TUBULAR AND FIBRILLAR COMPONENTS OF MATURE AND DIFFERENTIATING SIEVE ELEMENTS

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ABSTRACT

An ontogenetic study of the sieve element protoplast of *Nicotiana tabacum* L. by light and electron microscopy has shown that the P-protein component (slime) arises as small groups of tubules in the cytoplasm. These subsequently enlarge to form comparatively large compact masses of 231 ± 2.5 (SE)A (n = 121) tubules, the P-protein bodies. During subsequent differentiation of the sieve element, the P-protein body disaggregates and the tubules become dispersed throughout the cell. This disaggregation occurs at about the same stage of differentiation of the sieve elements as the breakdown of the tonoplast and nucleus. Later, the tubules of P-protein are reorganized into smaller striated 149 ± 4.5 (SE)A (n = 43) fibrils which are characteristic of the mature sieve elements. The tubular P-protein component has been designated P1-protein and the striated fibrillar component P2-protein. In fixed material, the sieve-plate pores of mature sieve elements are filled with proteinaceous material which frays out into the cytoplasm as striated fibrils of P2-protein. Our observations are compatible with the view that the contents of contiguous mature sieve elements, including the P-protein, are continuous through the sieve-plate pores and that fixing solutions denature the proteins in the pores. They are converted into the electron-opaque material filling the pores.

INTRODUCTION

It is well established that both mature and differentiating sieve-tube elements of higher plants contain characteristic protein components (3, 6–8, 14). Until we proposed to designate these components as P-protein (9), they were described as slime (including slime bodies and slime plugs) in most plants, and as irregular bodies (3), flagellar inclusions (14), and crystalline protein inclusions (14) in some Leguminosae. We intend to use the term P-proteins for all types of specific protein components in the sieve elements.

The P-proteins originate in the form of discrete bodies discernible with the light microscope, and they can be identified as protein by several cytochemical tests including mercuric bromphenol blue (e.g., 8, 14, 25). Some investigators report the presence of lipids (26) and ribonucleic acid (4) in the slime bodies, but the chief chemical component is protein. In mature cells in killed material, the P-protein is usually found as either dispersed through the cell or largely accumulated at one sieve plate in the form of a plug (slime plug). It remains a matter of controversy as to whether one of these two forms of distribution of P-protein, or any other, respresents the condition in the living protoplast.

Knowledge of the relationship of the P-protein component to the protoplast of the functioning sieve tube is of fundamental importance with regard to the elucidation of the mechanism of transport of nutrients in the phloem. The explanation of this relationship requires an understanding

of the origin and structure of the P-protein. The present paper describes the results of studies of the P-proteins in mature and differentiating sieve elements in the phloem of *Nicotiana tabacum* as observed with the light and electron microscopes.

MATERIALS AND METHODS

Nicotiana tabacum L. plants were grown from seed under greenhouse conditions. 1-cm pieces were cut from the petioles and midveins of differentiating leaves. The samples were placed in either 3% unbuffered glutaraldehyde; in 3% glutaraldehyde in 0.05 M phosphate buffer at pH 6.8; or in glutaraldehyde-formaldehyde according to the method of Karnovsky (13). Postfixation in each case was with 2% osmium tetroxide. The quality of the fixation obtained with the three fixation procedures was similar. The material was dehydrated through acetone solutions and embedded in Epon epoxy resin. Sections were cut with a diamond knife on a Porter-Blum MT2 ultramicrotome, stained with uranyl acetate and lead (18), and viewed and photographed with a Siemens Elmiskop I.

Comparable material was fixed for light microscope studies in the following fixatives: (1) mixture of chromic acid, acetic acid, and formalin (Craf III 27); (2) 10% acrolein; (3) 3% glutaraldehyde. The material was embedded in Paraplast (Scientific Products, Inc., Detroit, Michigan). For developmental studies, the sections were stained with Heidenhain's hematoxylin. In tests for protein, dewaxed sections were stained with mercuric bromphenol blue according to Mazia et al. (17).

OBSERVATIONS

Mature and differentiating sieve tube elements of *Nicotiana tabacum* were observed in transverse and longitudinal sections. The general organization of the cytoplasmic components and the degree of elaboration of the cell walls were used as criteria to judge the stages of differentiation of the sieve elements. We have shown previously that, as the elements differentiate, characteristic changes affecting all components of the cell take place (3, 8). In the present investigation, our main concern was an examination of the P-protein component. As has been shown with the light microscope for numerous species, the P-protein appears in the form of discrete bodies in young cells, but becomes dispersed as the cell matures. This transformation was studied with the electron microscope.

P-Protein Stained with Mercuric Bromphenol Blue

The proteinaceous nature of the P-protein component has been deduced from cytochemical studies for a number of species, but not specifically for that of *Nicotiana tabacum* sieve elements. In the present study, sections of *N. tabacum* phloem stained with mercuric bromphenol blue showed that the P-protein stained blue, a characteristic reaction of the proteinaceous cell components to the stain. The staining was intense in the compact bodies of young cells and in the accumulations on the sieve plates (slime plugs) of mature cells; it was somewhat weaker in the more or less dispersed P-protein. Material killed with the aldehydes, particularly the acrolein, proved more satisfactory for the test than was that fixed with Craf III.

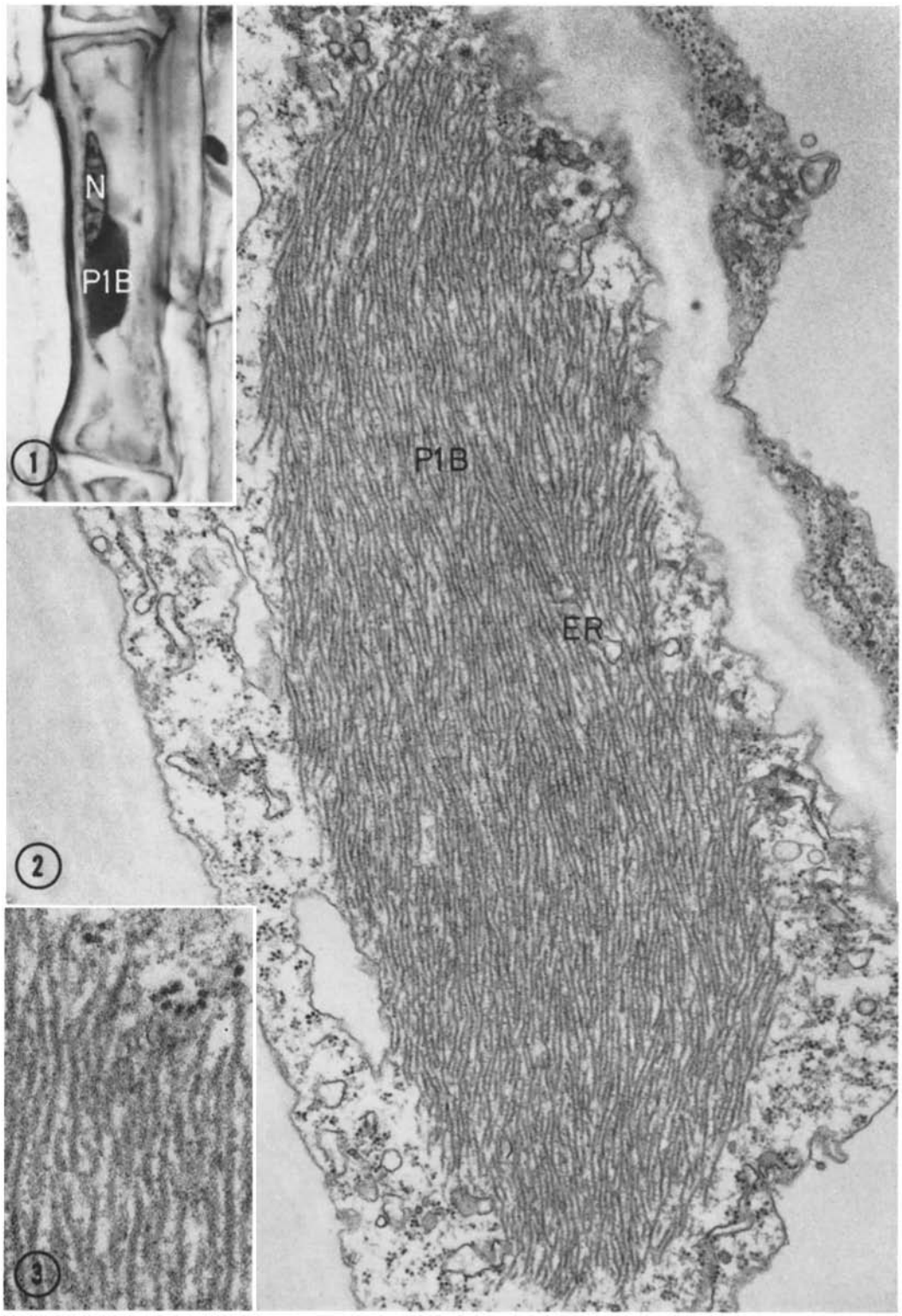
The P-Protein Bodies

As seen with the electron microscope, the P-protein first appears in the cytoplasm of young sieve elements in the form of compact bodies which consist of small aggregates of tubules. Observations of sieve elements at several stages of differentiation have shown a variation in the size of these bodies, and we assume that the small discrete aggregates subsequently enlarge. Although the sieve elements of tobacco usually contain a single protein body, we have seen no evidence of interconnections between small bodies or of their fusion. The bodies in different cells attain a fairly uniform size, approximately 15 by 6 μ, and usually assume an

FIGURE 1 *Nicotiana tabacum*. Light micrograph of a longitudinal section of a differentiating sieve element. It has a prominent nucleus (*N*) and a deeply stained P-protein (slime) body (*P1B*). × 900.

FIGURE 2 *Nicotiana tabacum*. Longitudinal section of a sieve element at an early stage of differentiation. The P-protein body (*P1B*) consists of a mass of more or less longitudinally oriented tubules. *ER*, endoplasmic reticulum. × 28,000.

FIGURE 3 *Nicotiana tabacum*. Longitudinal section of a portion of a P1-protein body similar to the one shown in Fig. 2. The tubules are evenly stained. × 72,000.


N
P1B
1
P1B
ER
2
3

ellipsoidal shape. They are visible with the light microscope, as shown in Fig. 1, in which the body is larger than the adjacent nucleus. At the electron microscope level, the body is seen to consist of numerous tubules which are oriented roughly parallel to the long axis of the body (Fig. 2). The bodies are not surrounded by a membrane and, although they are compact, the tubules around the periphery appear to be fraying out slightly into the surrounding cytoplasmic matrix. With the exception of occasional elements of endoplasmic reticulum, cytoplasmic components other than the tubules themselves are excluded from the bodies (Fig. 2). Cisternae of endoplasmic reticulum (*ER*) are frequently oriented parallel to the surface of the body (Figs. 2 and 4). These ER elements usually have their ribosomes arranged in groups (polysomes; Fig. 3). In longitudinal views, the tubules in the P-protein bodies reveal no substructure (Fig. 3).

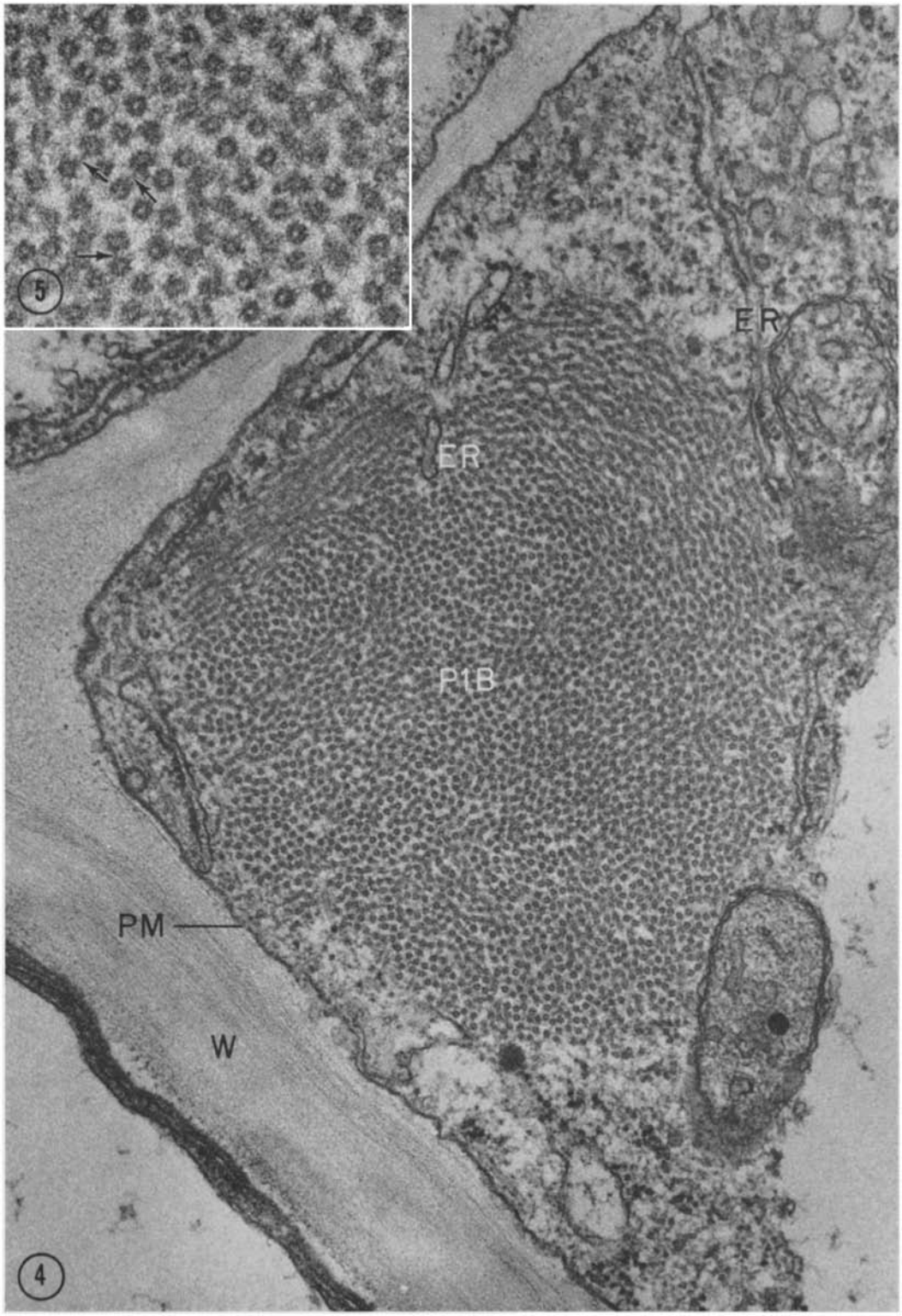
In transection, the P-protein bodies appear roughly circular. In view of the longitudinal orientation of the tubules in the P-protein bodies, a transection of the body also cuts most of the tubules in transection (Fig. 4). At higher magnifications, the tubules in transection are seen to consist of an electron-transparent lumen and an electron-opaque wall (Fig. 5). The tubules are not in contact with one another, but each is surrounded by a comparatively electron-transparent region and thus certain minimal distances appear to be present between the tubules. In some electron images, the individual tubules are seemingly connected by an electron-opaque fibrous component (Fig. 5, arrows). Tubules seen in transection give some evidence of substructure in the walls, but, so far, we have been unable to determine the number of subunits. The tubules are 231 ± 2.5 (SE)A(n = 121) in diameter and have a wall thickness of *circa* 60 A.

Dispersal of the P-Protein Bodies

It has been established that, during the differentiation of the sieve elements, the nucleus breaks down, the tonoplast disintegrates, the dictyosomes and ribosomes lose their identity, and the membranous components of the cells (*ER*) are reorganized. The P-protein bodies disaggregate at about the same time. This phenomenon is brought about by a separation of the component tubules so that the P-protein bodies lose their compact form. Eventually, the component tubules become dispersed in the cytoplasm which has become highly hydrated because of the loss of tonoplast. An early stage of disaggregation of a P-protein body is depicted in Fig. 6. The body has become elongated (it was twice as long as shown in the micrograph), the tubules are beginning to disperse, and cytoplasmic components have come to occupy spaces between groups of tubules. The adjacent nucleus is apparently still intact. Dictyosomes are present in the cell and are giving rise to coated vesicles with dense contents. Some of these vesicles are contained within the P-protein body. A more advanced stage of dispersal of a P-protein body is shown in Fig. 7. Groups of considerably dispersed tubules are associated with a second component of P-protein. This component consists of groups of fibrils with a striated appearance. We propose to distinguish the two morphologically distinct forms of P-protein by naming the tubular form P1-protein and the striated fibrillar form P2-protein. A higher magnification view of the tubules and fibrils is shown in Fig. 8. The tubules (*P1*) show indications of cross-striations, a feature even more conspicuous in Fig. 14. In view of this feature and the obvious replacement of the tubules by striated fibrils during the differentiation of the cell, we assume that the tubular form becomes reorganized into the striate fibrillar form; P1-protein becomes P2-protein. The striated fibrils are characteristic

FIGURE 4 *Nicotiana tabacum*. Transverse section of a sieve element at an early stage of differentiation. The large P-protein body (*P1B*) consists of a compact mass of tubules most of which are cut in transection. Cisternae of endoplasmic reticulum (*ER*) are applied to the body and one element occurs among the tubules. *PM*, plasma membrane; *W*, cell wall. × 53,000.

FIGURE 5 *Nicotiana tabacum*. Higher magnification view of a portion of the P-protein body shown in Fig. 4. The tubular nature of the protein can be clearly seen. There is some evidence of substructure in the walls of the tubules. Fine fibrils interconnect some of the tubules (arrows). × 165,000.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ER
ER
P1B
PM
W
4

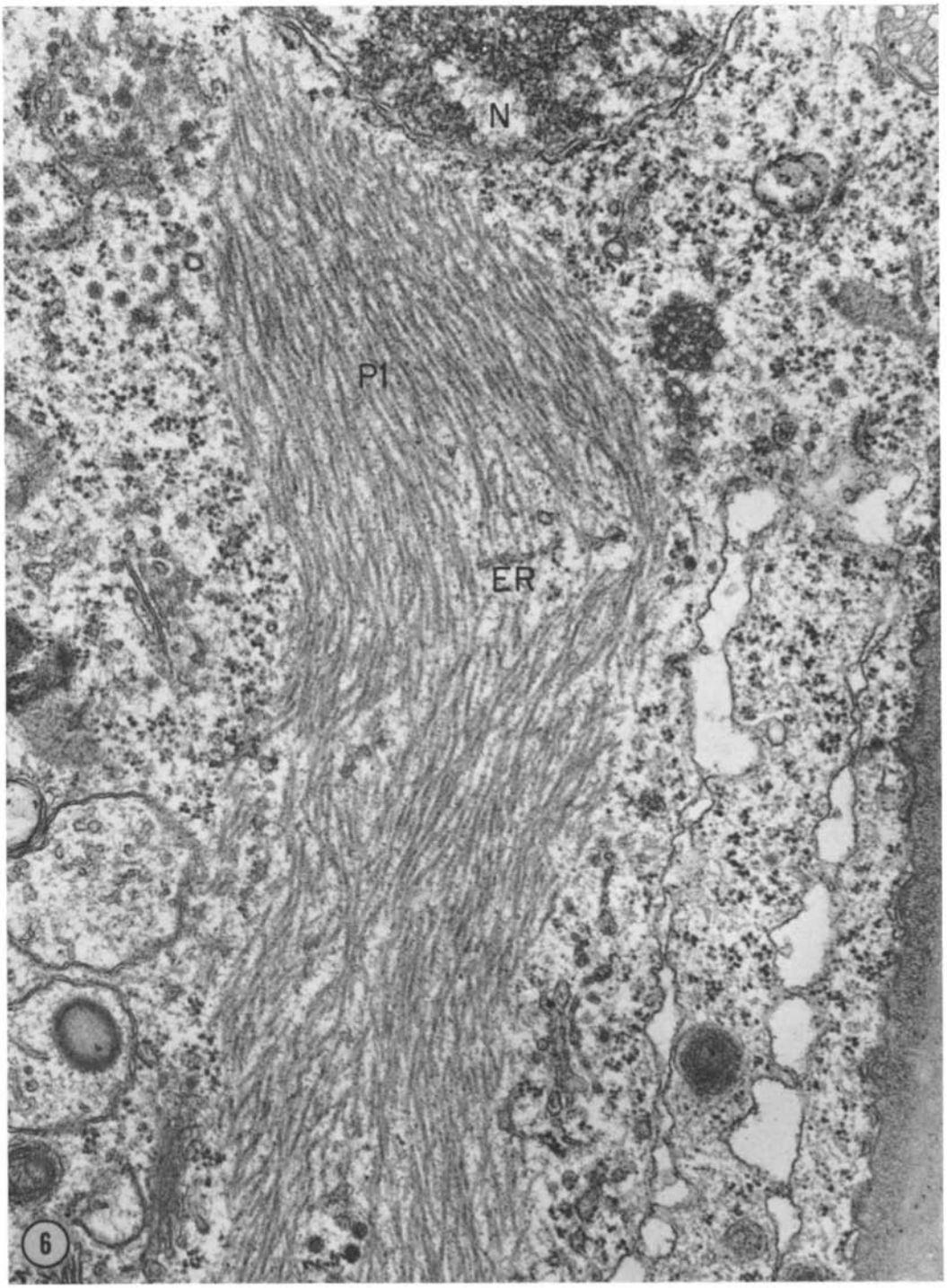


FIGURE 6 *Nicotiana tabacum.* Longitudinal section of a sieve element at an intermediate stage of differentiation. The nucleus is still intact (*N*). Adjacent to it is part of a P-protein body (*P1*) which has elongated (only one-half of the body is shown) and is starting to disaggregate. Surrounding cytoplasm contains plastids, mitochondria, endoplasmic reticulum, ribosomes, and dictyosomes. The dictyosomes appear to be giving rise to coated vesicles some of which occur among the tubules of the P-protein body. Elements of endoplasmic reticulum (*ER*) are also lodged within the body. × 33,000.

of the mature elements, especially in the region of the sieve-plate pores (Fig. 10). They are 149 ± 4.5 (SE)A(n = 43) in diameter, and the mean center-to-center distance of the stained bands is about 150 A. In transections they appear circular in outline (Fig. 9), with an indication of an electron-transparent center.

P-Proteins in Mature Sieve Elements

The pores of the sieve plates in the glutaraldehyde-fixed material of tobacco phloem we have examined thus far appear to be filled by an electron-opaque substance, which frays out into the lumen of the cells in the form of the 149-A striated fibrils of the P2-protein. This arrangement is illustrated in Fig. 10 in a longitudinal section of a portion of two sieve elements and a sieve plate. The fibrils in the central regions of the sieve-plate pores have lost their identity, and the contents of the pores are uniformly electron opaque. The plasma membrane (*PM*) can be seen to be continuous from cell to cell, lining the pore. The sieve elements in Fig. 10 are either at a late stage of differentiation or are mature. The membranous components (*ER*) of the cell have become rearranged adjacent to the plasma membrane. Dictyosomes and ribosomes have disappeared. Mitochondria and plastids persist, but are not shown in the figure. In the transectional view of a sieve-tube element at a late stage of differentiation in Fig. 11, the lumen of the cell contains dispersed P-protein mainly in the form of tubules. The P-protein which is located in the pore, however, is in the form of striated fibrils (Fig. 12). Some striated fibrils are also evident around the periphery of the cell (*P2*).

The P-protein component which is dispersed throughout the lumen may largely have the form of tubules (Fig. 11), or of striated fibrils similar to those which are packing the sieve-plate pores, or it may be a combination of the two forms together with intermediate forms between the two. A good example of a combination of two forms is shown in Fig. 13. The P-protein component which fills the sieve-plate pores is fanning out into the lumen of the cell mainly in the form of striated fibrils The P-protein component which is in the lumen of the cell is both in the form of tubules (*P1*) and in the form of striated fibrils (*P2*). The tubules and the fibrils are seen in longitudinal and in cross-sections.

Occasionally, the tubular form of the P-protein shows apparent structural periodicity in longitudinal section (Fig. 14). This feature has been observed only in more or less widely dispersed tubules and, as mentioned, may be used to relate the tubular form to the fibrillar. The fibrils are usually loosely arranged, but if they form bundles they show registry of the densities of electron-staining regions which results in a periodicity for the fibril bundle (Fig. 15).

Certain fibrous components observed in the sieve-tube elements could not be related to the P-protein bodies or the product of their dispersal, the fibrous P-protein component. Quantitatively, they are minor components and were seen in the form of very fine filaments (Fig. 16, arrow) or as bundles of long fibrils (Fig. 17) similar to those that have been described in *Avena* epidermal cells by O'Brien and Thimann (20).

DISCUSSION

Ontogenetic studies on sieve elements indicate that the P-protein component originates in the form of discrete bodies in the cytoplasm. The demonstration that the P-protein bodies in the sieve elements of *Nicotiana tabacum* consist of compact masses of tubules follows several recent studies which have shown the widespread occurrence of microtubules in the cytoplasm of both animal and plant cells (e.g., 5, 15, 28). The organization of the tubules in the differentiating sieve elements into discrete bodies seems to preclude the functions that have previously been suggested for the microtubular components of the cytoplasm, such as those of a cytoskeleton or one concerned with cytoplasmic movement. It could be, however, that the tubules contained in the P-protein bodies are merely at a storage stage and that their function is not realized until the cytoplasmic components are reorganized as the sieve element differentiates. During this differentiation, the tubules of the P-protein bodies disaggregate, and after this disaggregation they could, conceivably, have one or more functions previously suggested for the microtubules. The tubules, or their component fibrils, could form a structural component of the cytoplasm, maintaining its integrity at the time when it is subjected to rapid hydration after the tonoplast breaks down.

The tubular protein appears to become reorganized into a fibrillar form at about the time the tonoplast disintegrates. Possibly, this reorganization takes place because of the different chemical conditions that become established in the cyto-

plasm after its dilution with the vacuolar contents. The occurrence of tubular and fibrillar forms in the same mature cells (Fig. 13) may indicate that the reorganization is not necessarily complete. In the sugar beet, only a tubular form was encountered in mature sieve elements (10).

The demonstration of the two morphological forms of the P-protein component clarifies the relationship of the "persistent" crystalline protein bodies of the sieve elements of the Papilionatae (3, 14, 29) to the slime, or P-protein, which has been observed in the sieve elements of various other plant species. The striated P-protein component which we have described as P2-protein has been shown (Fig. 15) to be combined sometimes into larger fibrils which have lateral registry of the densely staining components. This image resembles that demonstrated by Laflèche (14) as being characteristic of the "persistent" protein body in *Phaseolus vulgaris* sieve elements. We think, therefore, that the P2-protein component corresponds to the protein in the "persistent slime body," or "flagellate body," characteristic of the sieve elements of Papilionatae. The P1-protein apparently has its counterpart in the Papilionatae also, for Laflèche (14) found tubular material, in addition to the persistent body, in the sieve elements of the bean. It is of interest that the type of structure characteristic of P2-proteins has been shown to occur in tonofilaments of epithelial cells in mice (12), and that other proteins such as collagen and fibrin show periodic repeat units when viewed with the electron microscope.

We suggest that differentiating sieve-tube elements are capable of producing a variety of protein components, some in large quantity, and that at least some of these components are related to the function of the sieve elements. The demonstration of the ontogenetic relationship of the P1 tubular component to the P2 striated fibrillar component indicates that the P-protein can exist in more than one structural form. The tertiary structure of the protein component must be such that several types of organization are possible and that there can be interconversion of the various forms. The idea of interconversion is supported by images such as the one depicted in Fig. 14, which shows that the tubular form of the P-protein has periodic staining densities along it. It is also supported by the observations of Northcote and Wooding (19) according to whom P-protein bodies in sieve tubes of *Acer pseudoplatanus* consist of either regularly arranged, closely packed fibers 180–240 A in diameter, or of more loosely organized, striated fibrils 90–100 A in diameter, or of both fibers and fibrils. During the disaggregation of the P-protein bodies, the 180–240-A fibers disperse to produce the 90–100-A fibrils which then fill the lumen and the sieve-plate pores of the mature sieve elements.

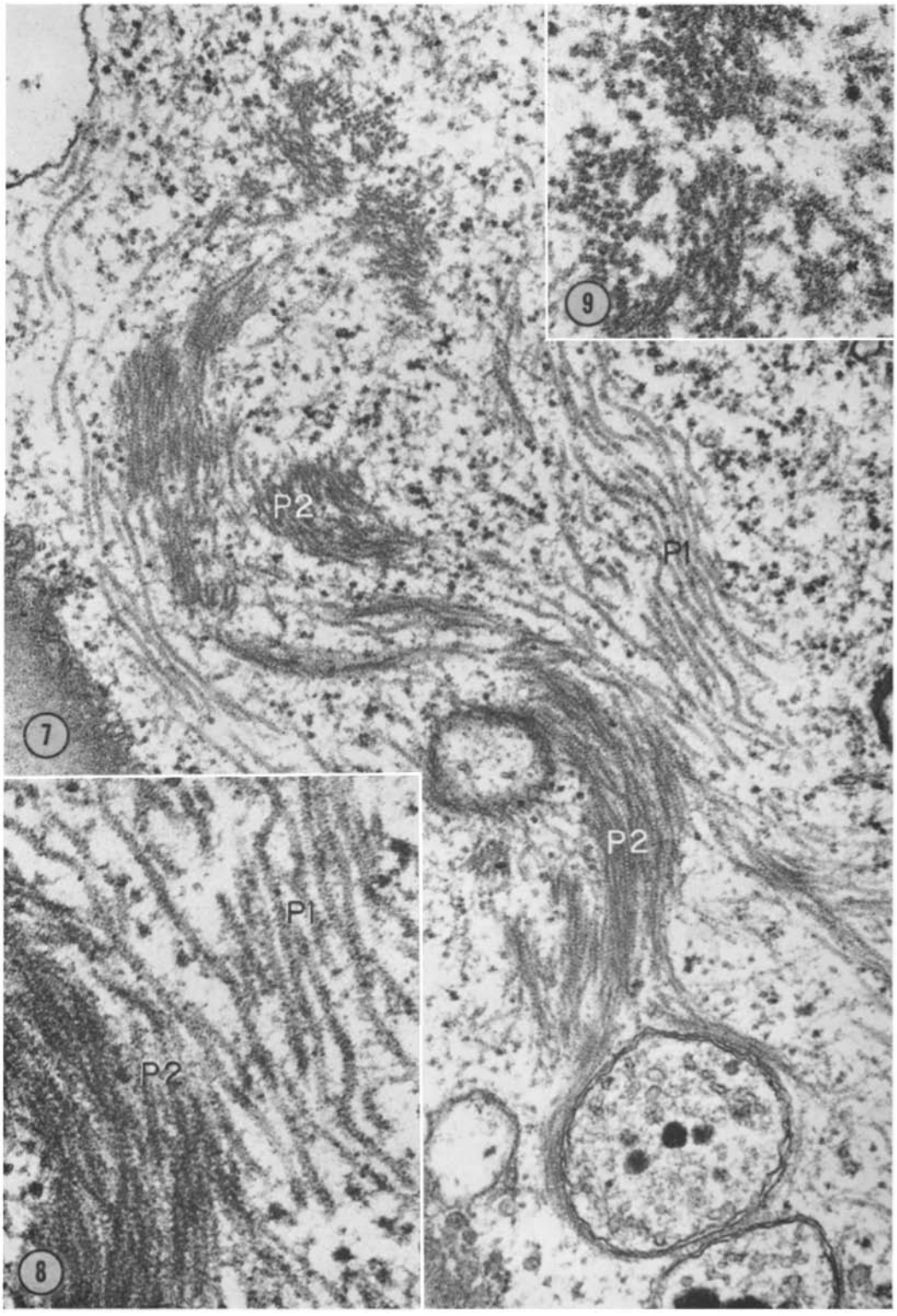
Other proteins in plant and animal cells are known to occur in various conformations and systems of subunits. Renaud, Rowe, and Gibbons (24) isolated the outer fibers of cilia of *Tetrahymena pyriformis* and showed that after precipitation the isolated proteins have an irregular form, but are composed, at least in part, of parallel protofilaments about 40 A in diameter. These authors postulated that the aggregated material in aqueous solutions of the outer fibers consists of oxidized or partly denatured protein. Protofilament subunits of microtubules have been shown in sections of cells in plants (16) and in *Sepsis* (23), as well as in isolated microtubules (1, 11, 21). Investigators working with rat optic nerves and flight muscle have described systems of microtubules and filaments and have advanced the concept that certain cytoplasmic filaments may be formed from microtubules (2, 22).

It seems pertinent to consider the common phenomenon of filling of the sieve-plate pores with P-protein in the light of our observations that, in sugar beet plants infected with the beet yellows

FIGURE 7 *Nicotiana tabacum*. Longitudinal section of a sieve element at a comparatively late stage of differentiation. The P-protein body is at an advanced stage of disaggregation. Two components of the P-protein are visible, the tubular form P1-protein (*P1*) and the striated fibrillar form P2-protein (*P2*). × 44,000.

FIGURE 8 *Nicotiana tabacum*. Higher magnification view of the P1 and P2 proteins shown in Fig. 7. × 80,000.

FIGURE 9 *Nicotiana tabacum*. Higher magnification view of the transection of P2-protein shown in Fig. 7. × 80,000.


P2
P1
7
P2
9
P1
P2
8

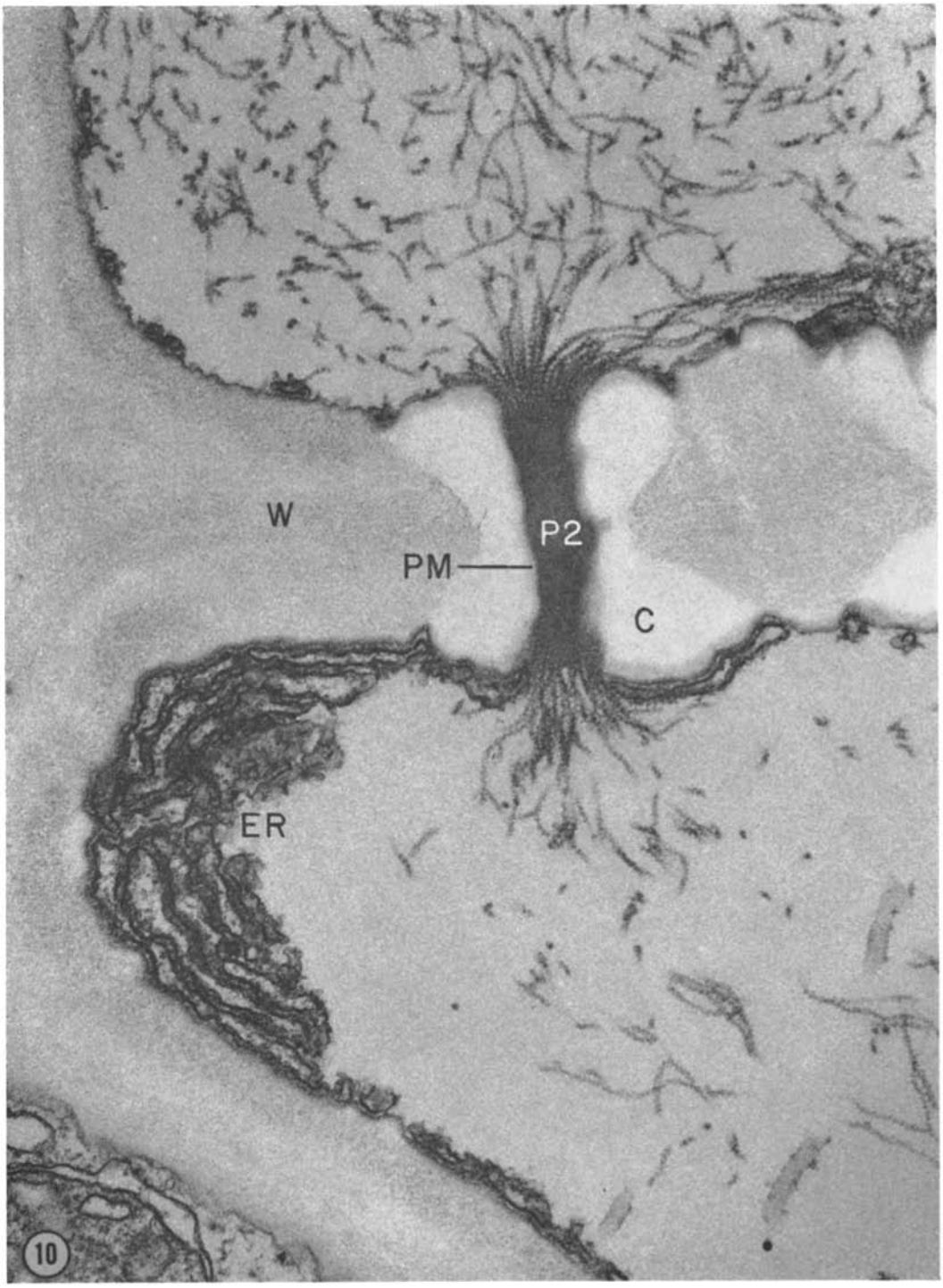


FIGURE 10 *Nicotiana tabacum*. Longitudinal section of a sieve element at a late stage of differentiation with a median section through a sieve-plate pore. The plasma membrane (*PM*), a triple-layered structure, is continuous through the pore. Pore is filled with an electron-opaque component fanning out into the cytoplasm as the characteristic striated P2-protein fibrils. Within the sieve elements, cisternae of endoplasmic reticulum (*ER*) are closely applied to the plasma membrane. *C*, callose; *W*, wall. × 62,000.

virus, the sieve-plate pores were occupied by virus particles (10). The virus particles appeared to have replaced the P-protein component in the pores. We interpreted the views as indicating a mass movement of the virus particles through the conduit together with the translocated photosynthate. If this interpretation is correct, one could assume that the presence of P-protein in the pores similarly indicates a movement of this component in the nutrient stream and its retention (possibly in a denatured state) *in situ* in the pores by the fixing solution. The difficulty with this assumption is that it requires an additional one: the protein is moving out of the sieve tube at some place. We have no evidence for such movement. Another possibility would be that the P-protein accumulates in the pores as a result of additional mass flow caused by pressure changes in the conduit induced by cutting, and is fixed there by the killing solution. This interpretation has been made often in the past, but it does not seem to be entirely compatible with the symmetrical arrangement of the P-protein component on the two sides of the pore as depicted in Fig. 10. If the protein component were moving in response to the cutting of the conduit, one would expect it to be more massive on one side of the sieve plate. The third possibility, that the filling of the sieve-plate pores with P-protein represents the normal condition in the functioning sieve tube, seems to be precluded by the observations on the relation of virus particles to the pores. Obviously, a satisfactory interpretation of the relation of the P-protein to the sieve plate (and the lumen of the cell) requires additional research.

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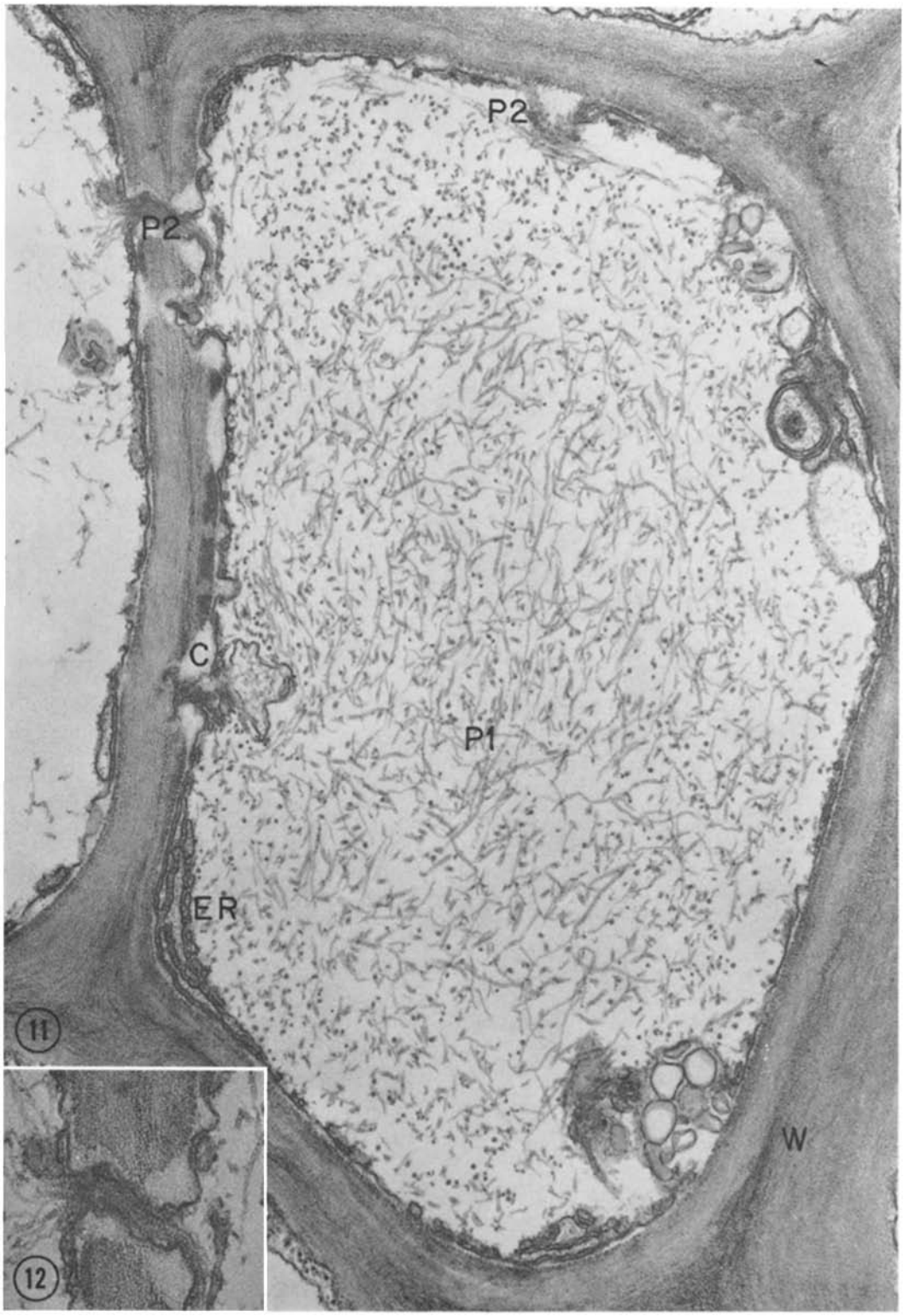
FIGURE 11 *Nicotiana tabacum*. Transverse section of a sieve element at a late stage of differentiation. Membranous components of the cytoplasm occupy the periphery of the cell. The cell lumen is filled with evenly dispersed units of P-protein. Most of the protein is in the tubular or P1 form (*P1*) although some P2-protein is evident (*P2*). The sieve-plate pore is filled with the P2 type protein. *C*, callose; *ER*, endoplasmic reticulum; *W*, cell wall. × 31,000.

FIGURE 12 *Nicotiana tabacum*. Higher magnification view of the sieve-plate pore shown in Fig. 11. The striated nature of the P2-protein component within the pore can be clearly seen. × 53,000.


P2
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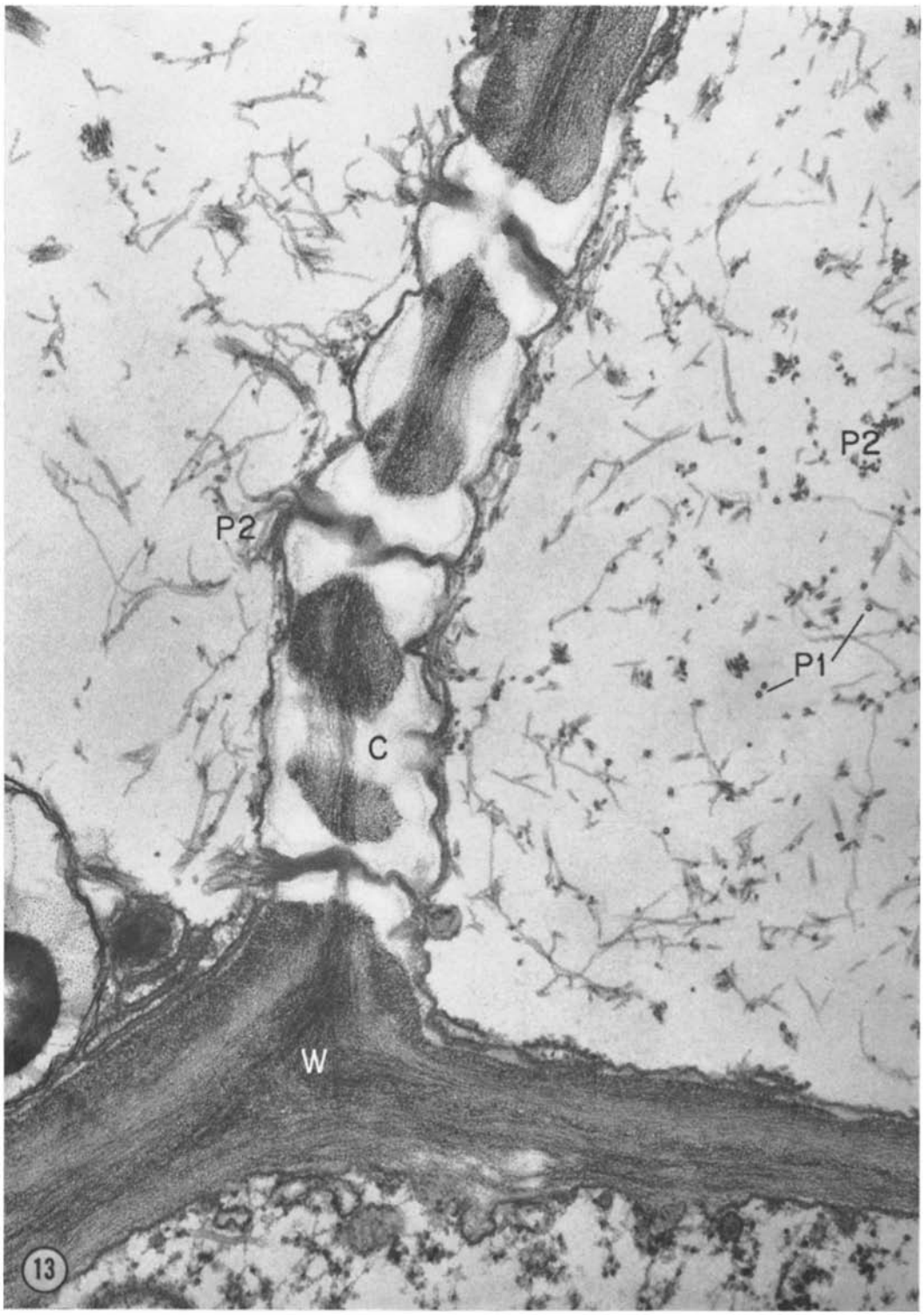

FIGURE 13 *Nicotiana tabacum*. Longitudinal section of a sieve plate between two mature sieve elements. Pores in the sieve plate are filled with the P2-protein which frays out in the form of the characteristic striated fibrils (*P2*). The lumina of the sieve elements contain P1 (*P1*) and P2-proteins (*P2*) appearing in longitudinal and cross-sections. *C*, callose; *W*, cell wall. × 45,000.

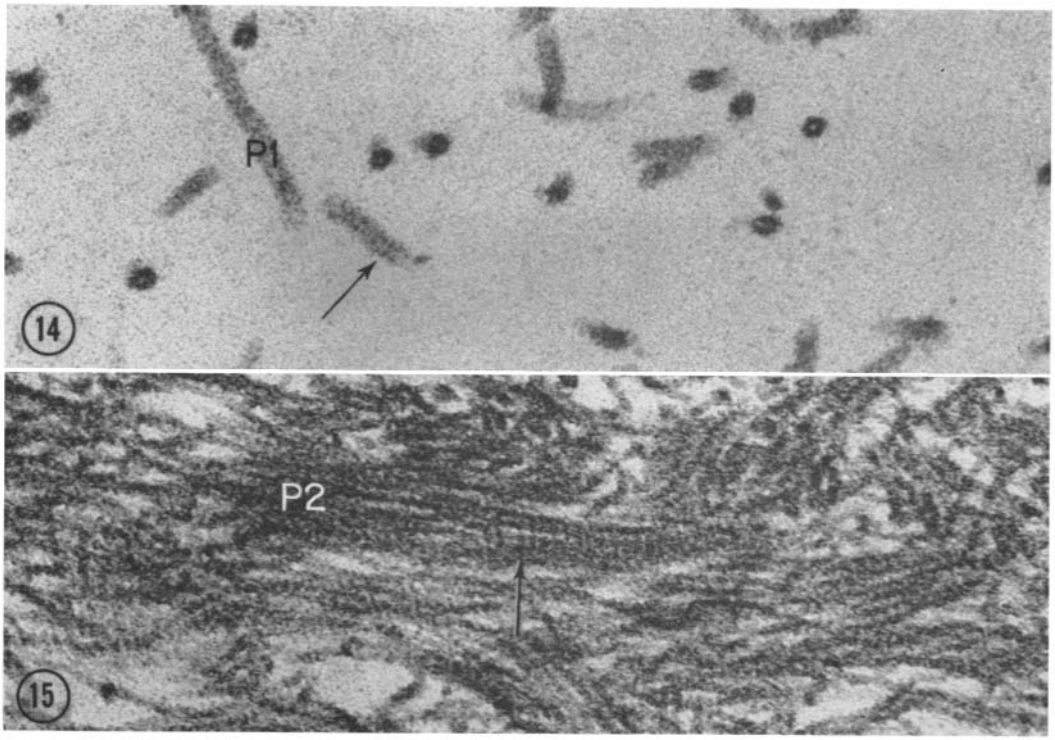

FIGURE 14 *Nicotiana tabacum*. Higher magnification view of some of the P1-protein units. At the arrow is a tubule of P1-protein which has a striated appearance. × 132,000.

FIGURE 15 *Nicotiana tabacum*. Higher magnification view of the P2-protein. Some of the P2-protein fibrils (*P2*) are closely aggregated and show a lateral registry of the electron-opaque bands (arrow). × 108,000.

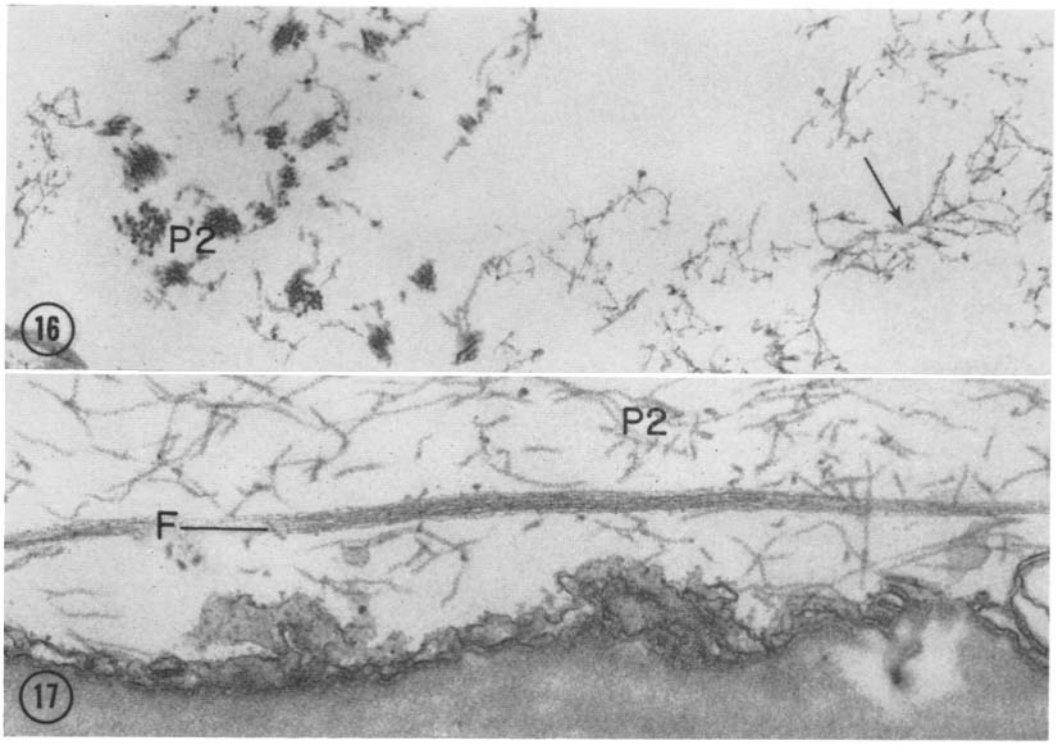

FIGURE 16 *Nicotiana tabacum*. Transections of the P2-protein component at *P2*, and a component consisting of fine fibrils indicated by an arrow. × 45,000.

FIGURE 17 *Nicotiana tabacum*. Tubules of the P2-protein component (*P2*) are seen together with a group of fine fibrils (*F*) in a mature sieve element. × 45,000.