A STUDY OF SIEVE ELEMENT STARCH USING SEQUENTIAL ENZYMATIC DIGESTION AND ELECTRON MICROSCOPY

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ABSTRACT

The fine structure of plastids and their starch deposits in differentiating sieve elements was studied in bean (*Phaseolus vulgaris* L.). Ultrastructural cytochemistry employing two carbohydrases specific for different linkages was then used to compare the chemical nature of "sieve tube starch" (the starch deposited in sieve elements) with that of the ordinary starch of other cell types. Hypocotyl tissue from seedlings was fixed in glutaraldehyde, postfixed in osmium tetroxide, and embedded in Epon-Araldite. Treatment of thin sections on uncoated copper grids with α-amylase or diastase at pH 6.8 to cleave α-(1 $\rightarrow$ 4) bonds resulted in digestion of ordinary starch grains but not sieve element grains, as determined by electron microscopy. Since α-(1 $\rightarrow$ 6) branch points in amylopectin-type starches make the adjacent α-(1 $\rightarrow$ 4) linkages somewhat resistant to hydrolysis by α-amylase, other sections mounted on bare copper or gold grids were treated with pullulanase (a bacterial α-[1 $\rightarrow$ 6] glucosidase) prior to digestion with diastase. Pullulanase did not digest sieve element starch, but rendered the starch digestible subsequently by α-amylase. Diastase followed by pullulanase did not result in digestion. The results provide evidence that sieve element starch is composed of highly branched molecules with numerous α-(1 $\rightarrow$ 6) linkages.

INTRODUCTION

The plastids of higher plant sieve elements are characterized by several distinctive types of inclusions. Among these, the starch deposits are the most widely distributed. It has been known for many years that these deposits differ from ordinary starch of other cell types in their reaction to the iodine stain (cf. 11). Other inclusions peculiar to the plastids of sieve elements are the crystalline proteinaceous bodies found in monocots (5) and the ring-shaped bundles of proteinaceous filaments apparently confined to certain families of dicots (6, 14, 15).

The starch in sieve elements (known classically as "sieve tube starch") is distinguished primarily by its reddish color response to the iodine stain, in contrast to the blue-black reaction of the ordinary starch found in the amyloplasts of parenchyma cells and other cell types (11). In this respect it resembles certain other polysaccharide deposits (12), including the waxy starch found in the endosperm of some members of the grass family, the floridean starch of red algae, and the glycogen of animal tissues. Ordinary starch contains two fractions, amylose and amylopectin. Amylose consists of α-(1 $\rightarrow$ 4) linked glucose residues and only a few α-(1 $\rightarrow$ 6) branches (21). Amylopectin is much more extensively branched, since it contains numerous α-(1 $\rightarrow$ 6) bonds that provide branch points. Waxy starch consists almost exclusively of amylopectin (37), and floridean starch and glyco-

gen are quite similar in composition (28, 30). There is reason to suspect, therefore, that sieve element starch may also be related structurally to amylopectin. Although conjectures as to its nature have been made in the past by students of phloem tissue (12, 36), its composition has remained obscure.

The starch grains of sieve elements cannot be isolated for study in vitro, since the elements are embedded among other cells that also contain amyloplasts. However, recent advances in carbohydrate enzymology have made it appear more attractive to attempt to explore the structure of starch and other plant polysaccharides in position within the plant cell. In planning the present work, it appeared promising to try to use α-amylase to digest starch in thin sections, since this enzyme cleaves α-(1 $\rightarrow$ 4) bonds and converts starch into breakdown products of various lengths. It also appeared likely that pullulanase, a recently discovered α-(1 $\rightarrow$ 6) glucosidase (1, 7), could be used in a similar way on thin sections. Since pullulanase is now being used in starch research to cleave the α-(1 $\rightarrow$ 6) linkages of amylopectin (2), it seemed feasible to attempt to use it sequentially with α-amylase to compare the digestibility of starch grains in sieve elements with that in other cells.

MATERIALS AND METHODS

Seeds of *Phaseolus vulgaris* L. cultivars Kentucky Wonder and Dwarf Horticulture (Olds Seed Co., Madison, Wis.) were germinated for 3 to 7 days in wet sand or vermiculite following an overnight presoaking. Segments about 1 mm long were cut from the seedling hypocotyl under 3% glutaraldehyde in 0.05 M potassium phosphate buffer at pH 6.8 and fixed for approximately 2 hr. The segments were then rinsed in several changes of 0.05 M phosphate buffer and postfixed in 2% OsO_4 in the same buffer for 2 hr. This was followed by dehydration through an acetone series and propylene oxide, then embedment in a resin mixture consisting of Araldite, Epon, and dodecenyl succinic anhydride (DDSA) in a ratio of 3:3:8 by volume. Silver sections were cut on a Porter-Blum MT-2 ultra-microtome (Ivan Sorvall Inc., Norwalk, Conn.), mounted on uncoated copper grids, and poststained with uranyl acetate and lead citrate before viewing in a Hitachi HU-11A electron microscope operating at 75 kv with a 30 μ objective aperture.

The content of thin sections was monitored with the light microscope by observing sections cut at a thickness of 0.5 μ and stained with toluidine blue O. Polysaccharide was demonstrated with the periodic acid-Schiff (PAS) reaction using plastic sections 0.5–1.0 μ thick. Starch was identified with an I_2KI solution (19), using both fresh material and tissue fixed in glutaraldehyde. The fixed tissue was frozen and sectioned at 16 μ in a model CTD cryostat (International Equipment Company, Needham Heights, Mass.) operating at -12 to -15°C. Protein was demonstrated in plastic sections 1 μ thick using mercuric bromophenol blue.

Silver sections mounted on uncoated copper, gold, or nickel grids were digested with bacterial α-amylase (Type IIA, Sigma Chemical Co., St. Louis, Mo.) or malt diastase (type VA, Sigma Chemical Co.) at 37°C for 1 to 48 hr at concentrations varying from 0.1 to 1.0% in 0.02 M phosphate buffer at pH 6.8. Diastase and α-amylase were used interchangeably, as no differences in effect were observed between them. Attempts to employ β-amylase on thin sections were unsuccessful, as described in the Results. Following digestion, grids were washed in a stream of distilled water and poststained in the usual manner.

The pullulanase, a gift from Dr. K. L. Smiley of the USDA Northern Regional Research Laboratory, Peoria, Ill., was a lyophilized preparation from *Aerobacter aerogenes*, 30 mg of which were capable of hydrolyzing the pullulan in 100–200 ml of a 1% solution in 1–2 hr. Copper, nickel, or gold grids bearing silver sections of material prepared in the usual manner were immersed in a 0.5–3.0% solution of this preparation in buffer at 37°C for 3–24 hr. Buffers used included 0.02 M phosphate at pH 6.8 and 0.05 M citrate at pH 5.4. Following digestion, grids were washed in a stream of distilled water and either poststained or subjected to further digestion by α-amylase or diastase for 1–24 hr.

For enzyme exposure of short duration (1–6 hr), digestion was carried out in covered depression slides. For longer exposures (6–48 hr), grids were incubated in the center well of covered micro-Conway diffusion vessels containing a few drops of toluene in the outer well. A number of controls were run with different enzyme and buffer exposures to test alternative explanations for the observed results.

Leaf material of timothy (*Phleum pratense*) was also examined in order to compare the histochemical properties and enzyme digestibility of the proteinaceous crystals in the plastids of monocot sieve elements with the same properties of starch grains in bean sieve elements.

RESULTS

Changes in Amyloplast Fine Structure During Ontogeny of the Sieve Element

The proplastids seen in the earliest recognizable sieve elements are spherical to ovoid and relatively small (Fig. 1). They are bounded by an envelope,

the inner membrane of which bears a few cisternal invaginations. Their stroma is moderately electron opaque and contains clearer regions in which a fibrillar material, possible DNA, is sometimes seen. Plastid ribosomes are also present in the stroma. No starch deposits are present at this stage of plastid development.

Changes in the plastids can be correlated readily with other events taking place during differentiation of the cytoplasm and walls of the sieve element. In the early stages of differentiation, the plastids enlarge and develop into irregular, elongate, and branching forms whose stroma contains one or more small starch grains (Fig. 2). These grains enlarge rapidly as differentiation of the sieve element continues. During their growth they are surrounded by a thin shell of finely granular material located in the adjacent matrix of the plastid (Figs. 3 and 4). Presumably this material is related to active synthesis and deposition taking place at the grain surface. As the grains approach mature size, the plastids containing them become roughly spherical (Fig. 4).

Degenerative changes in the plastids accompany the breakdown of other components of the cytoplasm in later stages of sieve element differentiation. The most obvious changes include the loss of granular material from the stroma, and the development of irregularities in the outline of the plastid envelope and in the spacing of its membranes (Figs. 5 and 6). Concomitantly the starch grains become fissured and coarsely granular (Fig. 6). Also frequently observed in the plastids are small clumps of granules that resemble the peripheral portions of the larger masses in appearance. These granules and granular clumps are similar in appearance to the glycogen inclusions observed in liver and muscle cells. The smallest granules are approximately 200 A in diameter, and thus are similar in size to the subunits of the glycogen β-particles observed by Wanson and Drochmans (39). In old sieve elements with perforated plates, the envelopes of many of the plastids have ruptured, and the starch bodies which were formerly in the plastids now lie free in the lumen (Fig. 7). Whether release of these bodies from the plastids occurs naturally during aging of the sieve elements or results from cutting and fixing the tissue has not been established (9, 10, 12, 24).

Fully developed starch grains in sieve element plastids (Figs. 3–5) commonly differ in appearance in several respects from those in parenchyma cell plastids (Fig. 8). The sieve element starch grains are generally smaller and more nearly spherical, and exhibit radial rather than concentric striations. Also, as has been reported previously (13, 24), their cores frequently appear electron lucent (Figs. 3 and 4). Otherwise the sieve element grains appear highly electron opaque (Figs. 2–7), whereas ordinary grains are more variable in this respect. Poststaining with the lead salt contributes most importantly to the electron opacity of the grains, as can be demonstrated by omitting the poststaining entirely (Fig. 9), and by comparing the effects of staining with lead and uranium singly and in combination. Finally, the stroma of the sieve element plastids remains closely associated with the starch grains (Figs. 2–5), and does not become separated by an electron-lucent region like that seen between stroma and starch grains in parenchyma cell plastids fixed in glutaraldehyde (Fig. 8).

Preliminary Histochemical Investigation of Sieve Element Starch

Preliminary attempts to identify the type of starch in sieve element plastids were made with standard light microscopic cytochemical techniques. From the results summarized in Table I it is clear that in *P. vulgaris* the deposits in the plastids correspond to classical "sieve tube starch." When the PAS test was applied to sections of plastic-embedded material cut at a thickness of 0.5 μ, the grains in the plastids gave a positive (red) response indicative of polysaccharide. In fresh and frozen sections treated with I_2KI solution, the grains in sieve elements were stained reddish brown, while those of plastids in the cortical parenchyma were stained bluish black. Grains in the vascular parenchyma stained reddish brown and thus showed a greater affinity in this test to the starch of sieve elements than to that in the similar parenchymatous type of cell located in the cortex.

Cuneate, crystalline inclusions are typical of the sieve element plastids of many monocots (5). When tests for the presence of protein were made on these bodies, using mercuric bromophenol blue applied to plastic sections of *Phleum pratense*, a strongly positive staining reaction was obtained, in agreement with the previous results of Behnke (4). Starch grains observed in *Phleum* guard cells and in *Phaseolus* parenchyma cells and sieve elements gave negative results in the same test.

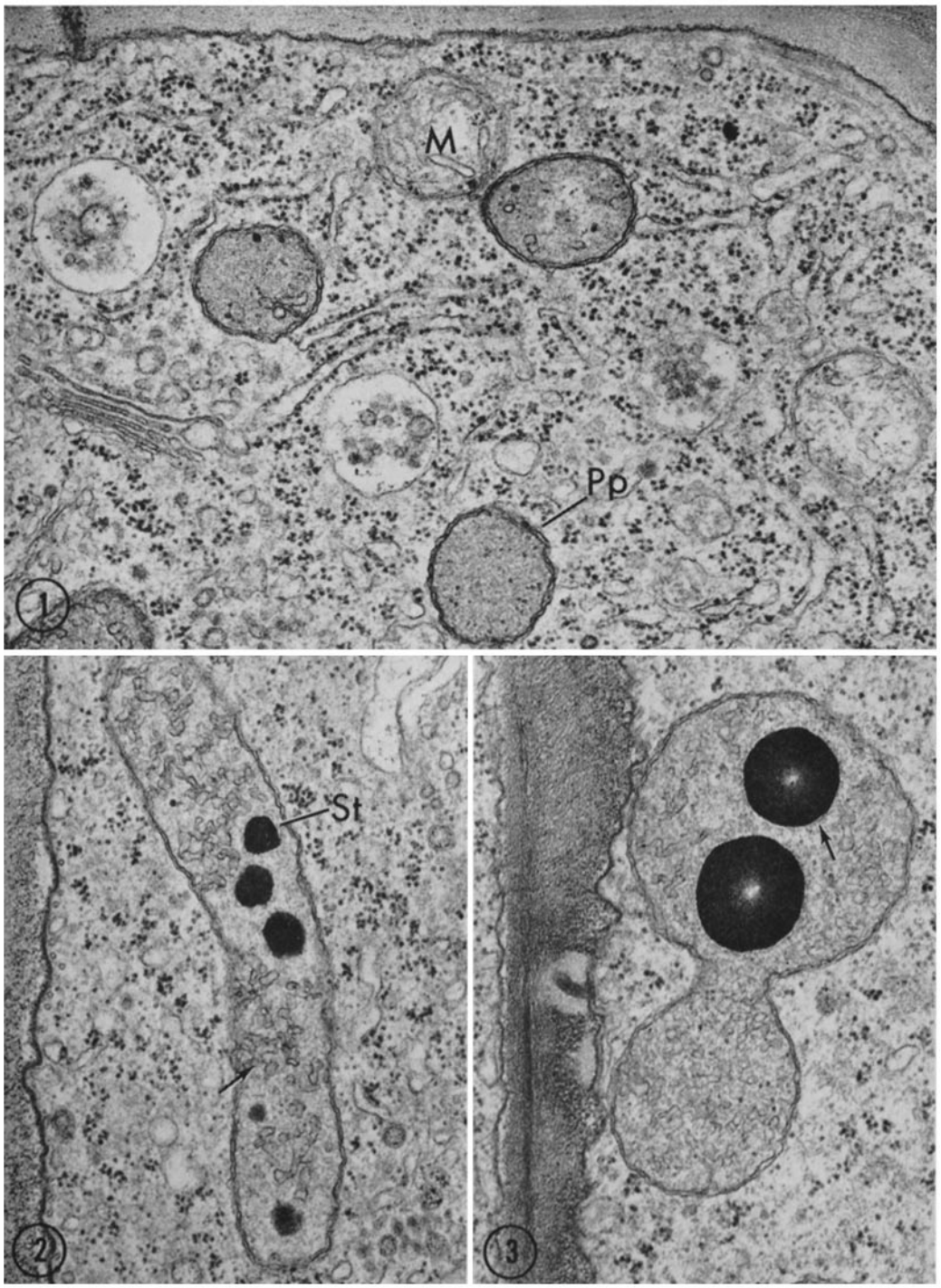

FIGURE 1 Proplastids (*Pp*) in a young, transversely sectioned sieve element of a bean seedling. Mitochondrion (*M*). × 42,000.

FIGURE 2 Elongate plastid in a young sieve element of a bean seedling. The plastid contains several small, electron-opaque starch grains (*St*) and numerous cisternae (arrow). × 39,000.

FIGURE 3 Sieve element plastid at a later stage of development than that shown in Fig. 2. A shell of granular material (arrow) surrounds each starch grain in the plastid. The nearby wall shows development of structure characteristic of sieve elements in bean. × 40,000.

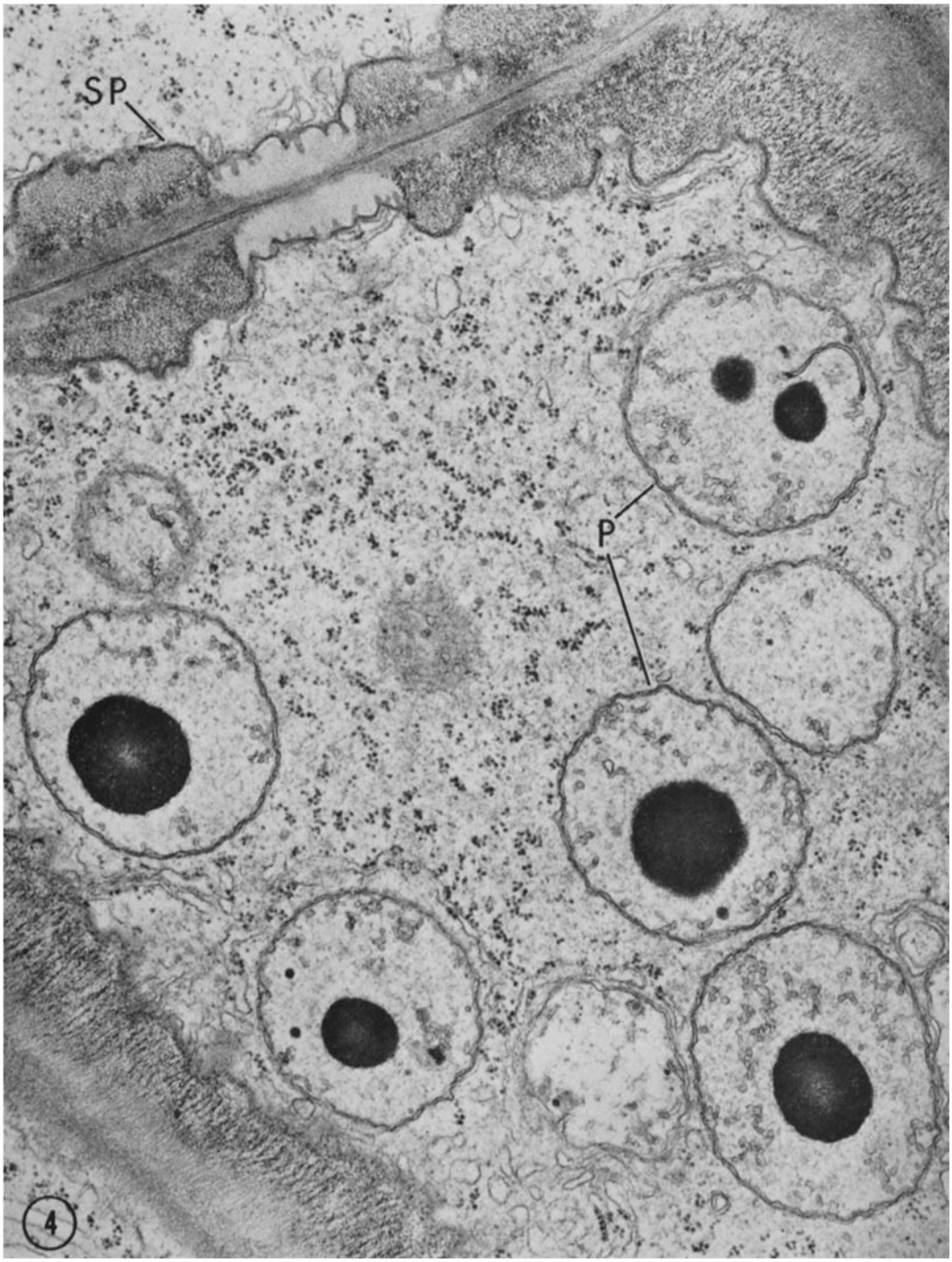

FIGURE 4 Longitudinal section through two developing sieve elements separated by a sieve plate (*SP*). Several plastids (*P*) with electron-opaque starch deposits are seen in the lower element. Shells of granular material surround the starch grains. × 35,000.

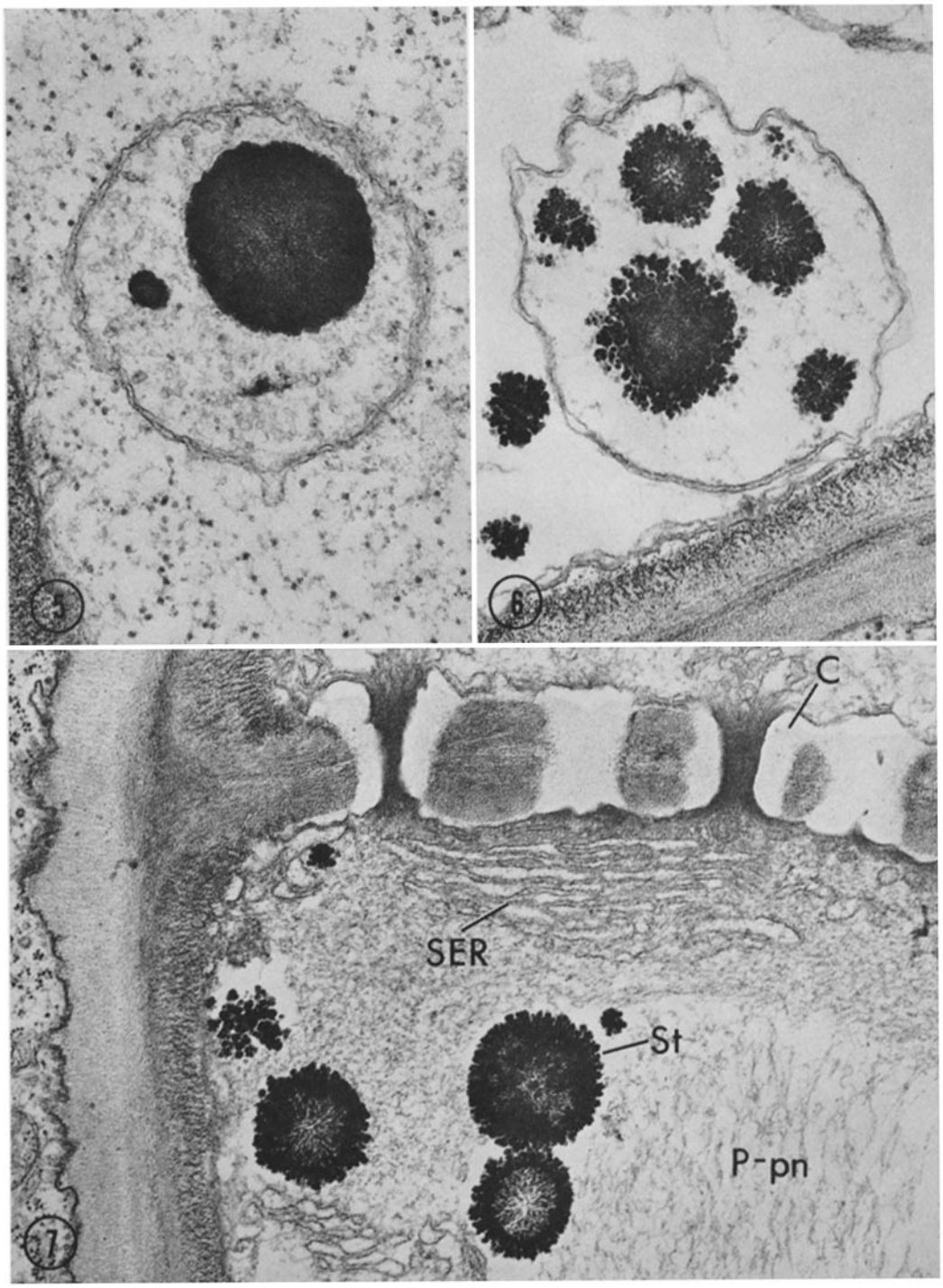

FIGURE 5 Plastid showing degenerative changes in a sieve element in a late stage of differentiation. The stroma of the plastid is relatively sparse and the starch grain shows incipient granulation. × 52,000.

FIGURE 6 Later stage of degeneration of both plastid and sieve element than that shown in Fig. 5. Note irregular outline of the plastid envelope, lack of evident stroma material, and granulated appearance of the starch deposits. × 41,000.

FIGURE 7 Longisection showing portions of two sieve elements at a late stage of differentiation. The elements are separated by a sieve plate containing pores lined with callose (*C*). Granulated and fissured starch bodies (*St*) lie free in the lumen of the element along with dispersed fibers of P-protein (*P-pn*) and cisternae of the sieve element reticulum (*SER*). × 29,000.

TABLE I

Results of Histochemical Tests on the Inclusions in Sieve Element Plastids

Reaction	Inclusion	Response
PAS	*Phaseolus vulgaris*	
	Starch grains of the ground parenchyma and starch sheath	+
	Sieve element starch grains	+
	Phleum pratense	
	Starch grains of guard cells	+
	Cuneate, crystalline inclusions in plastids of sieve elements	−
Mercuric bromophenol blue	*Phaseolus vulgaris*	
	Starch grains of the ground parenchyma and starch sheath	−
	Starch grains of sieve elements	−
	Phleum pratense	
	Starch grains of guard cells	−
	Cuneate, crystalline inclusions in plastids of sieve elements	+
I_2KI	*Phaseolus vulgaris*	
	Starch grains of the ground parenchyma and starch sheath	blue
	Starch grains of the vascular parenchyma	red-brown
	Starch grains of sieve elements	red-brown

Evidence for the Structure of Sieve Element Starch Based on Sequential Enzymatic Digestion

Sieve element starch and ordinary starch were differentially digested from thin sections by appropriate treatment with α-amylase (or diastase) and pullulanase preparations (Figs. 10–17). These enzymes acted in a highly selective manner without affecting other fine structural components. As the starch in a grain was digested, the grain first lost much of its electron opacity and developed a network of small holes (Figs. 11 and 14). After more complete digestion, the holes coalesced and the entire starch deposit was replaced by a single large hole (Figs. 10, 15, and 17). Precautions were taken to minimize the effect of the electron beam of the microscope on enlargement and coalescence of the holes by reducing the exposure of the sections to the beam as much as possible during examination.

The use of α-amylase and pullulanase readily distinguished between sieve element starch and the ordinary starch stored in plastids of other cell types. In experiments on tissue representing numerous embedments made over a 2 yr period, it was found consistently that 1% α-amylase or diastase at pH 6.8 digested ordinary starch but had no apparent effect on the starch in differentiating sieve elements (Figs. 10–12). Large starch grains in the numerous plastids of parenchyma cells were completely digested by treatment with 1% α-amylase for 1 hr (Fig. 10). Also, it was observed that 1% diastase for 1 hr removed the electron opacity of starch grains in the plastids of phloem parenchyma cells and destroyed the central portions of these grains, but did not affect noticeably the grains in adjacent sieve elements (Fig. 11). Even when the exposure to 1% α-amylase was lengthened to 24 hr (Fig. 12) or to 48 hr, starch deposits in sieve elements remained unaffected.

A 3% solution of pullulanase alone was also without apparent effect on sieve element starch grains. This is shown in Fig. 13 for material mounted on copper grids and exposed to pullulanase for 24 hr. However, if the section was treated first with pullulanase and then with 1% α-amylase or diastase, the sieve element starch was digested. An example of treatment with pullulanase for 24

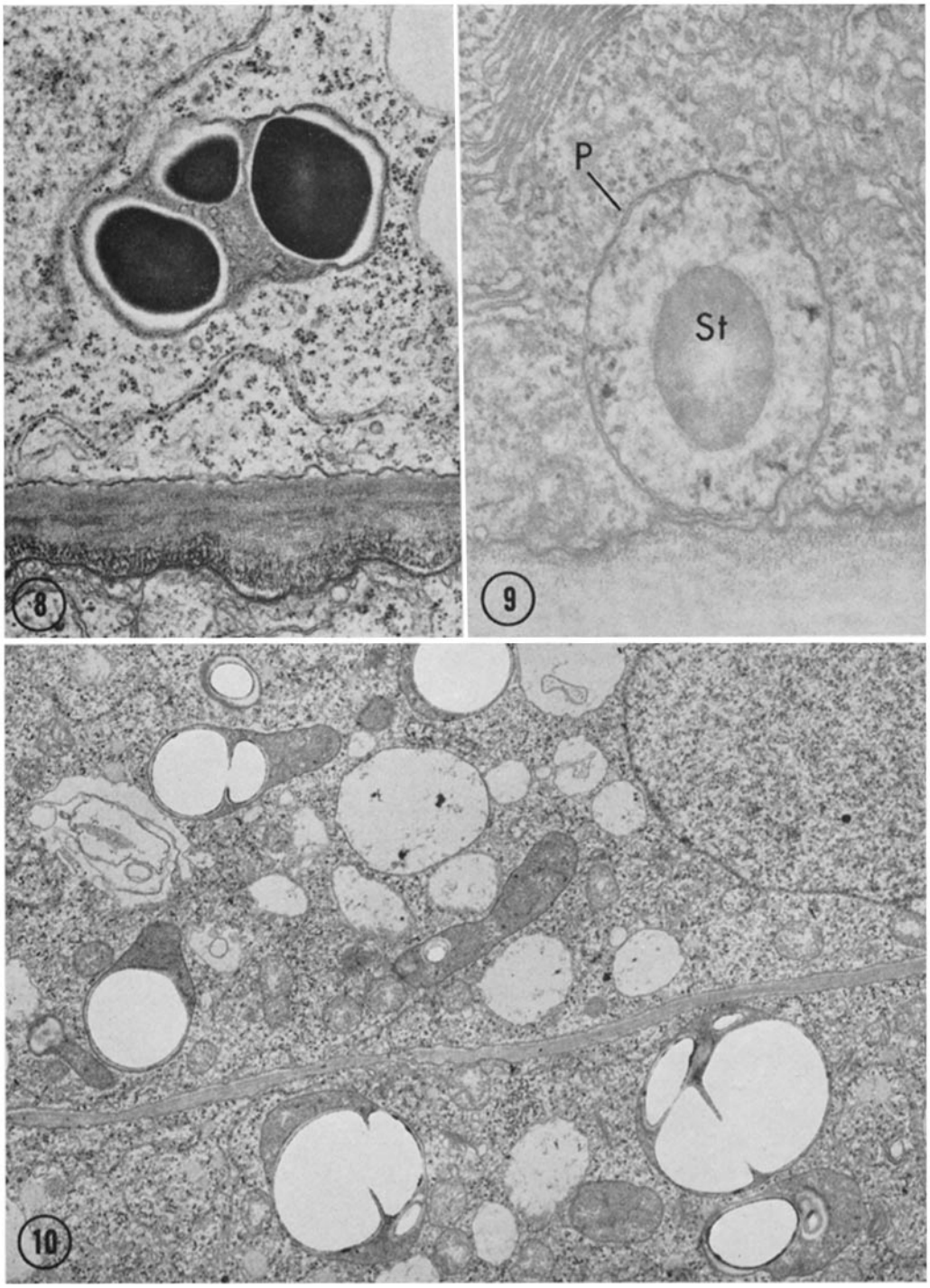

FIGURE 8 Amyloplast in a phloem parenchyma cell. An electron-lucent region separates the stroma from the three starch deposits in the plastid. × 25,000.

FIGURE 9 Appearance of a starch grain (*St*) in a section not poststained with uranyl acetate and lead citrate. The electron opacity of the starch in the plastid (*P*) is greatly reduced by omission of the poststaining. × 39,000.

FIGURE 10 Appearance of phloem parenchyma cells in a thin section after treatment of the section with 1% α-amylase at pH 6.8 for 1 hr at 37°C. The grains of ordinary starch in the amyloplasts have been completely digested, leaving holes in the plastic. × 14,000.

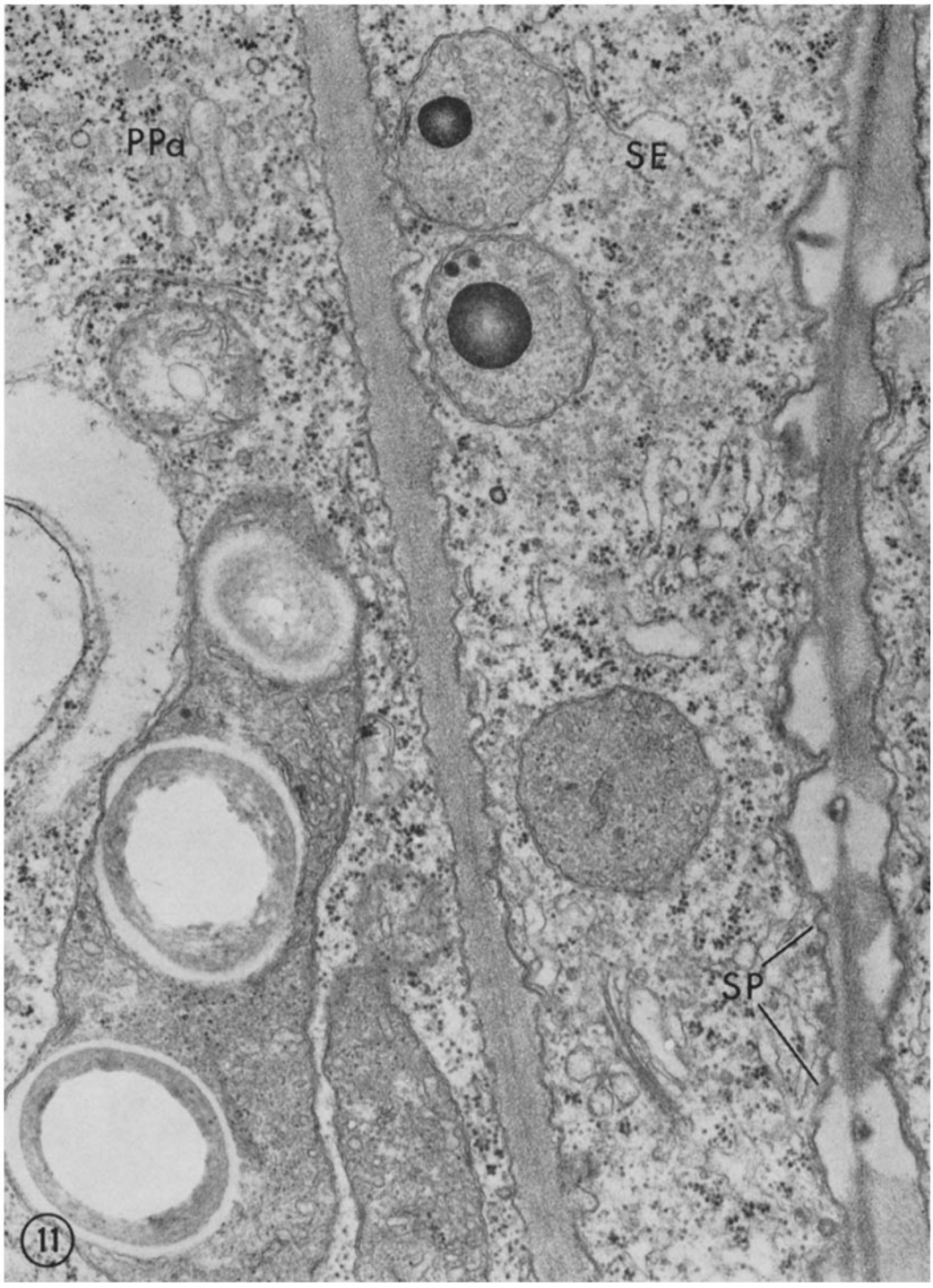

FIGURE 11 Comparison of the effect of diastase on amyloplasts in a phloem parenchyma cell (*PPa*) and an adjacent sieve element (*SE*). Incubation of section in 1% diastase at pH 6.8 for 1 hr. The starch grains in the phloem parenchyma cell show extensive digestion, while those in the sieve element appear unaffected. Note developing sieve plate (*SP*) on the right. × 41,000.

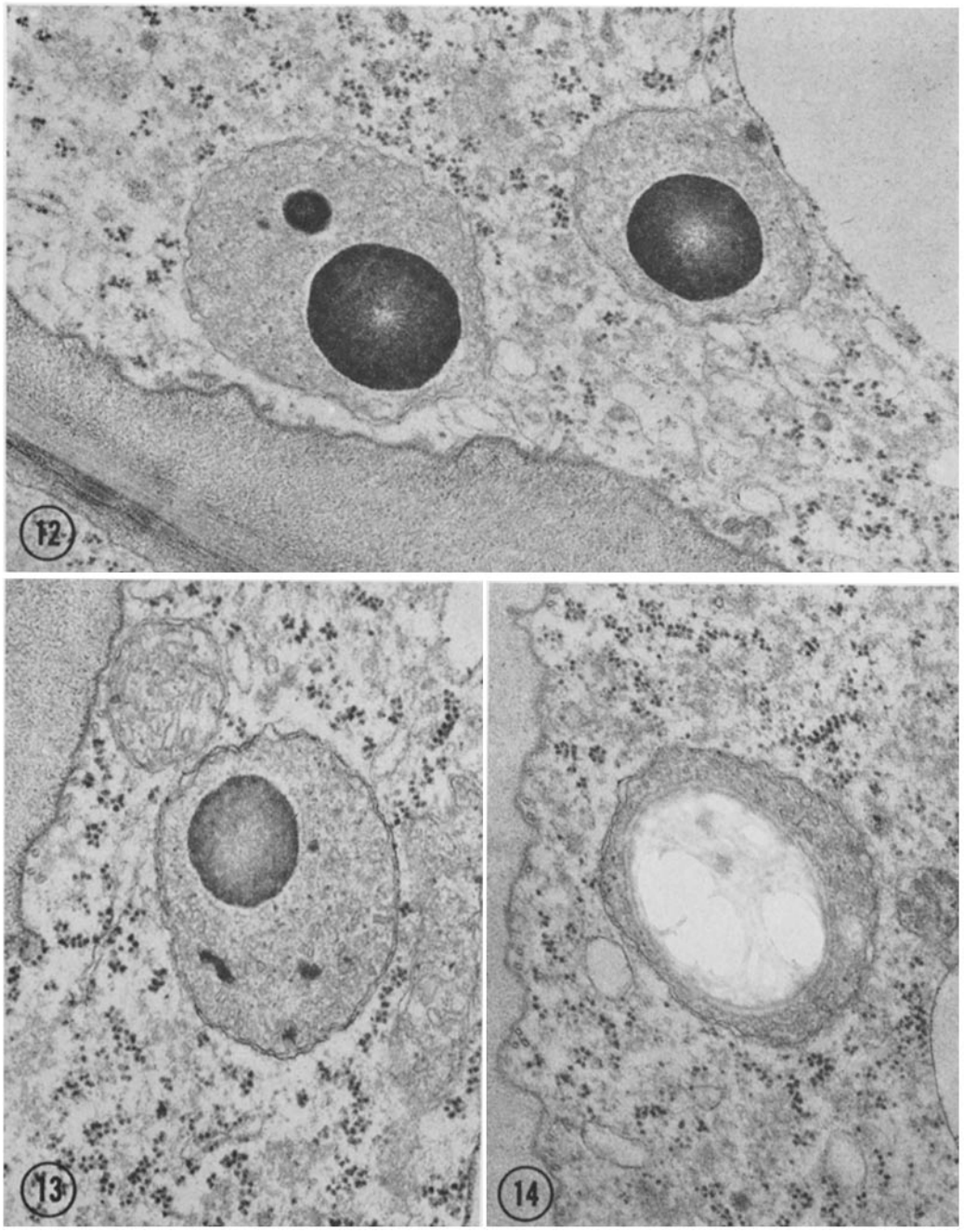

FIGURE 12 Appearance of starch grains in sieve element plastids after treatment of a section with α-amylase at pH 6.8 for 24 hr. The starch grains have not been digested. × 43,000.

FIGURE 13 Appearance of starch in a sieve element plastid after incubating a section in 3% pullulanase at pH 6.8 for 24 hr. The grain shows no digestion. × 45,000.

FIGURE 14 Appearance of a starch grain in a sieve element plastid after treatment with 3% pullulanase for 24 hr followed by 1% α-amylase for 1 hr. The starch grain shows extensive digestion. × 42,000.

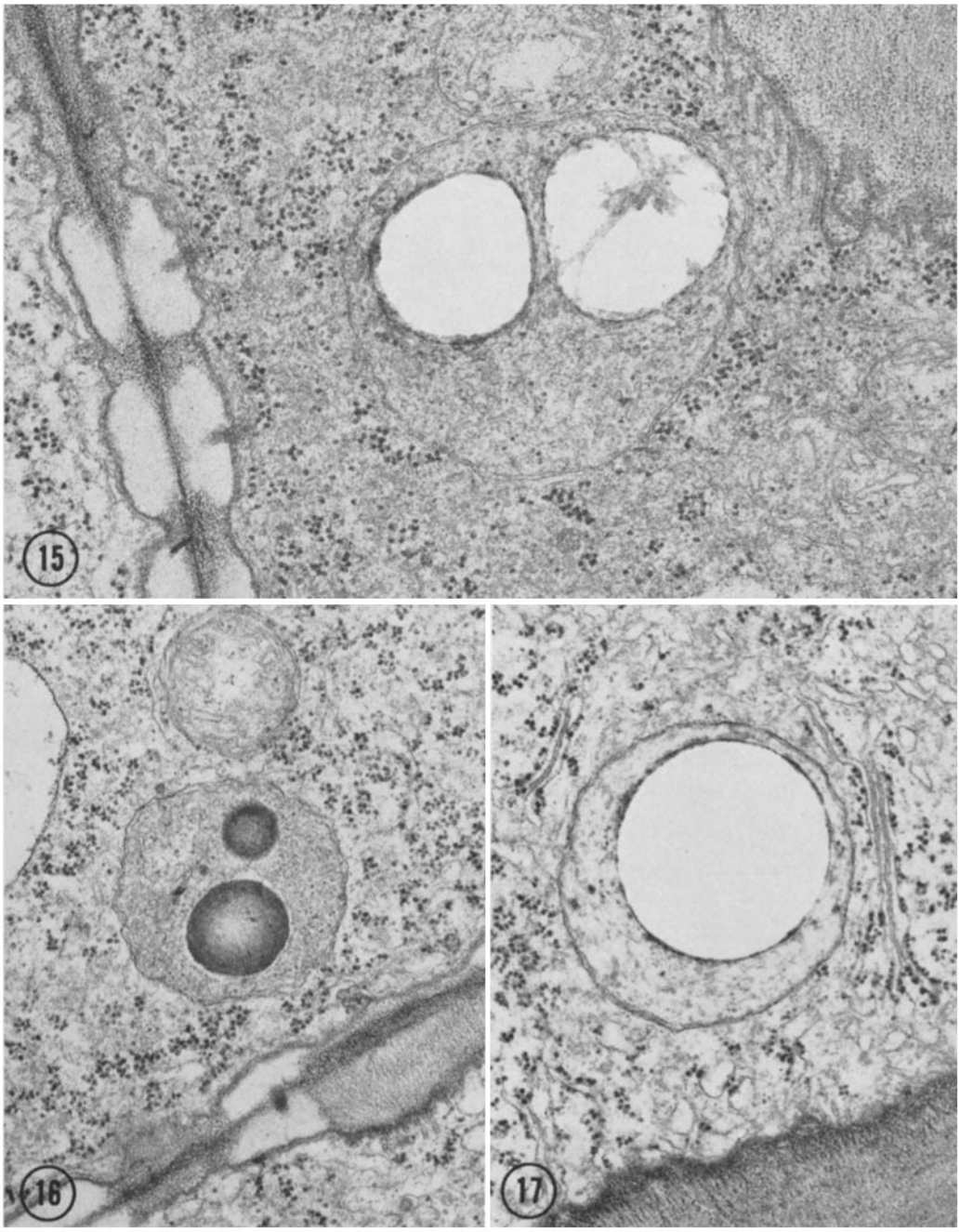

FIGURE 15 Almost complete digestion of starch deposits in a plastid of a sieve element brought about by incubation in 3% pullulanase for 24 hr followed by 1% α-amylase for 24 hr. Note young sieve plate on left. × 46,000.

FIGURE 16 Appearance of sieve element starch grains in a section incubated in the two carbohydrases in reverse order, i.e., 1% diastase for 2 hr followed by 3% pullulanase for 24 hr. No visible digestion of the grains has occurred. × 37,000.

FIGURE 17 Digestion of sieve element starch observed in a section mounted on a gold grid when the section was incubated in 0.5% pullulanase at pH 5.4 for 3 hr followed by 1% diastase at pH 6.8 for 2 hr. × 34,000.

hr followed by α-amylase for 1 hr is shown in Fig. 14: the deposit appears electron lucent and contains numerous holes. After a more complete digestion (pullulanase for 24 hr followed by α-amylase for 24 hr), little or none of the sieve element starch grains remained (Fig. 15). Exposure to 3% pullulanase for only 6 hr was inadequate, however, since subsequent treatment with amylase or diastase produced no digestion.

These results indicated that treatment with pullulanase renders the sieve element starch susceptible to subsequent α-amylolysis. This conclusion was reinforced by the results obtained when the order of exposure to the two enzymes was reversed in some of the above experiments. When sections were treated with α-amylase followed by pullulanase, no digestion of the sieve element starch was observed (Fig. 16).

A possible cumulative effect of amylase (i.e., of amylase possibly present as a contaminant of the pullulanase preparation, followed by the amylase of the posttreatment) was ruled out by showing that exposure to 1% diastase for a time equal to or greater than the total experimental digestion time (i.e., of pullulanase plus diastase) had no apparent effect on the sieve element starch grains.

Since the optimal activity of pullulanase occurs at pH 5.4 (7, 38), an attempt was made to obtain a more rapid rate of hydrolysis by lowering the pH of the digestions to this value. However, exposure of sections mounted on copper grids to 3% pullulanase in 0.05 M citrate buffer at pH 5.4 or 4.8 for periods up to 24 hr caused discoloration of the grids and apparently inactivation of the enzyme, since posttreatment with α-amylase yielded no digestion of the grains. On the assumption that the pullulanase was inhibited by copper ions resulting from the dissociation of copper from the grid surface at low pH, uncoated gold and nickel grids were substituted for copper. With these, it was found that at pH 5.4 a considerably lower enzyme concentration and shorter exposure time were required for starch grain digestion than with copper at pH 6.8. For example, using gold grids, 0.5% pullulanase at pH 5.4 for 3 hr followed by 0.1 to 1.0% diastase at pH 6.8 for 2 hr resulted in rather complete grain digestion (Fig. 17). At these respective concentrations, however, neither pullulanase alone for 3 hr nor diastase alone for 5 hr resulted in digestion.

In treatments of thin sections, the enzyme preparations proved to be highly specific for starch, as they did not noticeably digest other cellular structures even when the treatment was continued for as long as 48 hr (Fig. 15). Bounding membranes, organelles, ribosomes, and microtubules were not visibly altered. It is particularly noteworthy that the cell walls comprising a variety of polysaccharide components showed no digestion. This includes the developing sieve plate, whose prominent middle lamella and thickenings of callose remained intact (Figs. 11 and 15). Proteinaceous deposits, including the crystalline P-proteins of *Phaseolus* sieve elements and the crystalline inclusions in the phloem plastids of *Phleum*, also remained unaffected.

Incubation in the enzyme preparations was not totally without observable effect on the sections, however, since after poststaining the fine structure was not as electron opaque as it was in untreated sections. There was some indication that as exposure to the enzymes was continued, it resulted in a progressive loss of stainability. Prolonged exposure also caused the material to appear somewhat grainy (Fig. 15).

It was observed in early experiments that the pullulanase preparation caused partial digestion of ordinary starch. This effect resulted presumably from a small amount of α-amylase present as a contaminant. It was eliminated almost completely by extracting the preparation for 18 hr at 3°C in 0.05 M phosphate buffer at pH 6.8, centrifuging the suspension at 15,000 × *g*, and removing and using the supernatant (1; W. J. Whelan, personal communication). The pullulanase concentration of the supernatant was diluted to 0.5% with citrate buffer at pH 5.4 before use.

Rinsing the sections following incubation in the preparations of α-amylase, diastase, or pullulanase was sufficient to free them of contaminating deposits (Figs. 10–16). This was not true for the sections treated by β-amylase, however, as these always showed a coating of obscuring material when examined subsequently in the electron microscope. Since the contaminant was not removed by centrifuging and filtering the enzyme preparation before use or by washing the grids more thoroughly, the trials with β-amylase were discontinued.

DISCUSSION

Most starches consist of varying proportions of two fractions, amylose and amylopectin. Although it contains a few α-(1 → 6) branches (21), amylose is an essentially linear polymer of glucose. It can

be hydrolyzed by α-amylase, which cleaves α-(1 $\rightarrow$ 4) bonds at random, resulting in an increase in reducing power and in formation of oligosaccharides of shorter and shorter chain lengths. It can also be degraded by β-amylase, which cleaves α-(1 $\rightarrow$ 4) linkages in a sequential fashion from the nonreducing ends, yielding maltose.

Amylopectin, on the other hand, is a more extensively branched glucose polymer. Like amylose, it possesses α-(1 $\rightarrow$ 4) bonds that can be hydrolyzed by α- and β-amylase, but it has in addition numerous α-(1 $\rightarrow$ 6) linkages that provide branch points and reduce the extent of hydrolysis by α- and β-amylase (Fig. 16). The α-(1 $\rightarrow$ 6) bonds can be split by R enzyme, an enzyme commonly used in starch research, and by pullulanase, a recently discovered α-(1 $\rightarrow$ 6) glucosidase. Pullulanase is an inducible extracellular enzyme produced by certain bacteria, including *Aerobacter aerogenes*. For activity the enzyme specifically requires the presence of at least one α-(1 $\rightarrow$ 4) glucose bond on each side of the α-(1 $\rightarrow$ 6) branch points (1) (Fig. 18).

Largely on the basis of the iodine reaction, it was suggested some years ago that sieve element starch may be similar to an amylodextrin or dextrin (cf. 12). However, these substances are known only as hydrolytic degradation products of starch, and should not be confused with intermediates or end products in starch biosynthesis (18). It seems more likely that the reddish response to the iodine stain given by the starch of sieve elements is indicative of a branched, amylopectin type of molecule. Other starches that stain reddish with iodine are known to be of this type, including the starch in the endosperm of waxy maize, which consists entirely of amylopectin, and glycogen and floridean starch, both of which are quite similar to amylopectin. Furthermore, the iodine reaction has been studied for many years, and a correlation has been made between the observed color reactions and the molecular structure of the starch (16, 20). As the proportion of long straight chains of amylose molecules increases, more iodine molecules are bound inside these helically oriented glucose chains and the color response tends toward blue or black; on the other hand, as the chain length decreases or the ratio of branched-chain amylopectin components in the structure increases, the proportion of chain units in a helical configuration is reduced, resulting in less bound iodine and shifting the response toward red or reddish brown.

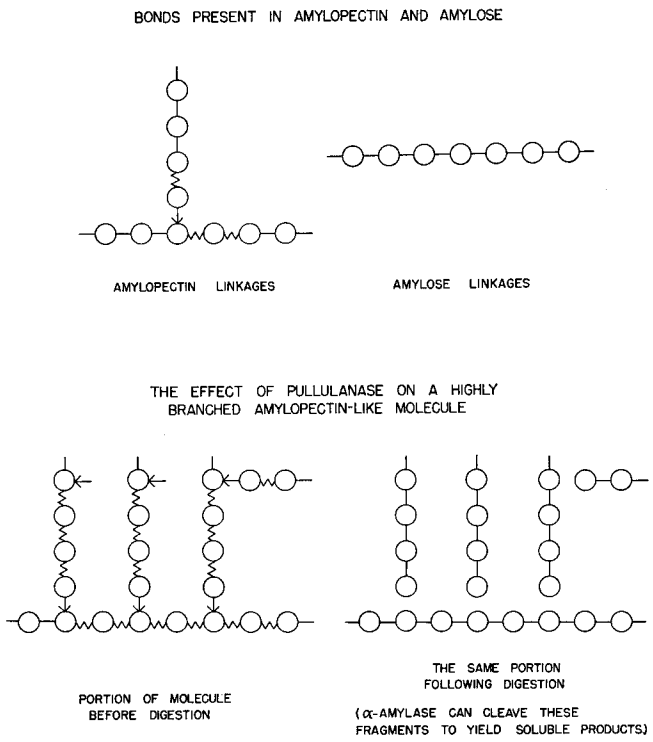

FIGURE 18 Diagrammatic representation of the types of linkages known to be present in amylopectin and amylose. Also shown is the effect of pul lulanase on these bonds and on subsequent digestion brought about by α-amylase or diastase (α-amylolysis). O, glucose residue; $\leftarrow$, α-(1 $\rightarrow$ 6) bond; —, α-(1 $\rightarrow$ 4) bond susceptible to α-amylolysis; MW, α-(1 $\rightarrow$ 4) bond resistant to α-amylolysis.

The response to iodine also shifts toward red as shorter molecules are produced during the progressive enzymatic degradation of starch of the blue or black staining type (31).

Roberts and Whelan (33) have shown that in waxy maize amylopectin and in glycogen the presence of the α-(1 $\rightarrow$ 6) branch points renders certain neighboring α-(1 $\rightarrow$ 4) bonds less susceptible to attack by α-amylase (see Fig. 18). Similar cases of resistance to digestion have been reviewed by Pazur (27). Consequently, if sieve element starch is highly branched like amylopectin, it should be less susceptible to digestion by α-amylase than starch with a lower proportion of α-(1 $\rightarrow$ 6) branches. In addition, pretreatment with the debranching enzyme pullulanase would be expected either to solubilize the sieve element starch grains or to render them susceptible to subsequent α-amylolysis.

The results of the present investigation support the hypothesis that sieve element starch is a highly branched molecule of amylopectin-type with numerous α-(1 $\rightarrow$ 6) linkages at the branch points. Whereas the ordinary starch grains of other cells are readily destroyed by α-amylase, those of sieve elements are not digested unless they are first rendered susceptible to α-amylolysis by prior treatment with pullulanase. The possibility that the effect of pullulanase might be due simply to a longer total exposure to amylase caused by the presence of the latter enzyme as a contaminant in the pullulanase preparation has been eliminated by showing that no digestion occurs when the order of incubation is reversed, i.e., when exposure to amylase precedes that to pullulanase. It has also been shown that an exposure to α-amylase as short as 1 hr is sufficient to cause obvious degradation when preceded by exposure to pullulanase, whereas exposure only to α-amylase has no visible effect even when prolonged for 48 hr.

Although there are conflicting reports (23), there is some evidence for the presence of a small number of α-(1 $\rightarrow$ 3) branch points in both amylopectin and floridean starch (28, 29, 40). While the techniques employed in our study do not reveal whether α-(1 $\rightarrow$ 3) bonds occur in sieve element starch, their presence would not be inconsistent with the observed results, since they would contribute to the high degree of branching indicated by the resistance to α-amylolysis.

Behnke (4) has shown that starch grains in the sieve elements of *Dioscorea* can be digested from thin sections with α-amylase alone. Although these results appear to be contradictory to ours, we have observed that starch grains in the plastids of old sieve elements with little remaining cytoplasm are digestible to some extent by α-amylase. We note in attempting to reconcile the two studies that the plastids in Behnke's micrographs also appear to be located in old sieve elements. We have not found the starch to be digestible by α-amylase alone in any of the earlier stages of sieve element development, nor when they occur free in the mature sieve element after their release from the plastid.

Our results also demonstrate that enzymatic digestions can be compared on the ultrastructural level so as to provide new information on the chemical nature of carbohydrate deposits in plant cells and allow a distinction to be made between closely related forms. Roberts and Whelan (33) have emphasized that in order to show relative differences in the susceptibility of chemical bonds to digestion, it is necessary to control the conditions carefully so as to achieve a low level of hydrolysis. In the present work the concentration and pH of the enzyme solutions, length of exposure of sections to the enzymes, and elemental composition of the grids were chief among the factors varied in adjusting the rates of hydrolysis so as to adduce evidence for the nature of sieve element starch and distinguish it from ordinary starch.

The present results indicate that hydrolytic enzymes can be used successfully to digest starch grains in plant material fixed in osmium tetroxide and embedded in Araldite-Epon. One of these enzymes, α-amylase, has been used previously in fine structural studies of starch deposits by Behnke (4) and Salema and Badenhuizen (35). It has also been used extensively in the electron microscopic investigation of glycogen deposition in animal tissues, but the variability of the results has raised questions about the effect of osmium fixation and plastic infiltration on enzyme activity (32). The most reliable procedure used in the studies on glycogen has involved treatment of the tissue with the enzyme before fixation and embedment (32), although glycogen in Epon-embedded material has been digested successfully with α-amylase in recent work by Rosa and Johnson (34).

It seems likely that interference from osmium has not proved to be a problem in our study because appreciable amounts of osmium are not bound by the starch grains. Most of the electron opacity of the grains results not from osmium fixation but from poststaining with lead. It also appears likely that the starch grains are digested readily because

very little plastic penetrates into their compact structure during embedment. This view is supported by numerous micrographs indicating that digestion starts first in the center of the grain and that it proceeds faster in large grains than in small ones. The latter fact is interpretable if the reasonable assumption is made that the degree of infiltration increases with decreasing size of the grain. Since glycogen is composed of relatively small granules it may not be readily digestible for the same reason.

Since amylose and amylopectin may be synthesized by separate metabolic pathways (3, 27), it can be assumed that variations in the composition of starch grains depend on the relative amounts or activities of the pertinent enzyme systems in the plastids. The synthesis of amylose is probably governed by the starch synthetase system utilizing UDPG and ADPG (22), while the synthesis and degree of branching of amylopectin appear to depend on the balance between the biosynthetic and degradative activities of phosphorylase and the branching activity of the Q enzyme. Presumably both synthetic pathways are operative in the plastids of parenchyma cells, but only the branching system is functional in plastids of the sieve element. A parallel example is afforded by the waxy maize mutant. The red-staining starch of waxy maize is not found in all tissues, but is limited to the endosperm, pollen, and embryo sac, while normal blue-staining starch is found in the embryo, seed coat, and other tissues (8). It appears that the amylopectin starch grains of the waxy maize endosperm have little or none of the starch synthetase system (17, 25, 26), although this system is present in the embryo and seed coat (26).

There is some evidence that there may not be an abrupt transition from synthesis of one type of starch in the sieve elements to another in other cell types, since the starch in the vascular parenchyma cells appears to be intermediate between ordinary starch and sieve element starch in its reactions. It stains red-brown rather than blue-black with iodine as was noted by McGivern (24) and confirmed in our study. It is also more slowly digested by α-amylase than is ordinary starch although since the grains of ordinary starch in cortical parenchyma cells are usually larger, a comparison of the relative rates of hydrolysis in the two tissue regions must be treated with caution.

Whether the indicated structural difference of sieve element starch from ordinary starch is important in the metabolism of the developing sieve element or in solute transport in the phloem is unclear at the present time.

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