P PROTEIN IN THE PHLOEM OF *CUCURBITA*

I. The Development of P-Protein Bodies

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ABSTRACT

Light and electron microscopical observations of the cells of the phloem of *Cucurbita maxima* have shown that two distinct types of P-protein bodies are formed: a larger type which arises as fine fibrils and a smaller type which apparently arises as groups of tubules. The tubules of the smaller type of body measure 242 ± 3.6 (SE) A (n = 48) and appear morphologically identical with the P1-protein tubules of *Nicotiana tabacum* L. In some of these P1-protein bodies the tubules are arranged in a regular manner with a center-to-center distance of 295 A. The P protein of the larger type of P-protein body is first apparent in the cytoplasm as small aggregates of fine fibrils. This P-protein component has been designated P3 protein. As the P3 protein accumulates it is organized into large bodies. Some of these bodies contain only P3 protein, others a tubular form of protein, and still others a combination of P3 protein and a tubular form. This variability indicates that there is a developmental sequence of the formation of tubules from the P3-protein fibrils. These tubules measure 179 ± 8.2 (SE) A (n = 31) and have been designated P4 protein.

INTRODUCTION

Our recent papers dealing with the cell components of sieve elements in *Nicotiana tabacum* have provided some clarification of the nature of the proteinaceous component usually called slime (5, 8). On the basis of these studies we proposed to name the substance P protein. In *N. tabacum* the P protein proved to have two forms: a tubular form characteristic of the structural elements of bodies (slime bodies) present in young sieve elements and a fibrous form with periodic cross-striations which appears during further differentiation of the sieve element, probably as a product of breakdown of the tubular form. We designated the tubular form P1 protein and the fibrous form P2 protein.

The occurrence of two forms of P protein in *Acer pseudoplatanus* may be deduced from the description of sieve-element ontogeny in that species by Northcote and Wooding (15). The authors characterized both forms as fibrous but differing in the diameter of the fibrils and suggested that the wider fibers, 180–240 A in diameter, which occur in compact masses (slime bodies), later disperse and produce the thinner, 90–100-A fibrils, spread throughout the cell. Along its length, the narrower fibril has alternating light and dark bands about 50 A long, an indication that this entity corresponds to our P2-protein form. Possibly the wider fibril constitutes a tubular form of the P1-protein type.

Two forms of proteinaceous inclusion were found also in *Pisum sativum* (2, 20) and *Phaseolus vulgaris* (12, 14). One form occurs as a compact, elongated, paracrystalline structure that has long been interpreted as the slime body in papilionaceous species. Its elements are fine fibrils with

cross-striations similar to those shown by P2 protein in *Nicotiana* (5). Additionally, Laflèche (12) depicted an inclusion with tubular components but she did not interpret it as slime and did not relate it to the paracrystalline body. Newcomb (14) identified as slime a fibrous and a tubular component in cells adjacent to the protophloem sieve tubes in roots of the bean.

The P protein has not been critically examined at the ultrastructural level although this material constitutes the most prominent component in immature sieve elements of Cucurbitaceae. At a certain stage of cell differentiation P protein occurs as numerous bodies resembling the chloroplasts in neighboring mesophyll cells in size and alignment along the wall in the parietal cytoplasm. Esau and Cheadle (7), using material fixed with potassium permanganate, observed that the texture of the P-protein bodies of *Cucurbita* was granular whereas the dispersed material appeared "granular, flaky, or fibrous." Buvat (4) found the bodies to be granular, with the granulation more pronounced after fixation with osmium tetroxide than after that with potassium permanganate, and he saw a similarity in this granulation between the P-protein bodies and the nucleoli. Buvat used this appearance, together with histochemical tests for RNA, to suggest that the P-protein bodies contained RNA. The use of glutaraldehyde–osmium tetroxide fixation by Evert et al. (10) did not result in an improvement of the resolution of the fine structure of the P-protein bodies in *Cucurbita*. Without good evidence the authors described the body as having "a combined granular-lamellar appearance."

In addition to the large P-protein bodies, an occasional small inclusion body with an orderly arrangement of component elements was detected with the electron microscope. Buvat (3) suggested that the body consisted of lamellae and described the complex as "lamelles myéliniformes." Eschrich (9) interpreted the elements composing the body as rods or filaments and proposed the term "filar body" (Filarkörper). His illustration (Fig. 5 on page 251 of reference 9) suggests that the filaments may be tubules and that these tubules tend to be in an orderly arrangement. Evert et al. (10) obtained no details on the structure of the small body and interpreted it as a portion of the large P-protein body projecting from the latter after the rupture of the presumed membrane covering the larger body. The slight suggestion of parallel lines in the "projection" was taken as an indication of the lamellar structure of the P protein.

The P protein of *Cucurbita* obviously required a reexamination The present paper reports on the fine structure of the P protein in *Cucurbita maxima*, relates it to light microscope views, and compares it with the fine structure of the P protein in *Nicotiana tabacum*.

MATERIALS AND METHODS

Cucurbita maxima plants were grown from seed under greenhouse conditions. 1 cm pieces were cut from young petioles and internodes and placed in glutaraldehyde-formaldehyde according to the method of Karnovsky (11). These samples were cut into smaller pieces after ½ hr, and fixation was continued for a total of 2 hr. Postfixation was done with 2% osmium tetroxide. 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, and viewed and photographed with a Siemens Elmiskop I.

Comparable material was fixed for the light microscope of Craf III (chrome-acetic-formalin formula III) (17) or 10% acrolein and 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. (13).

RESULTS

The general features of the ontogeny of the P-protein bodies at the light and electron microscope levels have been described and illustrated in many papers (see review in reference 7). Briefly, the P-protein component arises in the cytoplasm and accumulates into discrete P-protein bodies, which disperse during the later stages of differentiation of the sieve elements. In relation to this ontogenetic sequence a detailed description of the specific protein components of the sieve-element protoplast of *Nicotiana tabacum* has been presented, and the term P protein has been introduced (5, 8). We intend to continue the use of the term P protein for all types of specific protein components in the phloem.

The proteinaceous nature of the P-protein component has previously been deduced from cytochemical studies of *Cucurbita* (7). These results have been confirmed in the present study. Sections of *Cucurbita maxima* phloem stained with mercuric

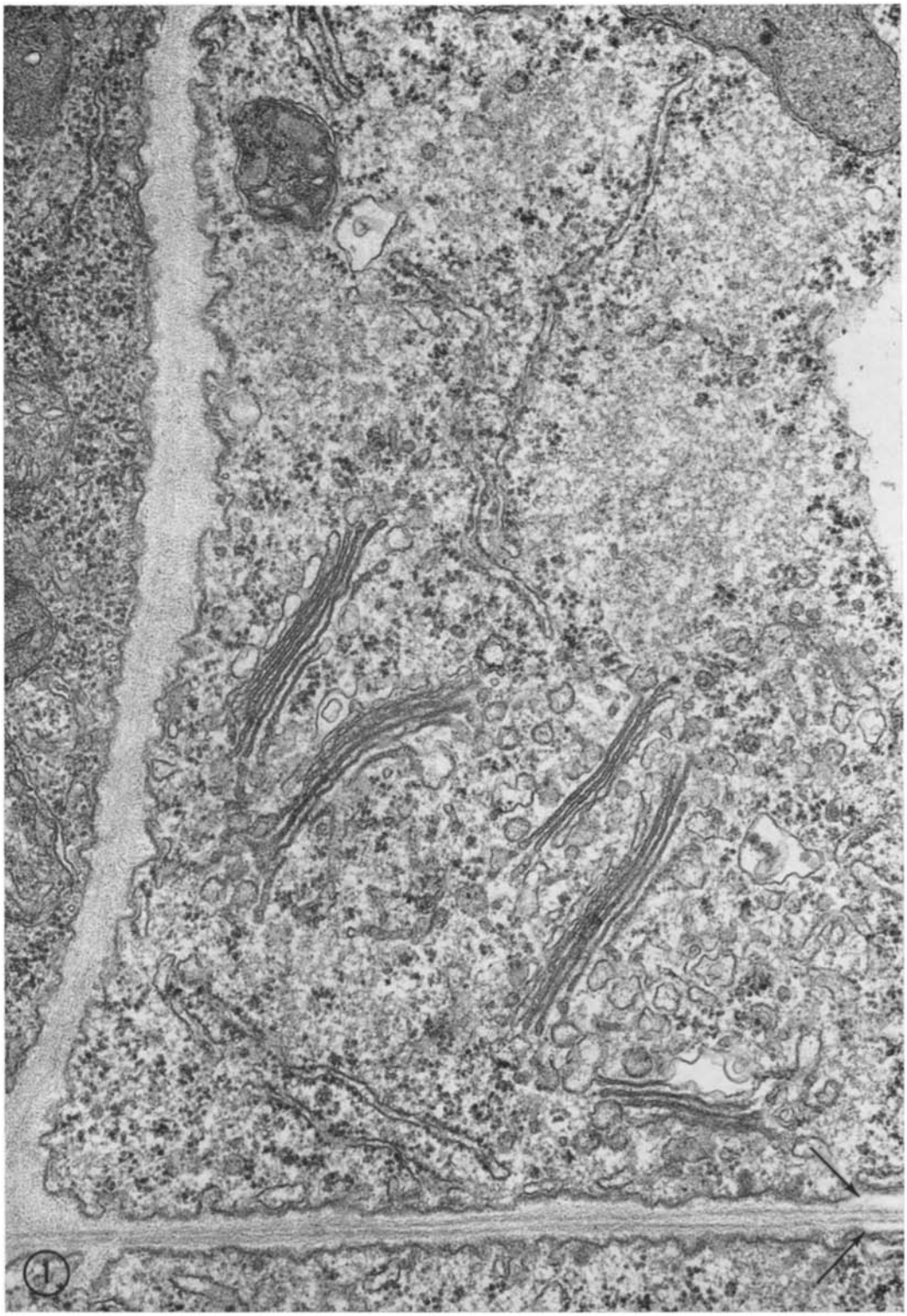


FIGURE 1 *Cucurbita maxima.* Longitudinal section of a portion of a sieve element at an early stage of differentiation. The P protein is present as groups of fine fibrils intermixed with the various organelles (lower left). In the larger accumulations of P protein (upper right) the center of the accumulation is free of other organelles. Several dictyosomes and cisternae of the endoplasmic reticulum are apparent in the region of P-protein synthesis. Arrows, callose platelets. × 39,000.

bromphenol blue showed intense staining of the compact bodies of differentiating cells and of the accumulations on the sieve plates of mature cells. A weaker staining reaction was noted for the dispersed P-protein components.

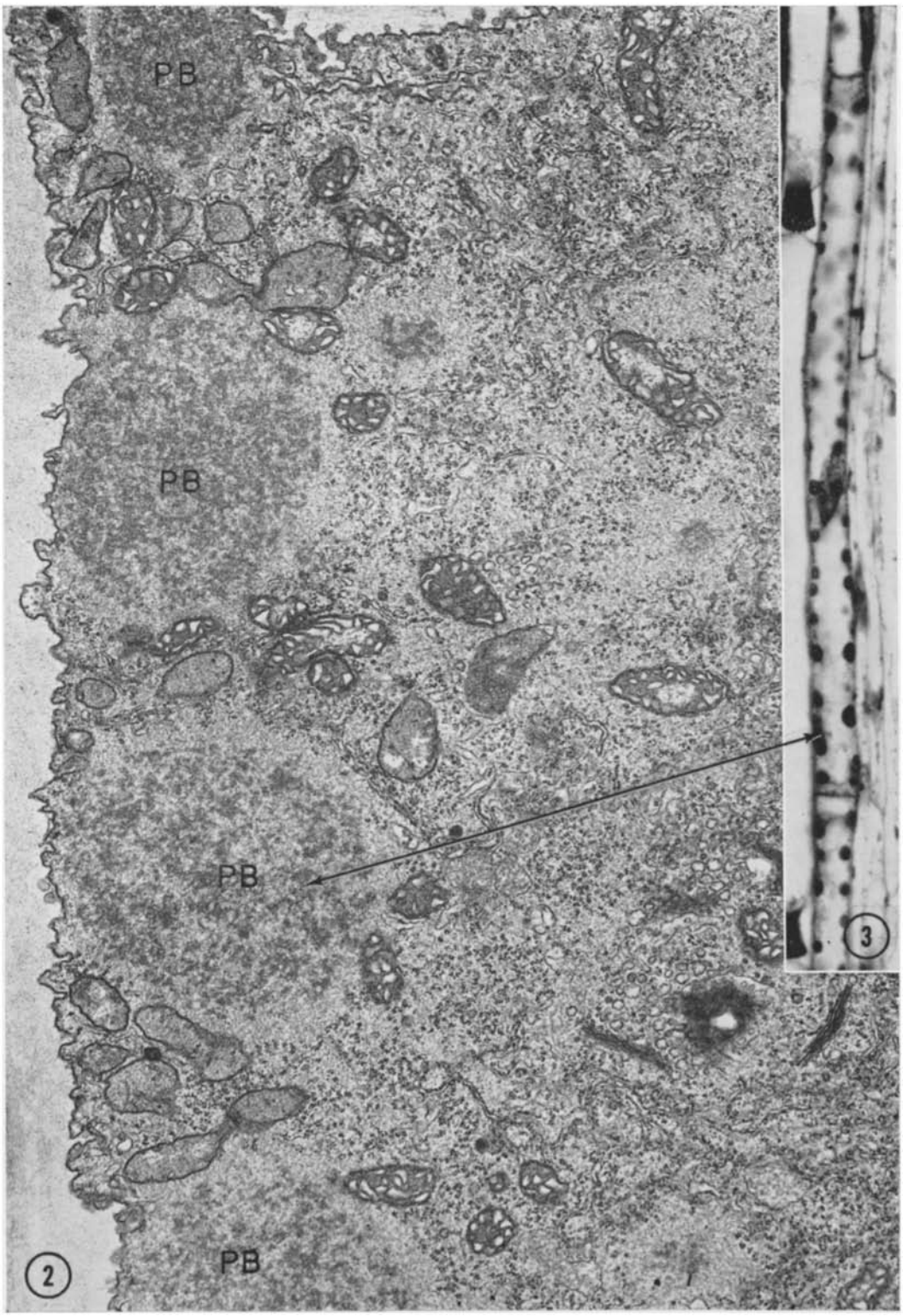
As seen with the electron microscope, two distinct types of P-protein bodies are evident in *Cucurbita maxima*, a larger type which arises as fibrils and a smaller type which has been seen only in tubular form. The larger type is considered first. The P-protein component of this type of body is first apparent in the cytoplasm as small aggregates of fine fibrils (to be called P3 protein henceforth) intermixed with various cellular organelles. Ribosomes, dictyosomes, and cisternae of the endoplasmic reticulum are prominent in these presumed regions of synthesis of P protein. The aggregates of fibrils contain also apparently circular or elliptical components. We have assumed these images to be transectional views of the fibrils although a granular component may be present as well. As the deposition of the P3 protein continues, the aggregates become larger and the cell organelles obviously are excluded from the center of the accumulations. Fig. 1 illustrates these early stages in the formation of the P-protein bodies. The section shows a very young sieve element with the future sieve plate just beginning to form callose platelets (arrows). Several dictyosomes with associated vesicles, cisternae of the endoplasmic reticulum, a mitochondrion, a portion of a plastid, and numerous ribosomes are included in the micrograph. Fine fibrils of the P3 protein are evident in the ground substance (lower left) and also as larger accumulations in localized regions (upper right). Organelles are excluded from the larger accumulations.

The P-protein material continues to increase in amount as cell differentiation proceeds, and the accumulations become organized into discrete bodies in the parietal cytoplasm (Figs. 2 and 3). These young P-protein bodies show an increase in the density of their components and rather consistently are surrounded by a comparatively electron-lucent region (Figs. 2 and 4). At no stage of their development are they membrane bounded although in some cases elements of the endoplasmic reticulum lie parallel to their surfaces and give the appearance of a bounding membrane (Fig. 5). Evert et al. (10) described the slime bodies in *Cucurbita* as membrane bounded but a close examination of their Fig. 11 reveals that the "membrane" shows interrupted profiles and attached ribosomes typical of sectional views of cisternae of the endoplasmic reticulum. Northcote and Wooding (15) depicted large accumulations of endoplasmic reticulum cisternae around a slime body in *Acer pseudoplatanus*. Young and mature P-protein bodies in *Cucurbita* occupy the peripheral cytoplasm of the cells and may be adjacent to the plasma membrane (Fig. 2). They are numerous (Fig. 3); we estimate that the number is 70–100 per cell. There is a variation in the size of the mature P-protein bodies but they are usually spherical or ellipsoidal and of the order of 20 μ in diameter. In any one cell the bodies are usually of a fairly uniform size. In both light and electron micrographs of longitudinal sections, the P-protein bodies appear in longitudinal rows, and in some cells over half the observable plasma membrane may be adjacent to the P-protein material (Fig. 2).

The sieve elements during the early stages of their differentiation have numerous dictyosomes which are frequently situated at the periphery of the developing P-protein bodies together with dictyosome-derived vesicles (Fig. 4). These vesicles often contain finely divided fibrous material which resembles the fibers of P3 protein (Fig. 4). The

FIGURE 2 *Cucurbita maxima*. Longitudinal section of a portion of a sieve element at an intermediate stage of differentiation. The P protein is organized in the form of discrete bodies (*PB*) which appear in a longitudinal row adjacent to the plasma membrane. Each of the P-protein bodies is surrounded by a comparatively electron-lucent region containing fibrils of the P3-protein material. The central part of the P-protein bodies is differentiated into more electron-opaque and more electron-lucent regions. × 14,000.

FIGURE 3 *Cucurbita maxima*. Light micrograph of a longitudinal section showing portions of sieve tubes one of which is at a stage of differentiation comparable to that depicted in Fig. 2. The section has been stained with hematoxylin and the P-protein bodies in the peripheral cytoplasm appear black. Arrow, P–protein bodies at light and electron microscopic levels. × 640.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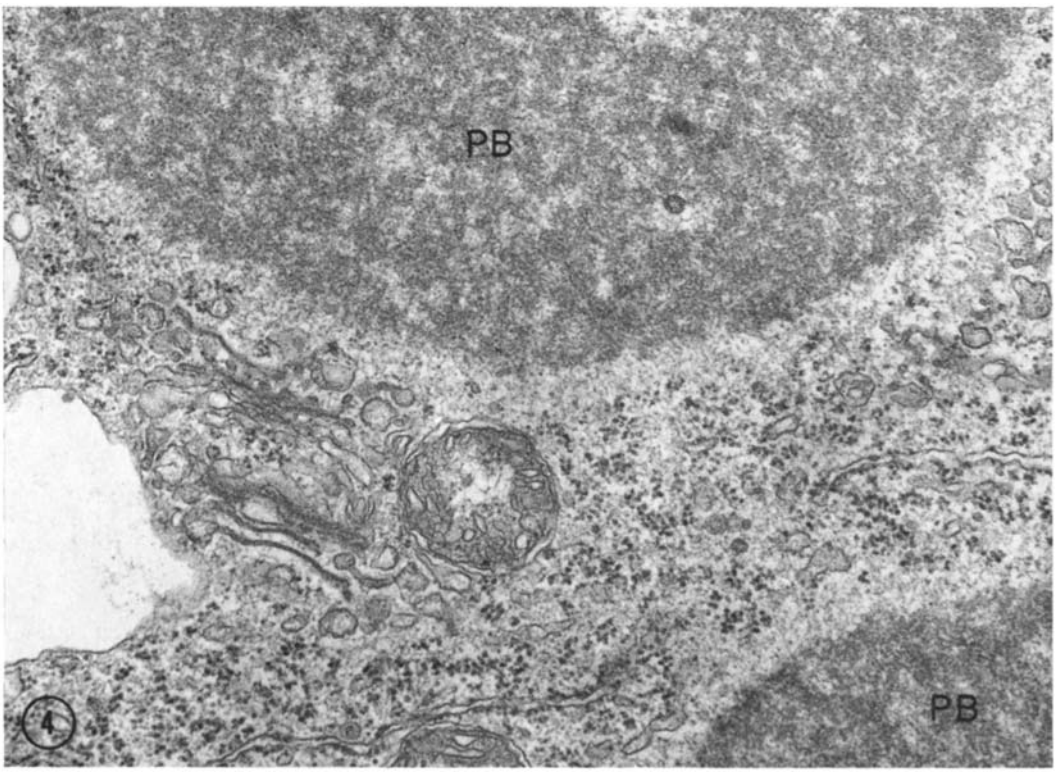

FIGURE 4 *Cucurbita maxima.* Section through a sieve element at an intermediate stage of differentiation showing portions of two P-protein bodies (*PB*) and the adjacent cytoplasm. The P-protein bodies are surrounded by a comparatively electron-lucent region. Adjacent to one of the P-protein bodies are a dicytosome and dictyosome-derived vesicles. The vesicles contain a fibrous component. × 30,000.

vesicles are most commonly smooth-surfaced although in some cells coated vesicles (1, 6) were observed both associated with dictyosomes and at the surface of the P-protein bodies (Fig. 6). In some cells the vesicles were localized in an apparent region of early synthesis of the P protein and were of the familiar coated type (see Figs. 1 and 7). In others the coat bore long extensions seemingly fibrillar in form (Figs. 5 and 7). The vesicles with the long extensions resemble the so-called spiny vesicles reported by Newcomb (14) in *Phaseolus.* Newcomb observed that the spiny vesicles appeared in procambial and pericyclic cells before and during P-protein formation. In *Cucurbita* they were evident in parenchyma cells (Fig. 7) and also in differentiating sieve elements (Fig. 5).

Many studies have indicated that the dictyosomes disappear as the sieve elements mature. Apparently their breakdown occurs at about the same stage of differentiation of the sieve elements as the breakdown of the vacuolar membrane and the nucleus, and their diappearance has been verified in the present study.

After its assembly or accumulation into distinct bodies, the P3-protein component may be structurally modified and the P-protein bodies may develop to show a range of internal structure. Most commonly, the interior of the P protein becomes differentiated into more electron-opaque and more electron-lucent regions (Figs. 2, 4, and 9). Older bodies may be uniformly electron opaque. Distinct vacuoles are often formed (Fig. 11). As previously stated, the various cell organelles are excluded from the bodies and are usually separated from the young bodies by a comparatively electron-lucent region, which appears as a halo in transection, consists of finely dispersed fibrils of P3 protein (Figs. 2, 4, and 8) and disappears as the P-protein bodies mature.

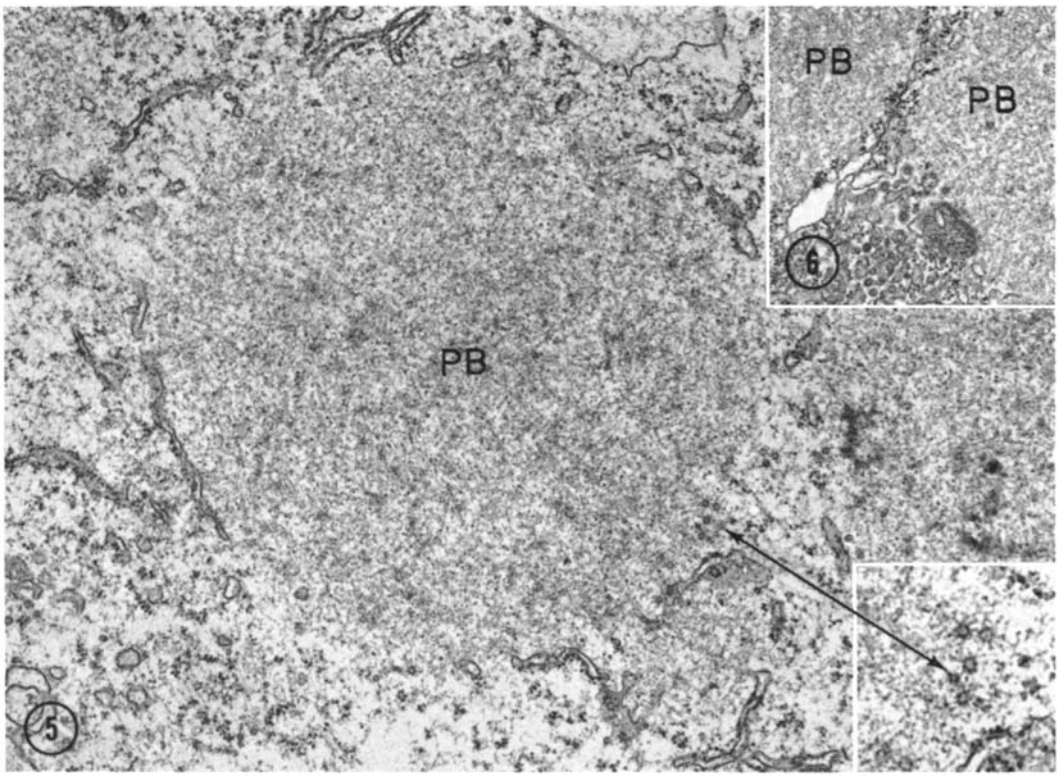

FIGURE 5 *Cucurbita maxima.* Section of a portion of a differentiating sieve element. One P-protein body (*PB*) and portions of two others are included in the section. They are of the finely fibrous P3-protein type. Coated vesicles of the spiny type are seen at the surface of one of the P-protein bodies (arrow). Some of the ER cisternae are parallel to the surface of the P-protein bodies. × 18,000. Insert, Higher magnification view of some of the coated vesicles shown in Fig. 5. × 37,000.

FIGURE 6 *Cucurbita maxima.* Section showing a portion of two slime bodies (*PB*); coated vesicles appear near the periphery of one. × 17,000.

The form of the P protein within the larger type of P-protein body varies from cell to cell but it is always fibrillar, or tubular, or a combination of the two. Whether the specific forms are real or artifacts of fixation, a variation in form no doubt exists, and we have concluded that the various electron micrographic images represent stages in a polymerization or depolymerization series. It appears that there is a developmental sequence from the fine fibrils of P3 protein to a larger component. This larger component usually becomes evident in the more electron-opaque regions of the P-protein bodies. In many transections these larger units have an electron-lucent lumen and an electron-opaque wall, an image indicating a tubular structure. We choose to refer to this component as P4 protein. It is illustrated in Figs. 9–11. The tubules of P4 protein measure 179 ± 8.2 (SE) A (n = 31). The formation of the P4-type protein component from the finely fibrillar P3 type does not take place in all P-protein bodies as is illustrated in Fig. 5. In the body shown in this figure the P-protein component apparently is remaining as the finely fibrillar P3 type.

The second, smaller type of P-protein body consists of an assembly of distinct tubules, which measure 242 ± 3.6 (SE) A (n = 48) and appear morphologically identical with the P1-protein tubules described in *Nicotiana* (5). Because of this resemblance the component is referred to as P1 protein. So far as we were able to observe, the smaller P-protein bodies are first apparent in the cytoplasm as groups of distinct tubules; in this regard they resemble the P1-protein bodies of

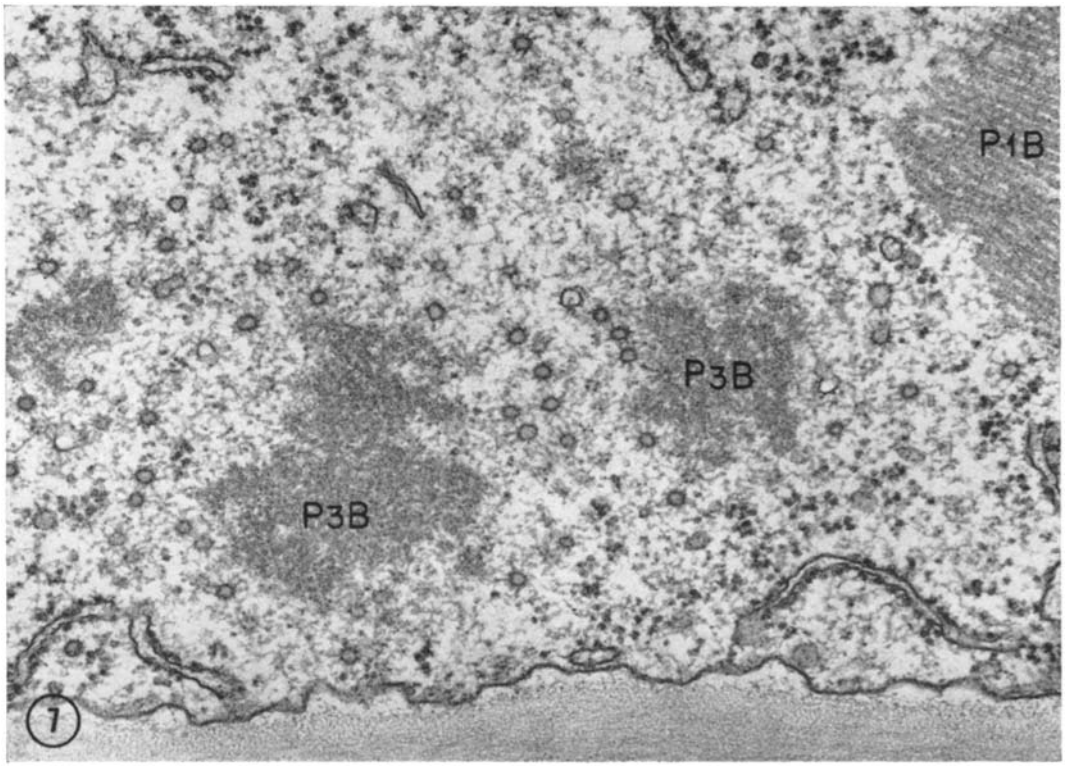

FIGURE 7 *Cucurbita maxima.* Section of a portion of a phloem-parenchyma cell. Four P-protein bodies are included in the section. One of these is of the tubular P1-protein type (*P1B*); the others are of the finely fibrous P3-protein type (*P3B*). Spiny vesicles are abundant in the cytoplasm surrounding the P-protein bodies. × 45,000.

Nicotiana. Most commonly the mature P1-protein bodies occur adjacent to the larger type of P-protein body ("satellite," Figs. 11 and 12). Often the tubules appear as an ordered array and are obviously equivalent to the lamelles myéliniformes described by Buvat (3). Fig. 13 shows these tubules organized in a lattice with a center-to-center spacing of 295 A. The parallel arrangement of the tubules in longitudinal section is shown in Fig. 14.

Accumulations of P protein are not restricted to differentiating sieve elements but are found also in companion cells (Figs. 15 and 16) and phloem-parenchyma cells (Fig. 7). The P proteins in these other cell types appear identical with those found in the sieve elements. The companion cell in Fig. 15 contains the normal cell components and in addition two P-protein bodies, a larger body of the P3-protein type and a smaller satellite body of the P1-protein type.

DISCUSSION

The present ontogenetic study on *Cucurbita* sieve elements has shown that the P-protein component arises in the cytoplasm in the form of fine fibrils or tubules and is assembled into discrete P-protein bodies of two sizes. The fibrils which are assembled into the larger bodies may be reorganized into tubules. Later, in most instances, these P-protein bodies disaggregate.[1] Essentially, this process is similar to that previously described for the sieve elements of *Nicotiana* (5). Several fundamental differences have been noted, however. There is commonly only one P-protein body per cell in *Nicotiana* (or one large one and a few small ones) whereas there are numerous bodies in *Cucurbita*. Further, in *Nicotiana* the body originates in the

[1] Cronshaw, J., and K. Esau. 1968. P protein in the phloem of *Cucurbita*. II. The P protein of mature sieve elements. *J. Cell Biol.* **38:** in press.

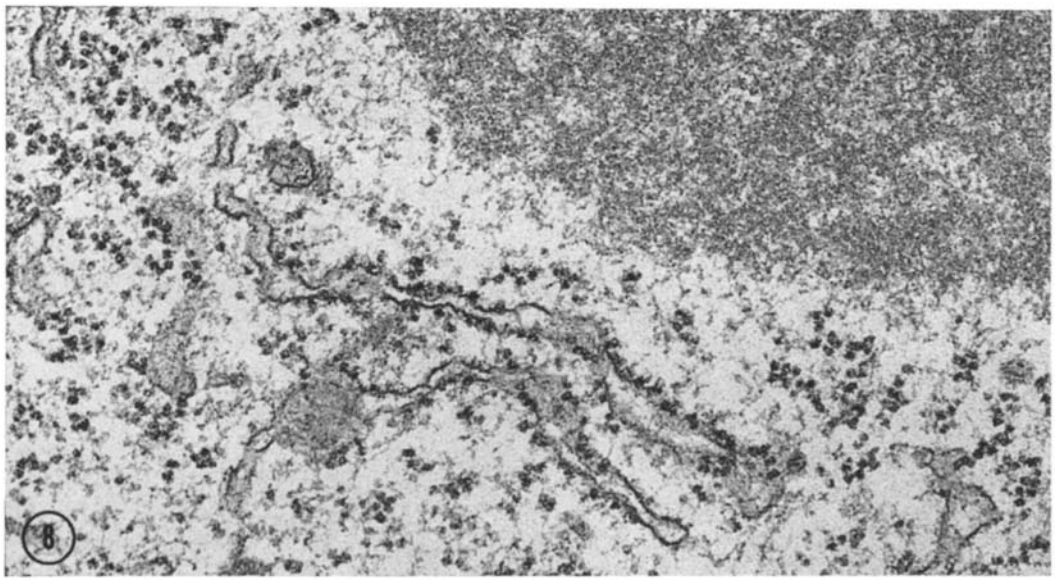

FIGURE 8 *Cucurbita maxima.* Section of a sieve element at an intermediate stage of differentiation showing a portion of a P-protein body. The comparatively electron-lucent region surrounding the P-protein body consists of finely dispersed fibrils. × 55,000.

cytoplasm as a small group of tubules whereas in *Cucurbita* the bulk of the P-protein component arises as fine fibrils of P3 protein. These fine fibrils first appear in the cytoplasm in a more dispersed state than the groups of tubules, being intermixed with other organelles. In the large type of P-protein body of *Cucurbita* the fine fibrils are assembled into larger units. The P-protein bodies in *Nicotiana*, on the other hand, consist of oriented tubules the form of which does not change as the P-protein body is organized. The small bodies in *Cucurbita* apparently show the same constancy of structure as do the bodies in *Nicotiana*.

The transformation of the fine fibrils of P3 protein into larger units may be related to the level of hydration within the P-protein bodies or may be a phenomenon of the self assembly of the unit particles of P3 protein. Tilney and Porter (19) have shown that, when *Actinosphaerium* is exposed to low temperatures or high pressures, the microtubules of the axostyle are completely disassembled and leave only a finely divided amorphous mass of material. When, however, the conditions inducing depolymerization are removed, the self assembly of unit particles begins again and the microtubules are reformed. This type of self assembly may take place in the *Cucurbita* P protein bodies.

The observation that the P1-protein tubules can form a square lattice is of interest from the point of view of molecular interactions and the subunit structure of the tubules. Evidence in favor of a model for the microtubules built from linear strands of 80 × 50-A subunits has recently been reviewed by Porter (16). The observation that the tubules of P1 protein, which are morphologically similar to cytoplasmic microtubules, can be organized into a square lattice indicates that the subunits are a particular cytoplasmic protein molecule and that they are most probably arranged in a helix with fourfold symmetry or linearly within the tubule. That the tubules have a center-to-center distance of about 295 A in the lattice indicates that the tubules and the protein subunits may be larger than is indicated by measurements of the electron-opaque wall.

In both *Cucurbita* and *Nicotiana* the P-protein bodies have been shown to be formed without a bounding membrane. The absence of the membrane presents certain problems regarding the interpretation of the growth and the form of the P-protein bodies. It is difficult to envisage a mechanism through which the integrity of the P-protein bodies is maintained. Mature sieve elements show no cytoplasmic streaming, and

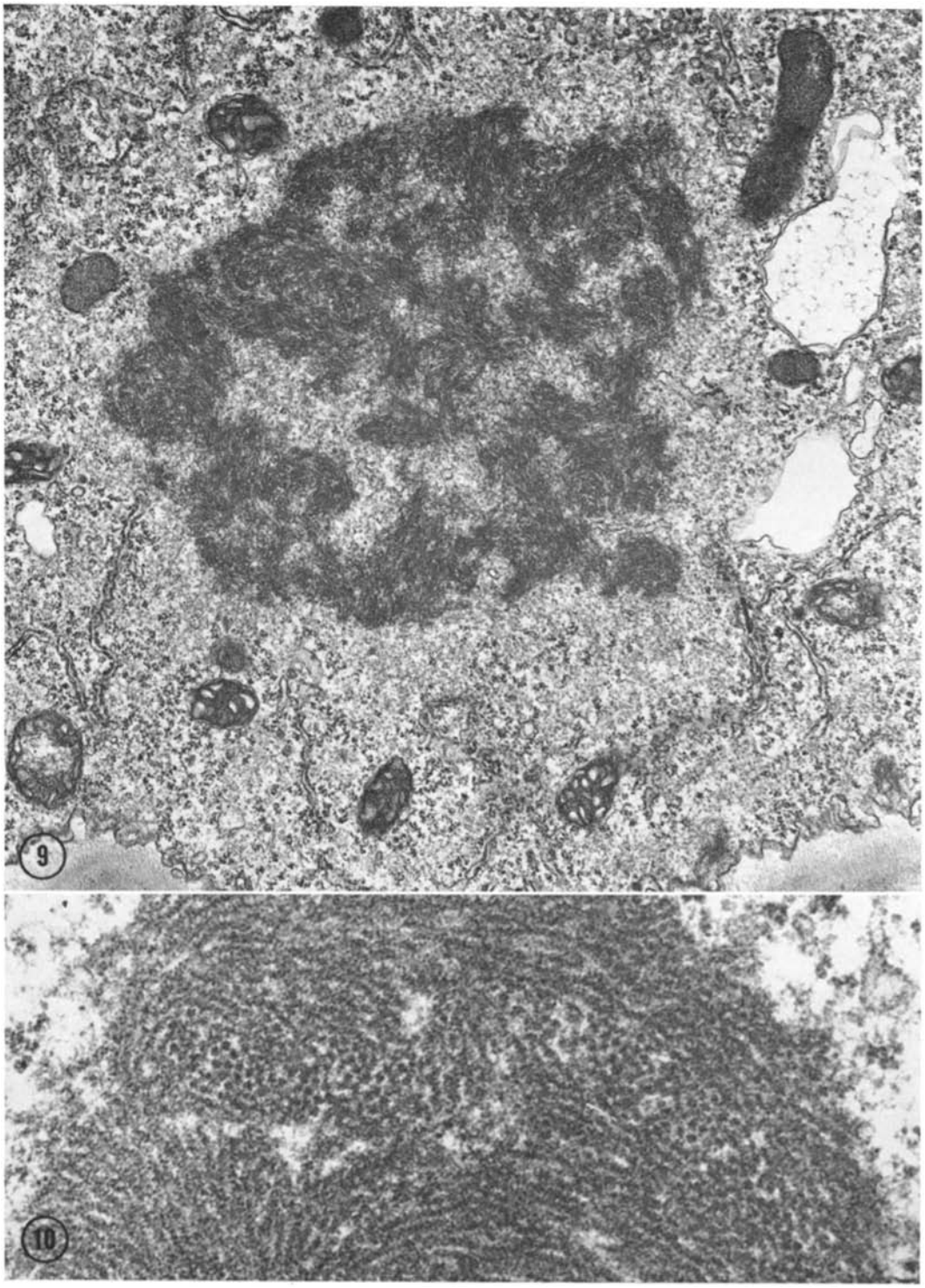

FIGURE 9 *Cucurbita maxima.* P-protein body in a differentiating sieve element in which the P-protein component has been largely organized into P4-type units. In some regions there is a tendency towards an orderly arrangement of the P4-type units. × 33,000.

FIGURE 10 *Cucurbita maxima.* Portion of a P-protein body similar to the one shown in Fig. 9. Where the P4-protein units are cut in transection, they are seen to have an electron-opaque wall and an electron-lucent core, indicating a tubular structure. × 74,000.

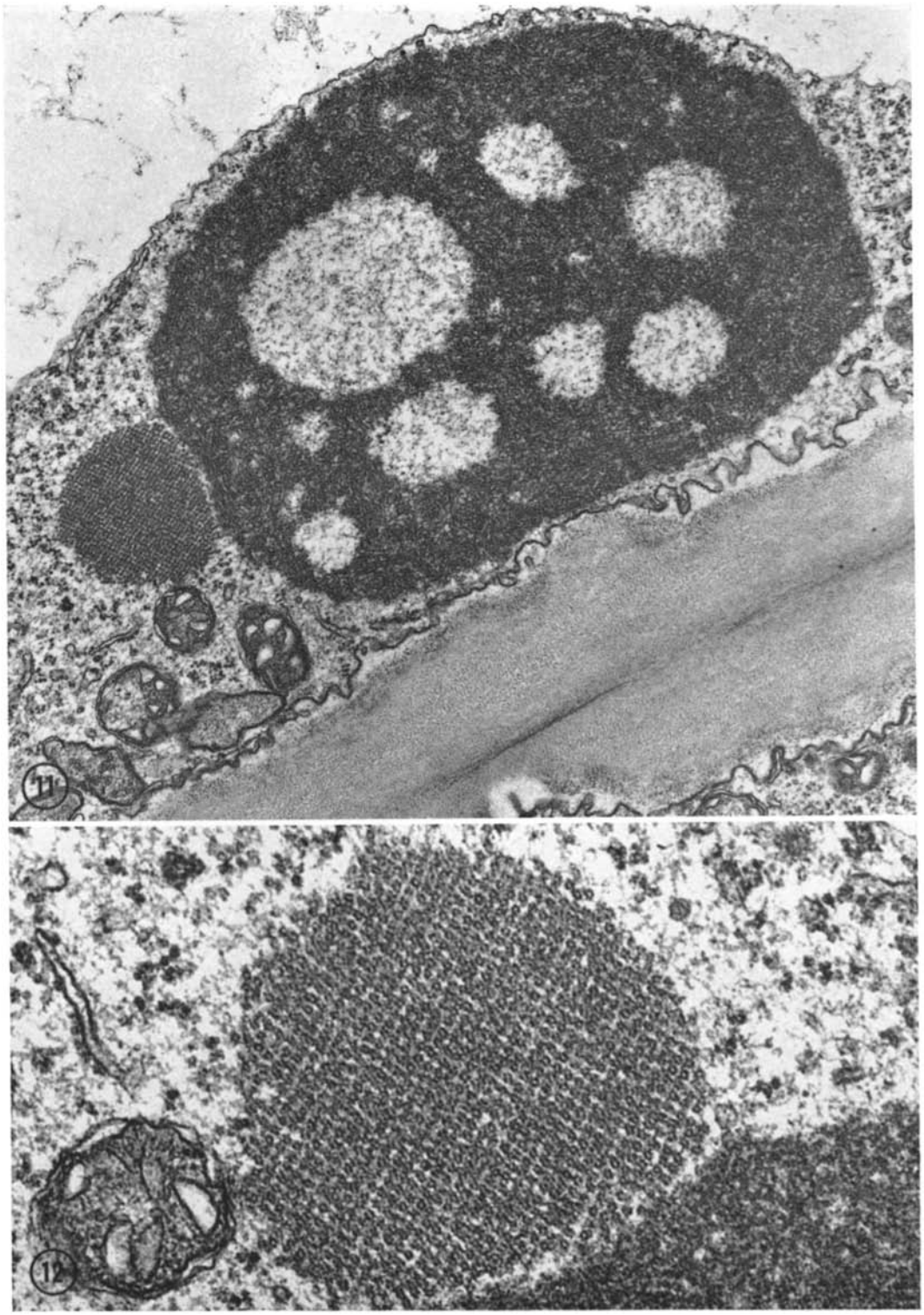

FIGURE 11 *Cucurbita maxima.* Longitudinal section through a differentiating sieve element showing a mature P3-protein body with a satellite body consisting of well oriented tubules. The P3-protein body has well-developed vacuoles. × 23,000.

FIGURE 12 *Cucurbita maxima.* Higher magnification view of a portion of the section shown in Fig. 10. The tubular nature of the P-protein component of the satellite body is shown. The tubules are oriented in a regular manner. × 65,000.

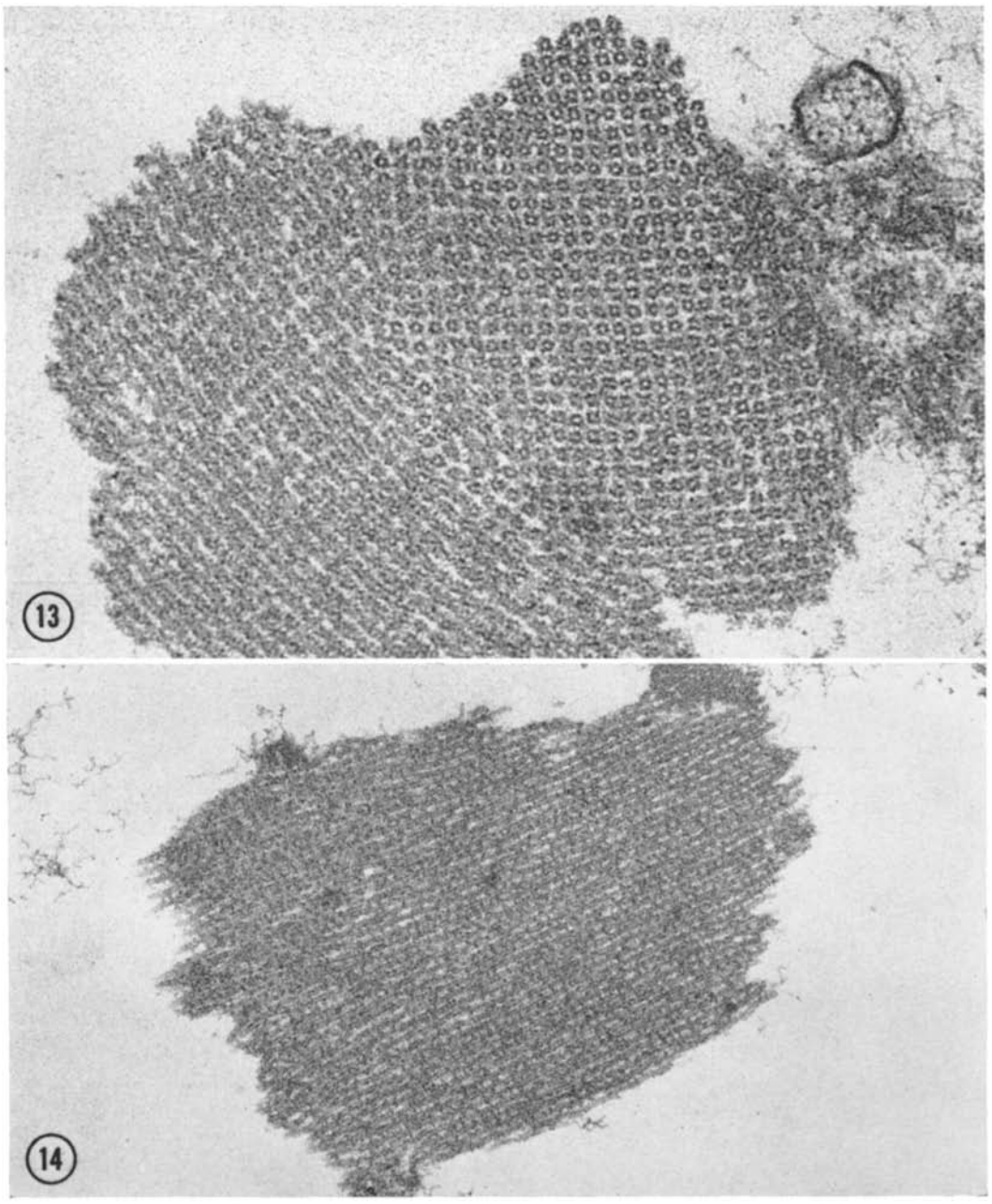

FIGURE 13 *Cucurbita maxima*. Section through a mature P-protein body in a sieve element shortly before dispersal of P-protein bodies. The tubules of the P1-protein component are organized into a lattice. × 85,000.

FIGURE 14 *Cucurbita maxima*. Section through a P-protein body in a sieve element as in Fig. 13. The P1-protein tubules are shown in longitudinal view. The tubules are arranged in a parallel manner. × 45,000.

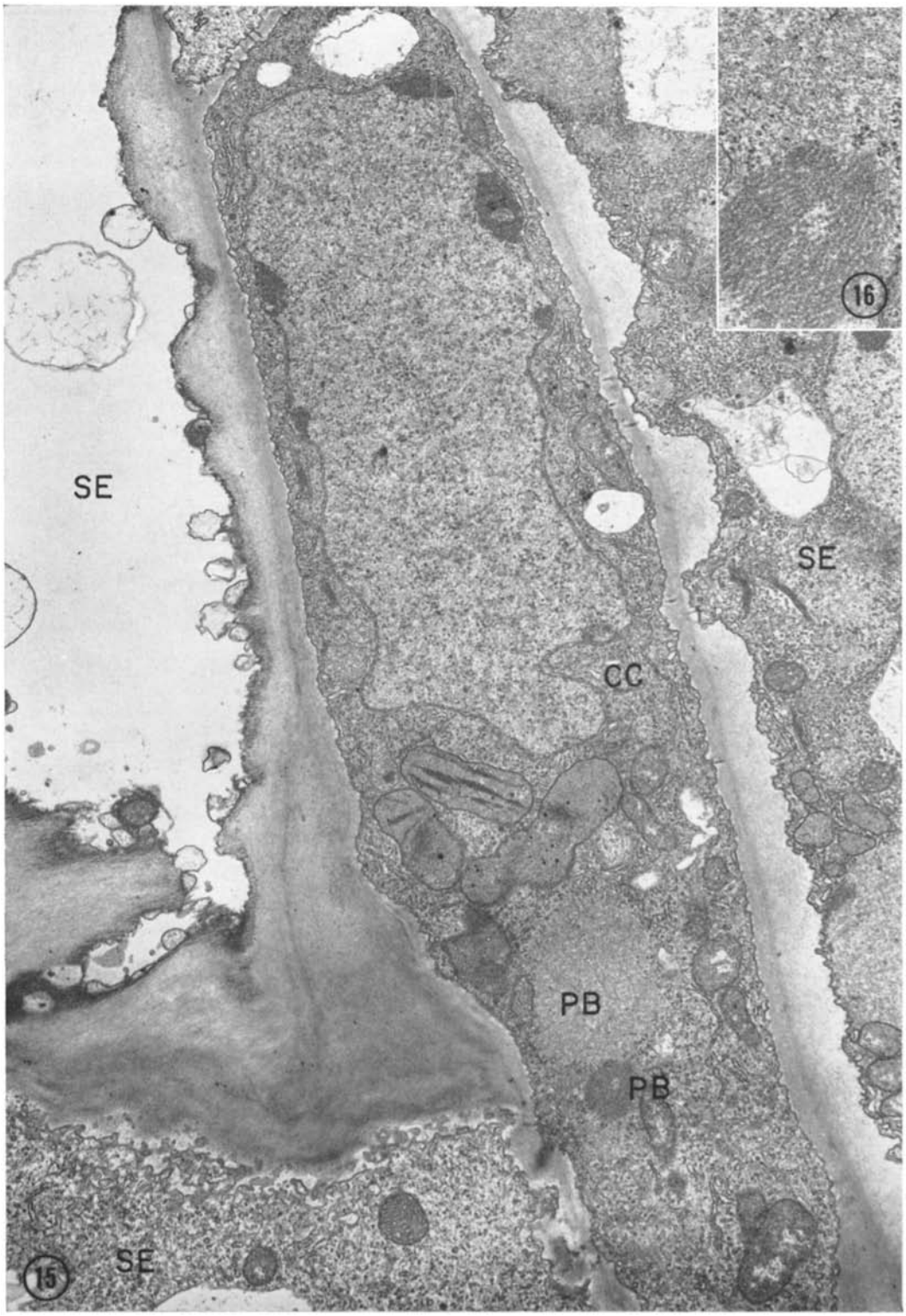

FIGURE 15 *Cucurbita maxima*. Longitudinal section through portions of three sieve elements (*SE*) and a companion cell. In the companion cell (*CC*) two P-protein bodies are evident (*PB*), one of which is of the fibrous P3-protein type and the other of the tubular P1-protein type. × 9,000.

FIGURE 16 *Cucurbita maxima*. Higher magnification view of the P-protein components shown in Fig. 15. × 29,000.

Strasburger (reference 18, page 285) found that in *Cucurbita* this streaming ceased with the appearance of the slime bodies. Absence of cytoplasmic streaming would be an important factor in maintaining the form of the P-protein bodies.

The absence of a membrane bounding the P-protein bodies poses problems related to the transport of material to the P-protein bodies and its transfer into them. The morphological association of dictyosomes and dictyosome-derived vesicles with the apparent regions of P-protein synthesis suggests that perhaps P protein is brought to the bodies by the dictyosome-derived vesicles. In all other cases of intracellular transport by membrane-enclosed vesicles, however, the vesicles fuse with a membrane surface, open out and discharge their contents to the other side of the membrane. If the P protein is brought to P-protein bodies via the dictyosome-derived vesicles, then there must be either a dissolution of the dicytosome-derived vesicle membranes or a rupture followed by a discharge of the vesicle contents into the P-protein bodies. The fact that in the early stages of differentiation the fine fibrils of P-protein material arise intermixed with the ribosomes and various other cell organelles is, on the other hand, a strong indication that the P-protein bodies can be formed, at least in their early stage, by a simple aggregation of this P3-protein material.

The P-protein bodies in both *Nicotiana tabacum* and *Cucurbita maxima* show no indication of an association with ribosomes as postulated by Buvat (4) for *Cucurbita pepo* slime bodies. In fact, during the development of the P-protein bodies ribosomes are conspicuously excluded from the aggregates of this protein.

The study was supported in part by National Science Foundation grants No. GB-5506 and GB-6271 and in part by faculty grant No. 308 from the University of California.

The authors acknowledge the assistance of Mr. R. H. Gill and Mrs. B. Osterhoff.

Received for publication 19 December 1967, and in revised form 4 March 1968.

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