuoles. Freed of uranyl and probably depolymerized, the carriers could be reused in a cyclic system of uptake. The driving force for such a process would be that postulated by Steward and Mott (19); the creation of complex macromolecules. This would require energy, and perhaps account for the report of high ATPase activity along the inner surface of the cell wall (18). In view of evidence that uranyl and calcium ions may bind to similar sites with uranyl being bound much more firmly, our results may represent an exaggerated and abnormal picture of a transport system which functions less conspicuously in normal plants (20).

11 ✓

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5. Intact germinated oat (*Avena sativa* L.) seedlings with roots 5-7 mm long were placed for various periods of time in solutions of uranyl acetate. After removal and desorption in three changes of distilled water, the apical 3 mm of the root was cut off and immediately fixed at 23-25° C in either 6 percent glutaraldehyde buffered with potassium phosphate (0.03 M, pH 7.4) for 1 hour, then washed twice in buffer and post-fixed in 2 percent osmium tetroxide in the same buffer for 2 hours or in aqueous 2 percent potassium permanganate for 2 hours. After dehydration in a graded ethanol series, the root tips were embedded in Epon (J.H. Luft, *J. Biophys. and Biochem. Cytology*, 9, 409. 1961). Thin sections were mounted on noncoated copper grids and post-stained for 5 minutes in 2% lead citrate (E.S. Reynolds, *J. Cell Biol.* 18, 208. 1963). The magnification standard was a 28,800 line diffraction grating replica. At least two and in most cases three or more roots of each of two oat cultivars, Compact (CI 8280) and a Victorgrain mutant (CI 7418), were examined for each

treatment described in the text and the illustrations are typical of results with these two varieties. Preliminary work with bean, barley, maize and wheat roots has yielded results similar to those with oats but are not extensive enough to justify conclusions about possible differences among species. The procedure for germinating oat seedlings has been published (H.H. Luke and H. Wheeler, *Phytopathology*, 45, 453. 1955) and other methods are described in the text.

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Legends for figures

- Fig. 1. Localization of uranyl crystals in outer secretory cells of the oat root cap. (A) Portion of a cell from an untreated root with lipid bodies (l), secretory vesicles (sv), an accumulation of secretory products (sp) between the plasmalemma (pl) and the cell wall (w) and intercellular space (is) filled with slime. (B-E) Similar to (A) but from roots exposed for 10 minutes (B) or 1 hour (C-E) to 1 millimolar uranyl acetate. Note distribution of uranyl crystals in cell walls, intercellular spaces and secretory products. Cell shown in (E) was desorbed in distilled water for 2 hours after exposure to uranyl. All other uranyl treated cells were desorbed for 30 minutes. Scale equals 1 micron.
- Fig. 2. (A and B) Outer secretory cells of the cap of oat roots exposed to 0.1 millimolar uranyl acetate for 10 (A) or 20 (B) hours. Note the absence of crystals in the vacuole (v) in (A) and the normal appearance of the nucleus (n) and mitochondria (m) in (B). Other symbols as in Fig. 1. (C-E) Portion of normally nonsecretory cells in the cap interior of oat roots exposed to 1 millimolar uranyl acetate for 1 (C and D) or 2 (E) hours. Note invaginations of the plasmalemma (pl) and presence of pinocytotic vesicles (pv) in C and transitions in membrane structures (arrows) in D. In E, the vacuole (v) contains a dense inclusion, many secretory vesicles (sv) are present and Golgi dictyosomes (g) are active. All roots were desorbed after exposure to uranyl for 30 minutes in distilled water. Scale equals 1 micron.

Fig. 3. (A-C) Cells from the cap interior of oat roots exposed to 1 millimolar uranyl acetate for 2 (A) or 6 (B and C) hours. (A) Unusual cell wall reaction without crystals but with heavy deposits of dense material. Note abundance of secretory vesicles (sv) and hypersecretory dictyosomes (g inset). (B) Massive deposits of uranyl crystals in walls of cells just beneath the outer secretory layers. (C) Highly distorted wall of cell near the center of the root cap with uranyl crystals embedded in masses of material which apparently were released from the wall. (D-F) Cells from oat roots exposed to 0.1 millimolar uranyl acetate for 10 (D and E) or 20 (F) hours. (D and E) Swollen cell wall (D) and vacuole with a dense deposit (E) in interior root cap cells. Arrows (E and enlarged area of D) indicate points of apparent membrane overlap in tonoplast (t) and plasmalemma (pl). (F) Cell on the left with uranyl crystals localized in secretory products (sp) is in the outer secretory region. Contrast with the normally nonsecretory cell on the right with crystals in vacuoles (v). All roots were desorbed after exposure to uranyl for 30 minutes in distilled water. Except for D inset, scale equals 1 micron.

Proposed cover illustration (Frontispiece).

Massive accumulation of secretory products between the plasmalemma and cell wall of outer root cap cells of oat roots exposed to 0.1 millimolar uranyl acetate for 20 hours. Potassium permanganate fixation. Magnification $\times 12,500$.

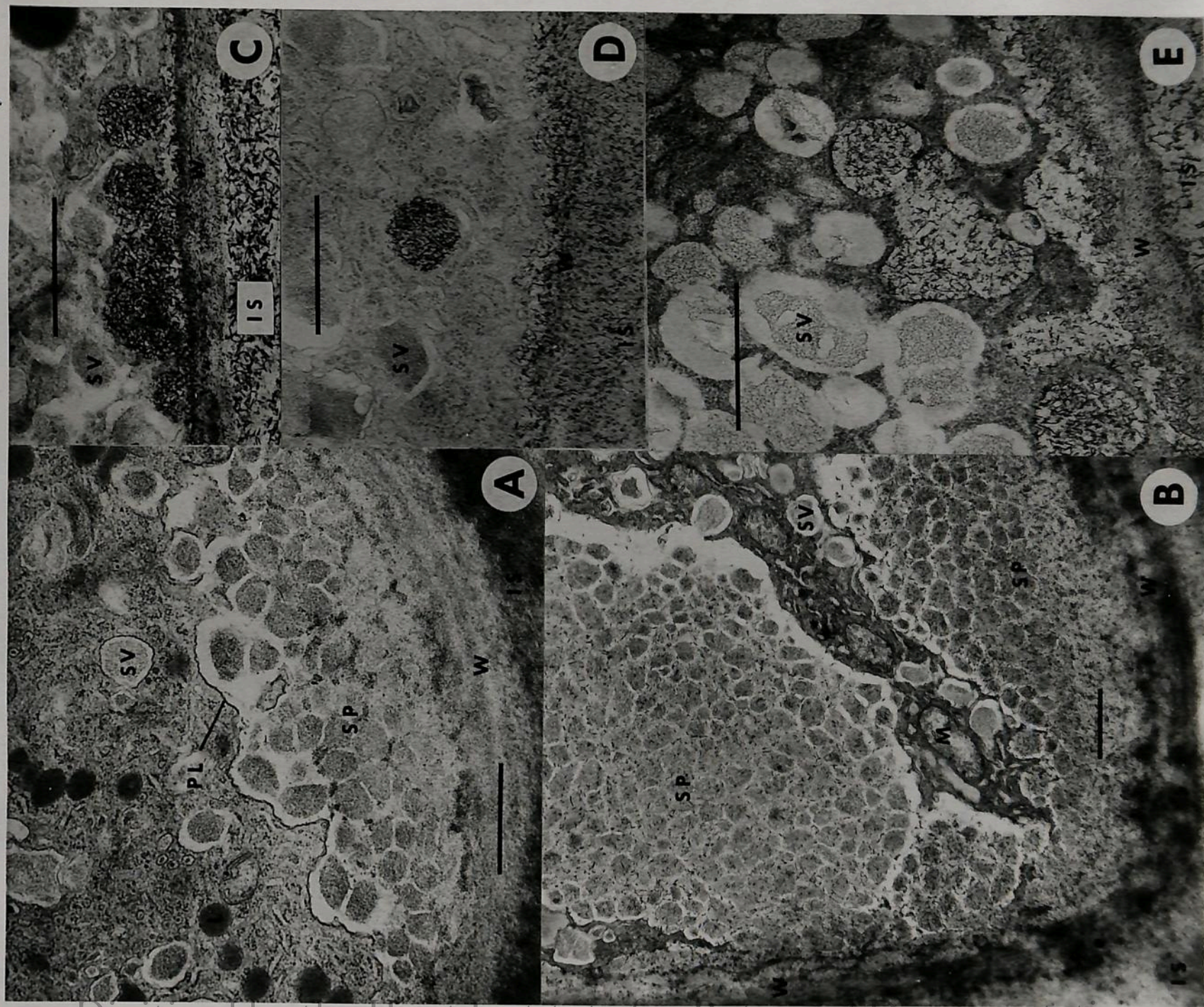


Figure 1. Reviewer's copy

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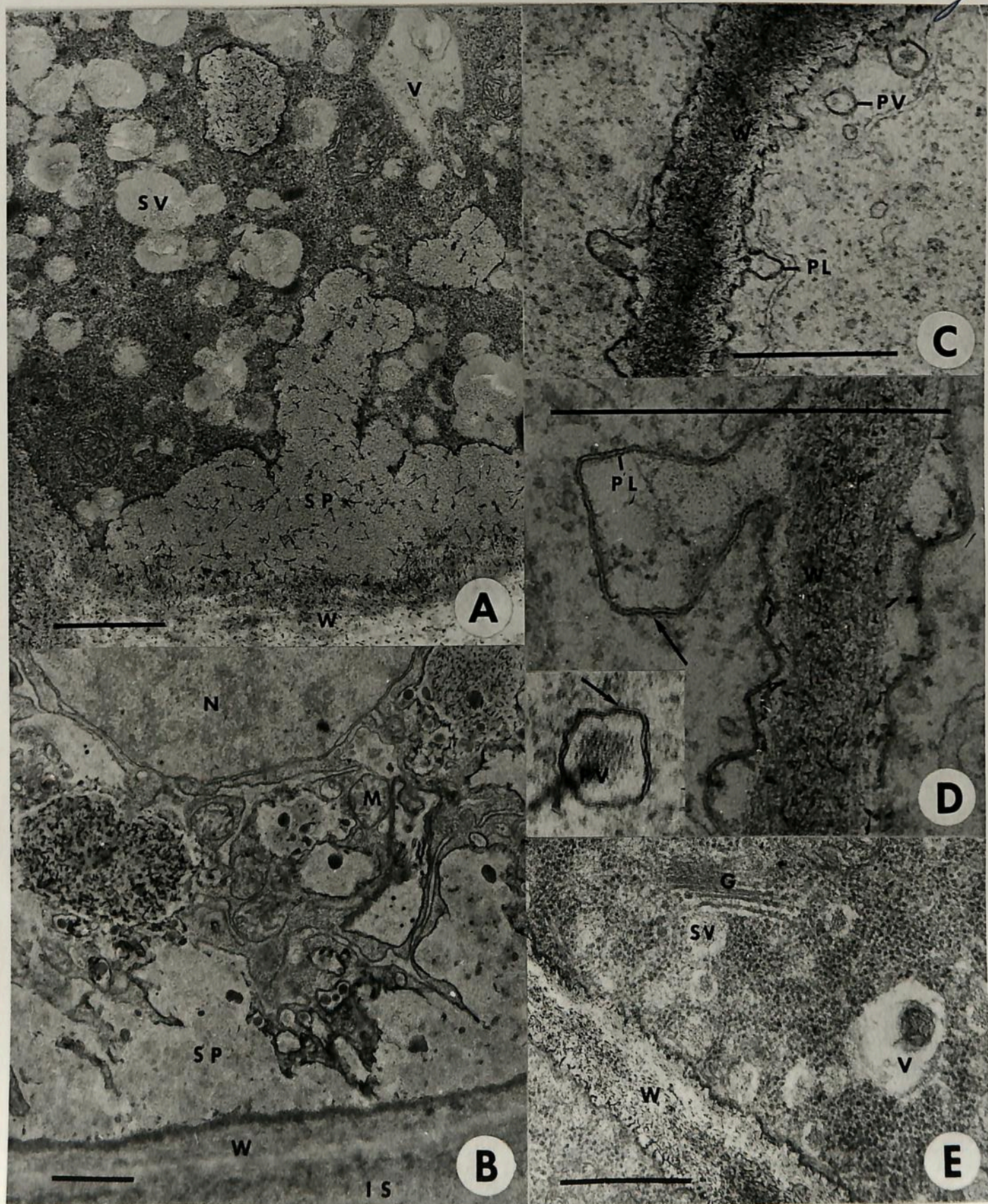


Figure 2. *Reverend copy*

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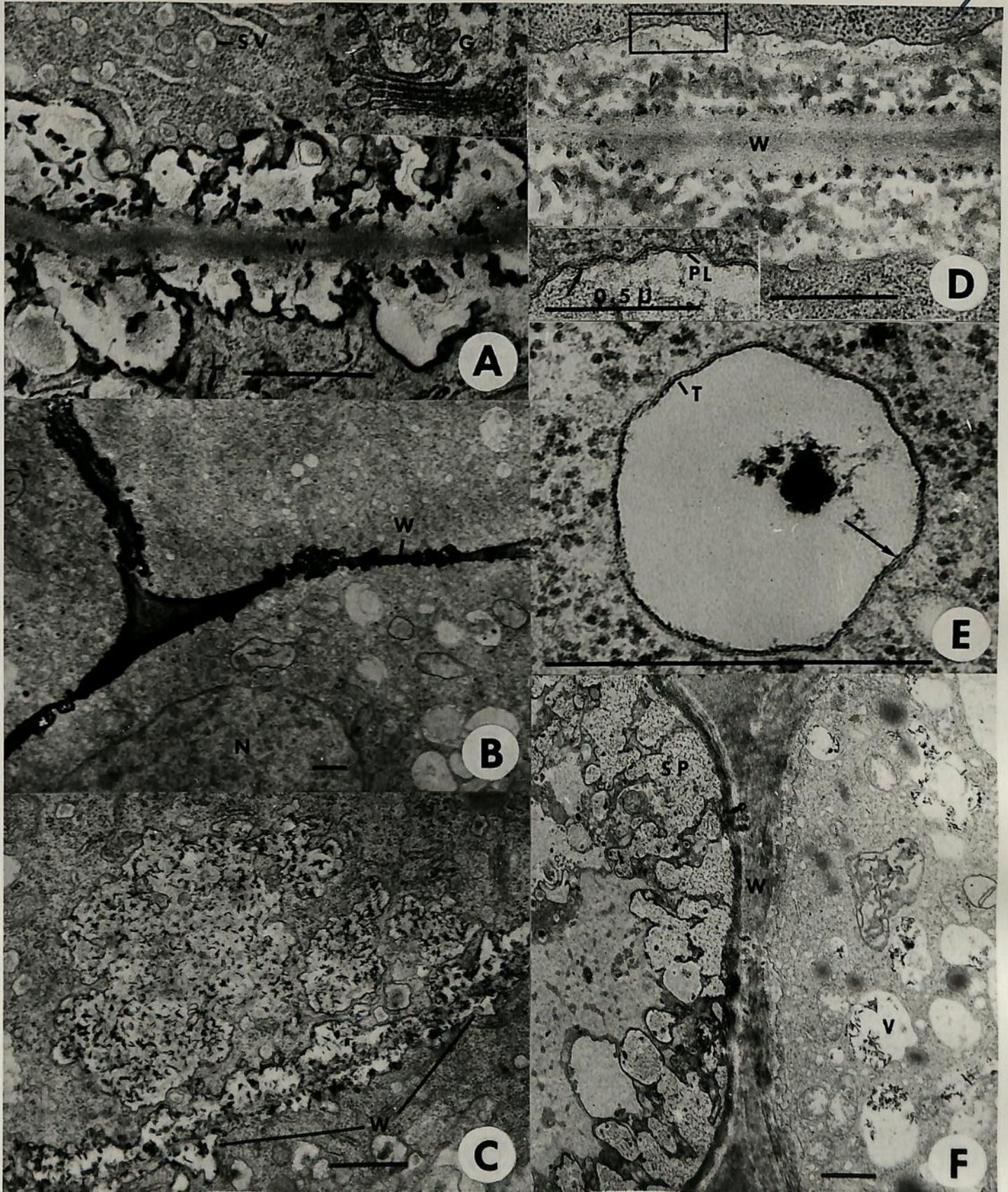


Figure 3. Reviewers Copy

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Wheeler & Hanchey



Proposed cover picture (frontispiece) Digitized by the *Hutchinson Institute for Ecological Documentation*

and atelectasis, these effects were not great enough to complicate the direct comparison of lung compliances.

A typical *P-V* curve from the control series is shown in Fig. 1. One filling-emptying air cycle required about 45 seconds. The virtually coincident third and fourth air curves were averaged to provide total lung compliance values. For the saline curves, a shift to the right in the *X*-axis was made to simplify registering the negative pressures on emptying. Three saline *P-V* curves were run; the second and third curves (B and C) were nearly coincident and were averaged. In contrast to the air curves, the inflation and deflation limbs are very similar. After *P-V* curves were obtained, lungs were sectioned and examined histologically.

Composite air and saline *P-V* curves for control and infected animals are shown in Fig. 2. To reduce error due to edema or atelectasis, the *Y*-axis was calibrated in terms of total lung volume instead of the filling volume. The total lung volume was obtained as follows. To each volume change due to filling or emptying, a volume representing the initial lung weight (assuming a density of one) and a volume representing retained gas (measured as buoyancy in 0.9 percent saline) were added. The resultant curves fall within similar volume ranges and, therefore, are directly comparable.

Except for pressures of 0 and 5 cm-H₂O during inflation, all points on the air curve, both inflation and deflation, were significantly lower for the infected animals ($P < .05$) than for the controls.

Table 1. Physical and microbiological characteristics of rats used in lung compliance measurements. Retained volume and body and lung weights are expressed as averages $\pm$ standard error of mean.

Item	Control	Infected
Animals (No.)	12	12
Mycoplasma from throats (No.)	0	12
Mycoplasma from lungs (No.)	0	6
Developing antibody to <i>M. pulmonis</i> (No.)	0	11
Body weight (g)	339.5 $\pm$ 30.1	331.3 $\pm$ 25.5
Lung weight (g)	1.93 $\pm$ 0.17	2.09 $\pm$ 0.19
Lung weight/body weight (g/kg)	5.75 $\pm$ 0.28	6.28 $\pm$ 0.24
Retained volume (ml)	0.38 $\pm$ 0.04	0.34 $\pm$ 0.02

Table 2. Effect of *M. pulmonis* infection on the compliance of rat lungs. Values are averages, (in milliliters per centimeter of water) of animals in each group $\pm$ standard error of the mean. The C_L is measured as the volume changes between the start (zero pressure) and end (20 cm-H₂O pressure) of filling. The C_{tts} was measured as the slope of the saline curve over a volume range similar to that found with the air curve. Inflation and deflation limbs were averaged.

Parameter (ml/cm-H ₂ O)	Controls	Infected
C_L (0-20)	0.22 $\pm$ 0.02	0.15 $\pm$ 0.01*
C_{tts}	1.33 $\pm$ 0.10	1.17 $\pm$ 0.09
C_{surt}	0.27 $\pm$ 0.03	0.17 $\pm$ 0.02*
Slope of air curves between 5 and 15 cm-H ₂ O		
Inflation C_L (5-15)	0.19 $\pm$ 0.02	0.14 $\pm$ 0.01*
Deflation C_L (5-15)	0.20 $\pm$ 0.02	0.13 $\pm$ 0.01†
Slope of saline curves		
Inflation C_{tts}	1.43 $\pm$ 0.11	1.25 $\pm$ 0.10
Deflation C_{tts}	1.23 $\pm$ 0.10	1.09 $\pm$ 0.10

* $P < .05$. † $P < .01$.

However, slopes of saline curves, determined as the average of inflation and deflation, of lungs from infected animals do not differ significantly from those of controls. These data indicate that infected lungs are more difficult to inflate with air and have less volume at any given pressure. The similarity between the saline curves indicates that the high resistance to filling does not reside within the tissue elasticity of the lung.

There was no histologic evidence of an acute inflammatory process in lungs, and we could not find significant pathologic changes even in rats where mycoplasmas could be isolated from the lungs (12). Rat lungs subjected to *P-V* studies did show some alveolar disruption, probably due to manipulations needed to obtain the *P-V* curves.

Total lung compliance was 33 percent lower ($P < .05$) in infected animals than in the control group (Table 2),

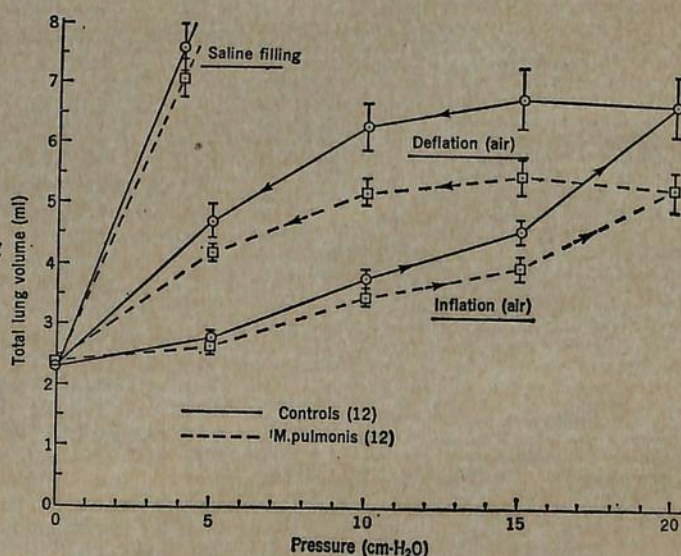
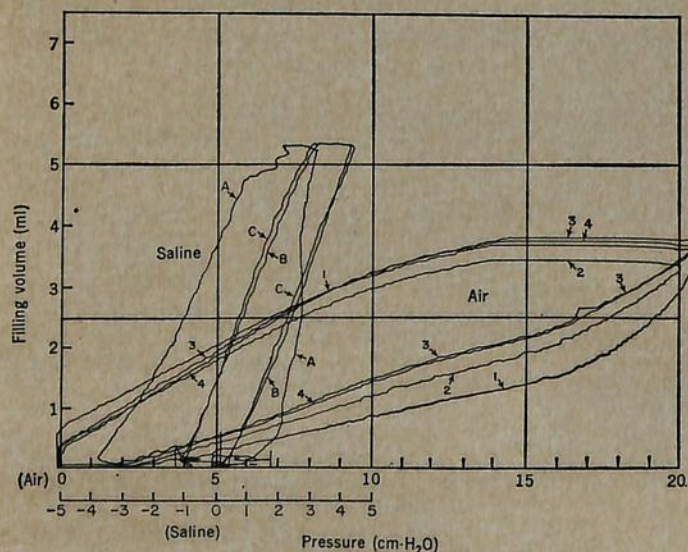


Fig. 1 (left). Compliance curves on an excised rat lung. Cycles of air inflation and deflation are numbered; saline cycles are lettered. Fig. 2 (right). Average pressure-volume (compliance) curves from excised rat lungs. The slopes of saline curves are averages of inflation and deflation limbs.

whereas compliance due to tissue forces was not significantly different ($P > .05$). The calculated C_{surf} was very significantly lower, 37 percent ($P < .01$), in infected rats. Within control and infected groups, the inflation and deflation limbs of the air P - V curves had similar slopes. This indicates that opening pressure was not the explanation of the lower compliance.

Our evidence indicates that altered pulmonary function in rat lungs infected with *M. pulmonis* is due predominantly to an increase in surface forces (13), implying disruption of the surfactant system.

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3. Innovar Injection (McNeil Lab, Fort Washington, Pa.) contains, per milliliter, 0.05 mg of fentanyl, a narcotic analgesic, and 2.5 mg droperidol, a narcotic agent. Rats received 0.075 to 0.1 ml, depending on their age and weight.
4. *Mycoplasma pulmonis* strain N3 had been obtained from J. Nelson of Rockefeller University. In our laboratory, it was passaged through rats to enhance its virulence, and the isolate used in these experiments had been recovered from the lungs of one of the animals. The organism was cultured and concentrated according to the method described by N. L. Somerson, W. D. James, B. E. Walls, R. M. Chanock, *Ann. N.Y. Acad. Sci.* 143, 348 (1967). Suspensions of organisms inoculated into rats contained at least 10^{10} mycoplasma colony-forming units.
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6. The time of killing was arbitrarily selected. Infected animals were killed within a period of 8 to 49 days after inoculation.
7. Minced lung tissue was inoculated into roller-tube cultures of rat embryonic kidney, WI-38, HeLa cells, and primary green monkey kidney cells. Two cultures of each type of cell were incubated at 37°C, and one at 33°C. Cell cultures were held at least 7 days, examined for cytopathic effects, and subjected to two additional passages.
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9. All cardiac tissue had been removed from the lungs. Lungs were tested for leaks by inflating them with air while submerged in saline; small leaks were repaired with Eastman 910 adhesive.

10. Buoyancy in 0.9 percent saline after the air P - V curves was used to estimate trapped or retained gas.
11. Antibody to *M. pulmonis* was measured in a fluorescent test described by P. Y. Ertel, I. J. Ertel, N. L. Somerson, J. D. Pollack, *Proc. Soc. Exp. Biol. Med.* 134, 44 (1970). Mycoplasmas were isolated from the lungs of one rat who did not show antibody. The animal had been killed 8 days after inoculation, probably before this antibody was produced.
12. The lack of microscopic pulmonary lesions contrasts with the findings of A. B. Organick and I. I. Lutsky [*J. Bacteriol.* 92, 1154 (1966); *ibid.*, p. 1166], who studied *M. pulmonis* infection in both conventional and gnotobiotic mice. They reported consolidation with a polymorphonuclear cellular response in the acute stage of infection. In some cases they showed

distension of alveolar spaces and disruption of mitochondria. Possibly the rats we used were more resistant than mice to *M. pulmonis*. However, D. F. Kohn and B. E. Kirk [in *Lab. Anim. Care* 19, 321 (1969)] reported bronchiectasis in rats given *M. pulmonis*. Perhaps our strain was not as virulent as the one they used.

13. We recognize that, theoretically, bronchoconstriction could mimic an increase in surface tension [see E. P. Radford, Jr., and N. M. Lefcoe, *Amer. J. Physiol.* 180, 479 (1955)]. However, there is no evidence that such a phenomenon occurs in mycoplasma-infected rats.
14. Supported by NIH grant AI 08587. We thank J. Pitt, K. Knore, and F. Elner for excellent technical assistance.

3 September 1970

Pinocytosis and Membrane Dilation in Uranyl-Treated Plant Roots

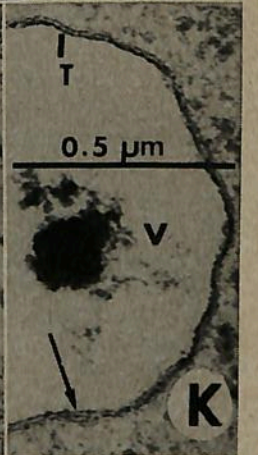
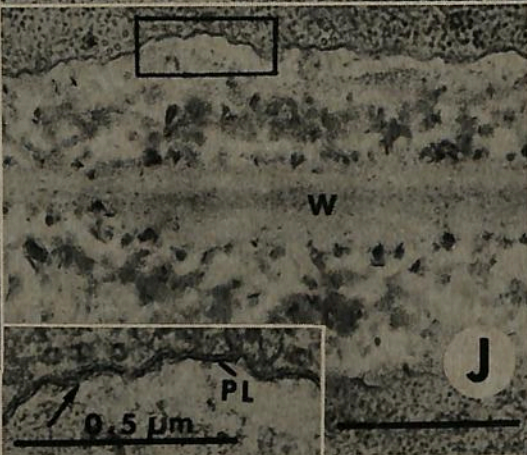
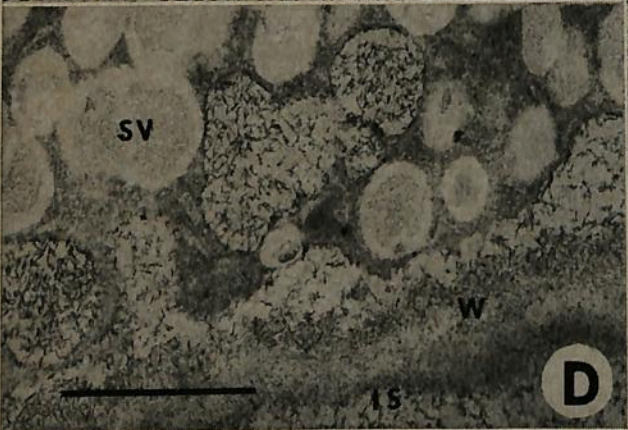
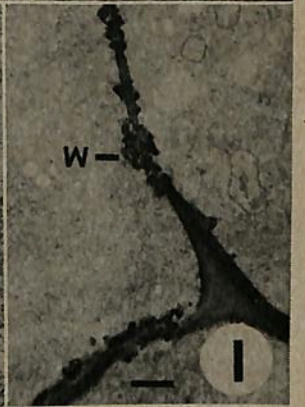
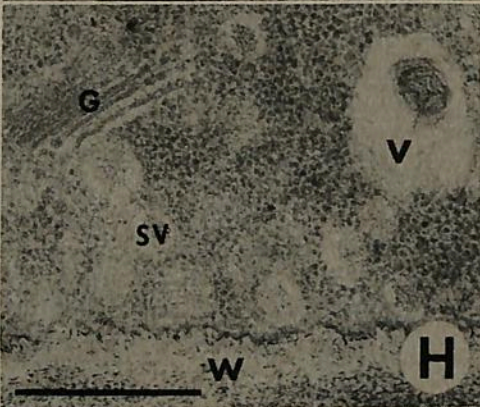
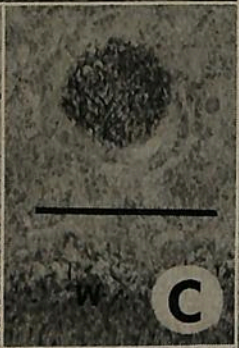
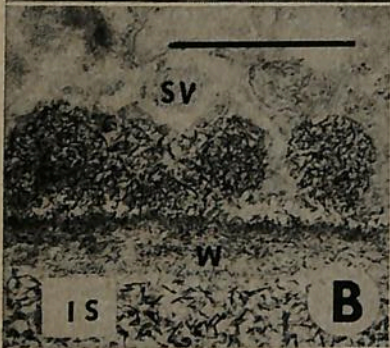
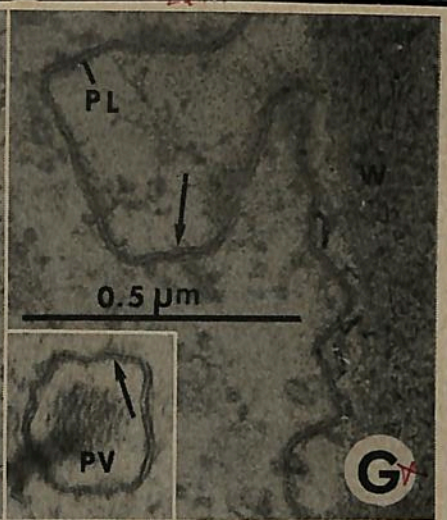
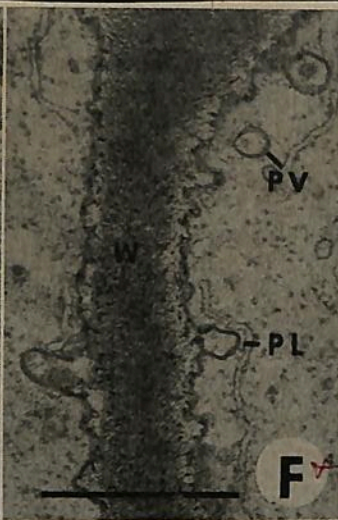
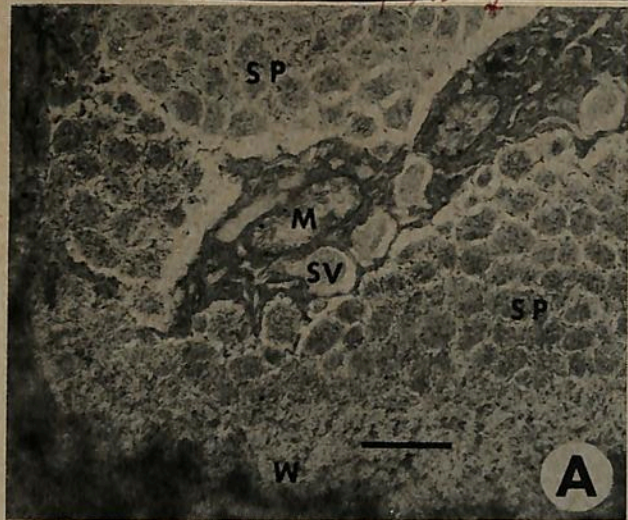
Abstract. Electron-dense crystals formed in plant roots exposed to uranyl acetate have been used to identify binding sites and to follow the pinocytotic uptake of uranyl in the oat rootcap. Before uranyl enters the protoplast, the plasmalemma is greatly dilated. After uranyl is sequestered in vacuoles, the tonoplast is similarly dilated.

When oat roots were placed in solutions of uranyl acetate, then fixed in phosphate-buffered glutaraldehyde, fixed in osmium tetroxide, and stained with lead citrate (1), very dense, needle-shaped crystals, easily visible in electron micrographs of thin sections, were formed. The crystals also formed in material fixed only in phosphate-buffered glutaraldehyde without additional fixation or staining. In view of this and their very high electron density, we conclude that they represent some type of uranium complex. They are probably not simply uranyl phosphate crystals since they were not found when aqueous or phosphate-buffered potassium permanganate was used as a fixative or when uranyl acetate was mixed in a variety of concentrations with phos-

phate buffer and deposited an collodion-coated grids. We have used these crystals to follow the binding and uptake of uranyl in cells of the oat rootcap (2).

The number and distribution of uranyl crystals in outer secretory cells of the oat rootcap varied with the concentration of uranyl solutions used and the length of time the roots were exposed. In one test, roots were exposed to 1 mM uranyl acetate for 5, 10, 20, and 30 minutes, then removed and rinsed in three changes of distilled water for a total desorption time of 30 minutes. Although the number of crystals increased as exposure to uranyl increased, uranyl crystals were, in all cases, sharply localized in the cell walls, intercellular spaces, and in secretory products in direct contact with cell

Fig. 1. Localization of uranyl crystals in cells of the oat rootcap. (A-E) Portions of cells in outer secretory layers of the rootcap from roots exposed for 10 minutes (A) or 1 hour (B-D) to 1 mM uranyl acetate. Secretory vesicles (sv) are common and in (A) secretory products have accumulated between the plasmalemma (pl) and the cell wall. Uranyl crystals are abundant in cell walls (w), intercellular spaces (is), and secretory products (sp). (E) Outer secretory cell exposed to 0.1 mM uranyl acetate for 20 hours. Note the normal appearance of the nucleus (n) and mitochondria (m). (F-I) Portions of normally nonsecretory cells in the cap interior of oat roots exposed to 1 mM uranyl acetate for 1 (F and G), 2 (H), or 6 (I) hours. Note invaginations of the plasmalemma (pl) and presence of pinocytotic vesicles (pv) in (F) and transitions in membrane structure (arrows) in (G). In (H), the vacuole (v) contains a dense inclusion, many secretory vesicles (sv) are present, and Golgi dictyosomes (g) are active. (I) Massive deposits of uranyl crystals in walls of cells just beneath the outer secretory layers. (J-L) Portions of interior cap cells from oat roots exposed to 0.1 mM uranyl acetate for 10 (J and K) or 20 (L) hours. (J and K) Swollen cell wall (J) and part of vacuole with a dense deposit (K) in interior root cap cells. Arrows (K and enlarged area of J) indicate points of apparent membrane overlap in tonoplast (t) and plasmalemma (pl). (L) Cell below with uranyl crystals localized in secretory products (sp) is in the outer secretory region of the rootcap. Contrast with the normally nonsecretory cell above with crystals in vacuoles (v). All roots were desorbed after exposure to uranyl for 30 minutes in distilled water, except for that in (D) which was desorbed 2 hours. Unless otherwise indicated, scale equals 1 μ m.



changes in membrane structure in uranyl-treated roots provide such markers. Because uranyl is toxic, our results do not establish pinocytosis as a normal mechanism of intake of material by plants. However, the best evidence of pinocytosis was with exposures to 1 mM uranyl for 1 hour (Fig. 1, F and G) or to 0.1 mM for 20 hours (Fig. 1E). Roots were not killed by these treatments; they grew at 30 to 50 percent of the normal rate when transferred to water.

In animals, salts and proteins induce pinocytosis (8), and this may account for active uptake of soluble macromolecular substances (9). In plants, a role for pinocytosis in active ion uptake has been postulated (10), and an increase in numbers of pinocytotic vesicles in roots exposed to salts has been reported (11). Chemically characterized plant-root secretions include acidic polysaccharides and protein-polysaccharide complexes of high viscosity (12). Such anionic polymers would not only bind UO_2^{2+} and other cations but also could account for cell wall swelling and invaginations of the plasmalemma. Released pinocytotically they could transport bound uranyl to vacuoles. Freed of uranyl, the carriers could be reused in a cyclic system of uptake. The driving force for such a process could be the creation of complex macromolecules (13). In view of evidence that uranyl and calcium ions bind to similar sites (5), our results may represent an exaggerated and abnormal picture of a transport system which functions less conspicuously in normal plants.

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References and Notes

1. Intact germinated oat (*Avena sativa* L.) seedlings with roots 5 to 7 mm long were placed for various periods of time in solutions of uranyl acetate. After removal and desorption in three changes of distilled water, the apical 3 mm of the root was cut off and immediately fixed at 23° to 25°C in 6 percent glutaraldehyde buffered with 0.03M potassium phosphate (pH 7.4) for 1 hour, then washed twice in buffer and fixed in 2 percent osmium tetroxide in the same buffer for 2 hours. After dehydration in a graded ethanol series, the root tips were embedded in Epon [J. H. Luft, *J. Biophys. Biochem. Cytol.* 9, 409 (1961)]. Thin sections were mounted on noncoated copper grids and stained for 5 minutes in 2 percent lead citrate [E. S. Reynolds, *J. Cell Biol.* 18, 208 (1963)]. The magnification standard was a 28,000-line diffraction-grating replica. At least two and in most cases three

- or more roots of each of two oat cultivars, Compact (CI 8280) and a Victorgrain mutant (CI 7418), were examined for each treatment, and the illustrations are typical of results with these two varieties. Preliminary work with bean, barley, maize, and wheat roots has yielded results similar to those with oats, but are not extensive enough to justify conclusions about possible differences among species. The procedure for germinating oat seedlings has been published [H. H. Luke and H. Wheeler, *Phytopathology* 45, 453 (1955)].
2. The ultrastructure of the cap region of untreated oat roots has been described (14) and is very similar to that of maize and wheat (15). The outer three to five layers of cells of the oat rootcap exhibit high secretory activity and, in this area, dictyosomes are hypersecretory, secretory vesicles are common in the cytoplasm, and intercellular spaces are filled with slime. Occasionally, accumulations of secretory products are found between the cell wall and plasmalemma in secretory cells of untreated oat roots. When treated with uranyl acetate, outer rootcap secretory cells undergo changes quite different from those in cells in the cap interior which normally do not exhibit secretory activity. Because of this, the effects of UO_2^{2+} on cells in these two regions will be discussed in separate sections. The rootcap was chosen for this study partly because its ultrastructural features were well known and partly because it represents a mass of cells isolated from the vascular system of the plant. We emphasize that the course of events found in the rootcap may not apply to other regions of the root. In preliminary work, crystals have been found localized in vacuoles in the root hair region of roots exposed to uranyl for as little as 10 minutes. The fragility of root hairs and their tendency to rupture when placed in hypotonic solutions preclude any conclusion as to how such localization occurred.
3. We will use pinocytosis as a general term for the intake of water or solids by invagination of the plasmalemma as defined by F. A.

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Developmental Variation in the Isoenzymes of Human Liver and Gastric Alcohol Dehydrogenase

Abstract. *The isoenzyme patterns of alcohol dehydrogenase from human liver and stomach are different in the fetal, newborn, childhood, and adult periods of life. This provides additional evidence that the fetal and adult alcohol dehydrogenases are qualitatively different. Gastric and hepatic isoenzyme patterns also differed from each other.*

Our understanding of the mechanism of differentiation may be advanced by studies which use specific developmental markers such as proteins that are present in the wild type of a species during a restricted period of development. Developmental markers already known in man include embryonic and fetal hemoglobins (1), the lactate dehydrogenases (2), the esterases (3), and creatine phosphokinases (4).

The activity of liver alcohol dehydrogenase (LADH) increases with fetal development in rats, guinea pigs, and man (5, 6). Values of the Michaelis constant (K_m) and optimum values of pH differ between the human fetal and adult forms of this enzyme (6), and developmental variation in the isoenzymes of human LADH has also been reported (7). This study shows that the developmental variation of human LADH and

human alcohol dehydrogenase from gastric mucosa (GADH) continues up to puberty.

Tissues were obtained 12 to 36 hours after death. Specimens from spontaneously aborted fetuses were obtained 12 to 24 hours after passage. An occasional fetal specimen was obtained by hysterotomy. All fetuses used had no visible external or internal malformations and tissues were analyzed immediately or stored at -20° to -25°C.

Tissues were homogenized at 0° to 4°C. The homogenate was diluted 1:5 with buffer prior to enzyme assay (8) and was applied directly to agarose gel for electrophoresis. Agar and agarose gel electrophoresis was performed according to a modification of the method described by Ursprung (9), and protein concentration was measured by a modification of the Lowry method (10).

Table. 1. Number and proportion of adult, newborn, and fetal samples in different ranges of LADH activity. The mean and range of LADH activity in milliunits per milligram of protein in adults was 79.8 (3 to 319), in newborns was 45.5 (2 to 164), and in fetuses was 15 (1 to 24).

Range of LADH activity in milliunits per milligram of protein	Analysis					
	Adult		Newborn		Fetal	
	No.	%	No.	%	No.	%
0-14	15	30	7	31.8	12	80
15-79	16	32	11	50.0	3	20
80+	19	38	4	18.2	0	00

$$\chi^2 = 17.378, .001 < P < .01.$$

The electrophoresis bands of alcohol dehydrogenase were identified by comparison with partially purified enzyme and by comparison of staining intensity with and without ethanol. Agar or agarose gel was used to reduce the likelihood that differences in banding patterns might be due to the separation of different enzyme-coenzyme complexes through the sieving properties of starch gel (11).

Fifty percent (weight/volume) homogenates from adult human brain, muscle (skeletal and cardiac), skin, spleen, leukocytes, fibroblasts, stomach, and liver were tested, but only homogenates of gastric tissue and liver showed readily defined patterns after the electrophoresis.

The agar or agarose gel pattern of human fetal LADH consisted of two anodal zones and a single, slow moving cathodal zone (Fig. 1, channel 1). Fifty fetuses (21 to 260 mm crown-to-rump; estimated gestational age, 45 to 184 days) showed this pattern (LADH 1) at pH 6.0 to pH 9.0. Later in gestation, a second, less intense, but more cathodal band appeared (LADH 2) (Fig. 1, channel 2). This band was also found in the patterns of most premature infants (870 to 2000 g birth weight). A third, still more cathodal band (LADH 3), varying in intensity, appeared at full term and was also detected in older infants (Fig. 1, channel 3). One of the 50 fetal specimens showed, on repeated study, a single cathodal band with slightly lower mobility than the normal fetal band, and this slower band might represent a structural variant of the "fetal" LADH.

Additional evidence that variation in banding pattern was related to age came from studies of liver homogenates obtained at autopsy from one Caucasian family consisting of the two parents and their six children (12). One child (aged 3 years) had a pattern similar to that seen in newborn infants, showing LADH bands 1, 2, and 3 (Fig. 1, channel 3). Three of the children (aged 5,

7, and 8½ years) had patterns showing LADH 1, 2, and 3 as intense bands and in addition a less intense band (LADH 4), which had the greatest mobility toward the cathode (Fig. 1, channel 4). The two other children (aged 11 and 14 years) had patterns like those of their parents, with weak LADH bands 1 and 4 and a strong LADH 2 band (Fig. 1, channel 5). Specimens from two children (aged 4 years) unrelated to the above-mentioned family had patterns like that of the 3-year-old child (Fig. 1, channel 3).

Two common LADH isozyme patterns were found in homogenates from different adult livers. Both types showed two weak anodal zones of LADH activity. The most frequent pattern showed three cathodal zones (Fig. 1, channel 5) corresponding in mobility to LADH 1, 2, and 4. In this pattern

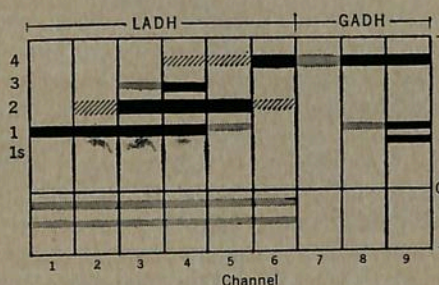


Fig. 1. The electrophoretic banding pattern of LADH and GADH in homogenates of human livers and stomachs at different ages on agarose gel at pH 8.7. As much blood as possible (blood does not contain alcohol dehydrogenase activity) was removed, and the tissue was weighed. A 20 percent (weight to volume) proportion was used. Tissues were homogenized in 0.25M KCl, pH 7.4, containing $10^{-3}M$ β -mercaptoethanol and $10^{-4}M$ diisopropyl fluorophosphate. One milligram of NAD was added to each 100 ml of agarose gel. Channel 1, fetus; channel 2, premature infants; channel 3, full-term, newborn infants up to 5-year-old children; channel 4, 5- to 9-year-old children; channel 5, 11-year-old children to adult (most common type); channel 6, adult (less common type); channel 7, fetus; channel 8, newborn infants; channel 9, adults.

the LADH 2 band always showed the most intensity, and prolonged electrophoretic runs suggested that the LADH 1 in some adults moved slightly faster than LADH 1 in fetal specimens. The other frequent adult LADH pattern had only two bands, LADH 2 and 4 (Fig. 1, channel 6). In this pattern LADH 4 was always most intense, and an LADH 1 band was not present.

Homogenates of adult liver specimens obtained by needle biopsy or open surgical biopsy showed the same electrophoretic patterns as autopsy specimens from adults. Any given specimen showed the same banding pattern over a 1-year period; and no interconversion of banding pattern was produced by storage, addition or removal of nicotinamide adenine dinucleotide (NAD) (13), heat, or proteolytic enzymes. Mixing homogenates of adult and fetal livers produced the sum of the individual banding patterns.

Developmental variation was also noted in the GADH isoenzymes (Fig. 1, channels 7, 8, 9). Homogenates of gastric specimens from embryos (100 to 195 mm crown-to-rump) had a single, low intensity, rapid-moving cathodal band with a mobility like that of the LADH 4 band in adult liver homogenates (Fig. 1, channel 7); and therefore this band has been designated GADH 4. In the newborn, a second cathodal GADH band, which moved like LADH 1, appeared and the GADH 4 band increased in intensity (Fig. 1, channel 8). The fetal, newborn, and adult gastric isoenzyme patterns were all different from the hepatic pattern at the corresponding ages. The adult pattern of GADH consisted of one very rapid and two much slower cathodal bands, the slowest of which was slower than LADH 1 and has been called GADH 1s.

No qualitative change in GADH banding pattern was noted after storage for up to 7 months at $-20^{\circ}C$. The intensity of GADH bands was strikingly reduced beyond 18 hours postmortem, but again no qualitative change in banding pattern was noted.

Our study, in contrast to an earlier report (6), found no linear correlation between LADH enzyme activity and the crown-to-rump length of the fetuses, weight of the newborns, or age of the adults. This difference may be related to the fact that fetal tissue was obtained by therapeutic abortion in the Scandinavian study, while spontaneous abortions comprised the majority of our samples.

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