THE DISSOLUTION AND REACCUMULATION OF STARCH GRANULES IN GRAPE VINE CANE

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Summary

Starch granules may be observed to break down *in vivo* either by corrosion spreading inwards from discrete points on the surface or by a decrease in size without change in shape due solely to surface action. The latter way may be termed reverse apposition because apposition growth of granules is observed as an increase in size without change in shape. There are grounds for suggesting the hypothesis that amylases are responsible for corrosion and some other enzyme system for reverse apposition.

This experiment was done to test this hypothesis. Small potted grape vines were grown and parent canes sampled at planting and at intervals up to 105 days afterwards. Starch content decreased from 14 to 3% and finally rose to 10% of cane dry weight. The number of starch granules, counted by light microscope methods, fell and rose in a similar fashion. The results indicated that all granules decreased in size during granule dissolution. Electron microscope observations indicated that granules at all times were free of corroded appearance and irrespective of size, had a clear rounded outline. When granules were treated *in vitro* with amylase and then examined, typical corrosion effects were found. Similar results were obtained with starch from grape vine leaves. It is concluded that amylase is not responsible for granule dissolution *in vivo* in tissues such as leaves, stems, roots, and tubers.

I. Introduction

There is evidence from various studies that starch granules grow by apposition (Badenhuizen and Dutton 1956; May and Buttrose 1959; Porter, Martin, and Bird 1959), with either starch synthetase involving a nucleotide diphosphate–glucose pathway (Leloir, de Fekete, and Cardini 1961; Recondo and Leloir 1961) or phosphorylase (de Fekete 1968) as responsible enzymes. Starch granules break down either by a reversal of apposition growth or by corrosion originating at localized surface points and spreading inwards. The enzyme systems responsible for starch breakdown are generally regarded as being either amylases or phosphorylase (Akazawa 1965). Reverse-apposition breakdown is typical of leaf starch (Matheson and Wheatley 1962; Badenhuizen 1966) and potato tuber (Chandorkar and Badenhuizen 1967), which are living tissues and in which many workers have noted that plastid membranes remain intact around starch granules under all normal conditions. Corrosion breakdown is typical of seed endosperms and occurs both *in vivo* during germination and *in vitro* in the presence of amylases (Buttrose 1960). Endosperm is dead tissue and plastid membranes are disrupted, as is also the case in cotyledons of

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germinating peas (Chandorkar and Badenhuizen 1967) when corrosion of starch is also observed (Bain and Mercer 1966).

There is much confusion as to whether amylase is responsible for dissolution of starch granules *in vivo* (Swain and Dekker 1966; Murata, Akazawa, and Fukuchi 1968), and there is slight hope that this question can be settled by chemical studies. However, the hypothesis is now suggested that the morphological difference between granules degraded by reverse apposition (combined with intact plastid membranes) on the one hand and corroded granules (combined with disrupted membranes) on the other was associated with correspondingly different forms of enzyme dissolution.

The present paper reports a morphological study of the breakdown of starch granules of perennial medullary ray cells of grape vine, both *in vivo* and in the presence of amylase *in vitro*.

II. Material and Methods

(*a*) *General*

From a number of 15-cm-long cuttings of grape vine (*Vitis vinifera* L. cv. Muscat Gordo Blanco) which had been stored at 4°C for 3 months after collection in the field, 250 were selected for uniformity of diameter, and their individual fresh weights taken. They were then planted into vermiculite contained in plastic buckets positioned in a glasshouse. A further 20 cuttings were weighed before and after drying at 105°C, and from the resulting estimate of water content dry weights of all cuttings were calculated. At bud burst 3 days later (day 0) 15 canes were harvested and each was treated as follows: from the middle of the cane a 1-cm portion was removed and, after weighing, was stored in a deep-freeze for subsequent starch granule counts. A second 1-cm portion was taken for fresh weight and dry weight determinations (moisture content). Next a thin slice was removed from the cane and prepared for electron microscopy. The remainder of the cane was dried at 55°C to constant weight, weighed, and then portions ground in a laboratory mill for starch assay. The carbohydrate status of canes could be assessed between harvests by comparing dry weight of sampled canes with their calculated initial dry weights at planting (see Buttrose 1966*a*). Subsequent harvests, involving the foregoing procedure, were made on days 20, 35, 65, and 105. The plants for these harvests, as well as additional spares, were grown, after bud burst, in 15-cm porous earthenware pots containing John Innes compost.

(*b*) *Counting of Starch Granules*

The 1-cm portion was sliced and macerated with a razor-blade, then transferred to a glass mortar with water. The macerate was ground with a pestle to a creamy consistency (few cells left intact) and then transferred to a centrifuge tube and the pellet resulting from centrifugation diluted to 10 ml with 0·2% iodine in 2% potassium iodide. After thorough suspension, a drop was transferred to a haemocytometer counting slide and the coverslip immediately applied. One field was photographed at ×100 magnification. Five further drops were photographed in this way, and starch granules, identified as dark spherical to oval bodies, were counted in a known area on resulting prints at a total magnification of ×550. In the day 0 sample there were approximately 60 starch granules counted on each photograph. Granules on the photographs were assigned to diameter size groups of 0–1, 1–2, 2–3 mm, etc. For each harvest there was a total of 90 photographs examined in this way. Results were calculated as the mean number of starch granules per gram cane residual dry weight; the latter was the weight of cane minus its starch content (measured by chemical analysis), and approached a common unchanging tissue mass.

(*c*) *Starch Assay*

Starch was determined by chloral hydrate extraction, acetone precipitation, and assay as glucose following acid hydrolysis (Buttrose 1966*a*).

(*d*) *Microscopy*

For light microscopy of tissue, free-hand sections were photographed in bright-field illumination. For electron microscopy, small pieces of cane were fixed in (1) 2% aqueous potassium permanganate for 2 hr at 20°C, or (2) 4% glutaraldehyde in 0·2M phosphate buffer at pH 7·0 for 2 hr at 4°C, followed by 4% osmium tetroxide in the same buffer at 4°C overnight. Following dehydration in acetone the tissue pieces were embedded in Araldite or Epon and sections viewed in an electron microscope. For studies on isolated starch granules, preparations of starch were obtained and treated with salivary amylase (the supernatants obtained by centrifuging saliva at 4000 *g* for 10 min) or 2·2N hydrochloric acid at 38°C (Buttrose 1966*b*). Treated granules were dehydrated, embedded in methacrylate, and sections viewed in an electron microscope after staining with potassium permanganate.

(*e*) *Amylase Activity*

Short lengths of day 0 canes (approximately 1 g fresh weight) were cut into thin slices, macerated with a razor-blade, and ground in a chilled mortar with acid-washed sand and 0·5M sodium citrate buffer (10 ml) at pH 5·6. The resulting suspension was made to 25 ml with additional citrate buffer, centrifuged at 4000 *g* for 45 min at 4°C, and aliquots of the supernatant used for enzyme assay. Reaction mixtures contained enzyme extract or water (1 ml), 1% starch solutions (B.D.H. Analar, 1 ml), citrate buffer as above (1 ml), and toluene (0·1 ml). To certain incubates 0·2 ml of 10^{-5}M mercuric chloride was added to inhibit enzyme activity. Following incubation for up to 12 hr at 30°C reducing sugar was assayed using a copper reagent (Somogyi 1952). The increase in reducing sugar as a result of enzyme activity was expressed as glucose equivalents.

III. Results

The change in starch content of canes is shown in Figure 1, and in starch granule numbers in Figure 2. The size frequency distribution of granules changed as shown in Figure 3. By day 65 there were relatively few large granules and a correspondingly large proportion of small granules. This indicates that all granules had become smaller in diameter, in addition to the total dissolution of a portion.

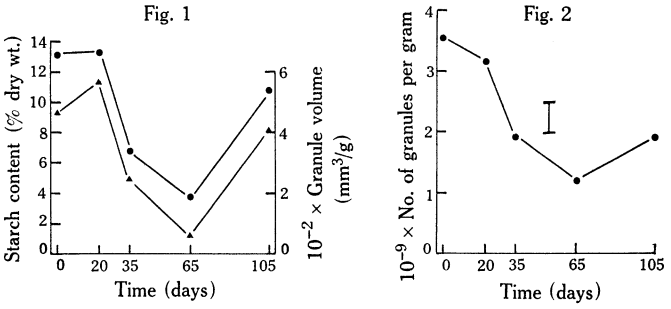


Fig. 1.—Changes in starch content of canes (●) and in calculated total granule volume (▲).

Fig. 2.—Changes in total number of starch granules. Vertical bar represents least significant difference ($P = 0·05$).

Granules were assumed to be perfect spheres, and resulting calculations of total starch volume are shown in Figure 1. Although absolute volumes are in error, relative volumes are meaningful. From day 20 onwards there was a close similarity between changes in dry weight of starch and in calculated volume. This is evidence that starch granule measurements were reliable.

Figures 4 and 5 show the appearance of medullary ray cells from day 0 and day 65 canes respectively. The section chosen for Figure 5 was from a cane devoid of starch.

An electron micrograph of portion of a ray cell, such as seen in Figure 4, is shown in Figure 6. The thick cell walls impeded penetration of fixatives and embedding media, and good-quality electron micrographs were difficult to obtain. For this reason plastid membranes are not obvious in this micrograph; however, whenever fixation was good, as judged by the preservation of membranes of other organelles, starch granules were invariably found to be contained within a plastid membrane. There was normally only one granule per plastid. Granules were smooth in outline. The dark spokes inside the granules and the dark peripheries are artefacts resulting from action of the electron beam on starch, and may be noted in many published electron micrographs. A typical micrograph from day 65 material is shown in Figure 7. The granules are about 1 μm in diameter, the predominant size for granules at this harvest. They have a smooth periphery, have no internal corrosion, and are surrounded by intact plastid membranes. The same observations, except for variations in granule diameter, were made for all samples at all harvests.

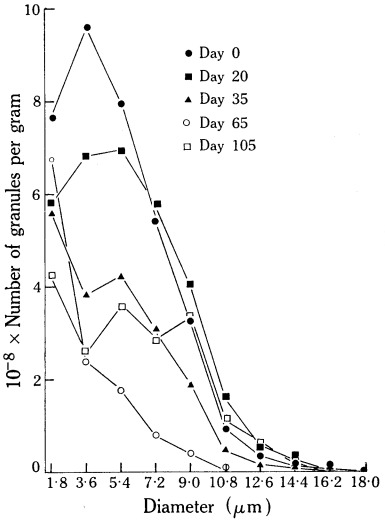


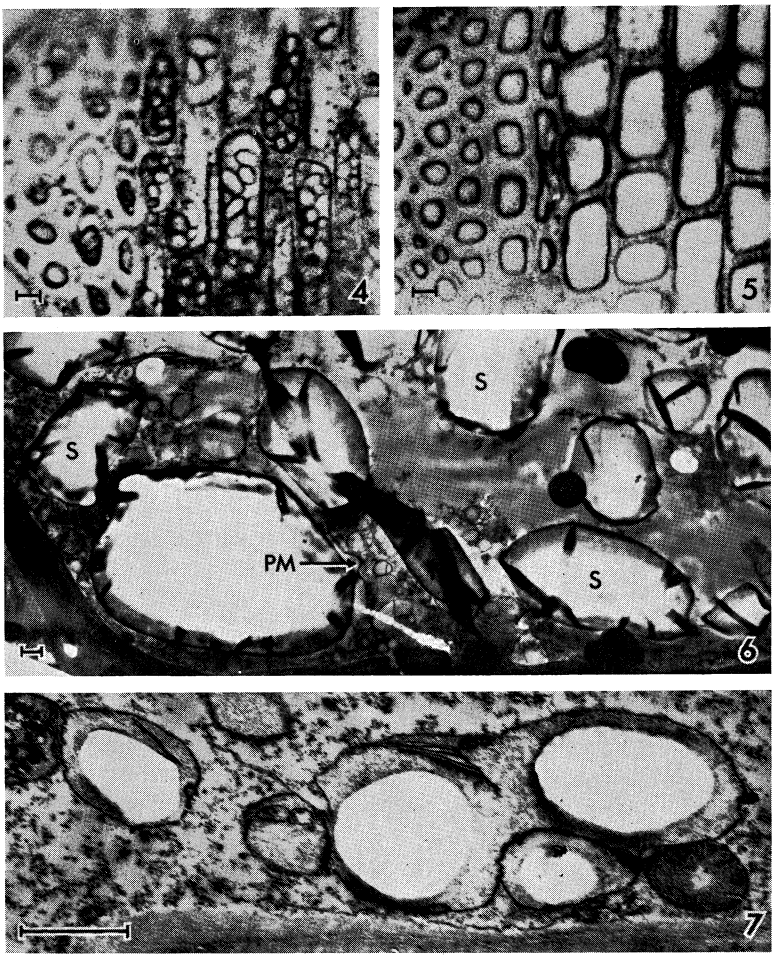
Fig. 3.—Size frequency distribution curves for starch granules at different harvests.

Figure 8 shows the appearance of day 0 granules following treatment *in vitro* with amylase. The upper granule section shows evidence of advanced corrosion, the lower of attack at various points on the periphery. It should be noted that during isolation of granules the surrounding membranes are disrupted.

Starch granules isolated from leaves of grape vine behaved similarly, as shown by Figures 9 and 10.

Figure 11 shows the appearance of portion of a vine cane granule after partial hydrochloric acid hydrolysis. Over 20 concentric rings could be counted, and it is clear that these granules, like those from all other tissues that have been examined, have a shell structure.

Amylase activity was found in extracts of day 0 cane, and averaged 1·7 mg glucose equivalents produced per gram cane dry weight per hour (0·16 μmole/g/min). If amylase had been hydrolysing starch *in vivo* at this rate all reserve starch could theoretically have been lost within 8 days.



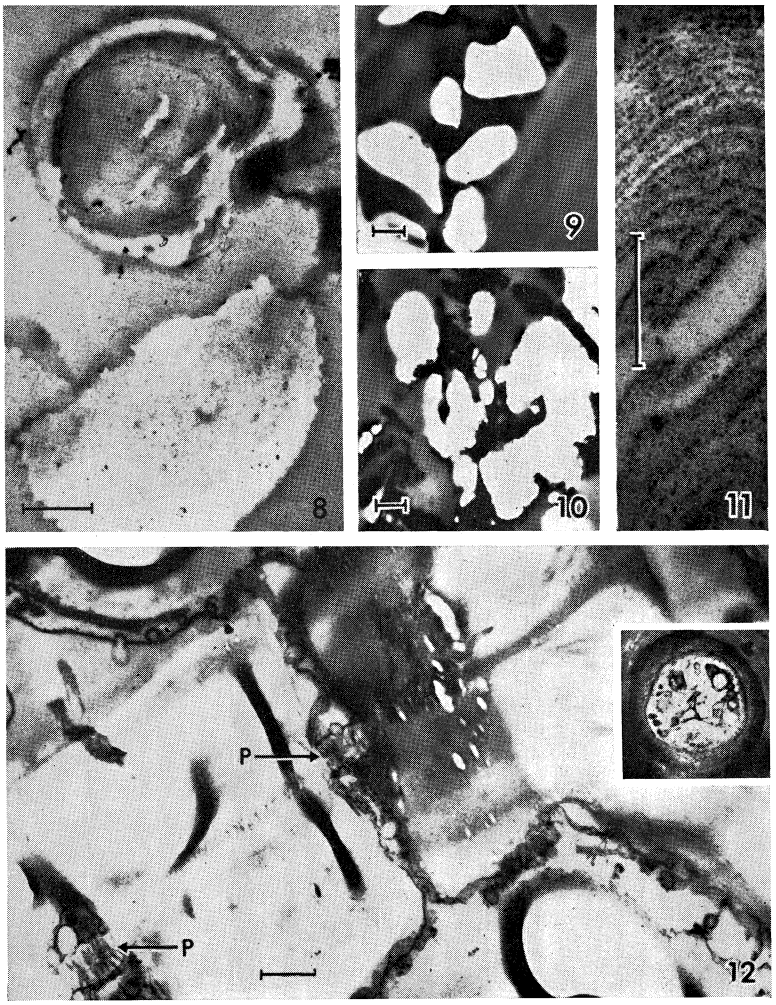
Figs. 4 and 5.—Photomicrographs of a section of a day 0 (Fig. 4) and of a day 65 cane (Fig. 5). In Figure 4 medullary ray cells are filled with starch, whilst in Figure 5 no granules are visible. ×350. Bars represent 1 μm.

Figs. 6 and 7.—Electron micrographs of a portion of a day 0 (Fig. 6) and of a day 65 (Fig. 7) medullary ray cell. *S*, starch; *PM*, plastid membrane. *6*, ×3,000. *7*, ×14,000. Bars represent 1 μm.

The dark lines traversing some of the medullary ray cell walls in Figures 4 and 5 were observed in the electron microscope to be pits (Fig. 12). Pits were approximately 1 μm in diameter and they have frequently been observed to be spaced about 5 μm apart, as in Figure 12. Approximately 2·5% of the cell wall surface could be penetrated by pits.

IV. Discussion

Light and electron microscope observation indicated that during starch depletion *in vivo* all granules decreased in diameter without changing their general



Figs. 8–11.—Electron micrographs of starch granules. Bars represent 1 μm in all cases. *8*, Isolated cane granule following treatment *in vitro* with amylase. ×9,000. *9*, Isolated vine leaf granules. ×4,000. *10*, Isolated leaf granules following treatment *in vitro* with amylase. ×4,000. *11*, Portion of a cane granule following hydrochloric acid treatment. The centre of the granule section was just below the bottom of the micrograph. ×17,000.

Fig. 12.—Transverse section of portion of a cell wall between two medullary ray cells. Two pits are visible in longitudinal section and the primary cell wall remaining is seen to be traversed by numerous plasmadesmata (*P*). × 7,000. Inset: a pit seen in transverse section. × 14,000.

shape, and they were surrounded by intact plastid membranes. Thus their dissolution was by reverse apposition. When treated *in vitro* with amylase their breakdown was

by corrosion. These observations are advanced as evidence that, *in vivo*, the dissolution of starch granules in both cane and leaf of grape vine was not mediated by an amylase enzyme, despite the presence of an amylase activity. The presence of a plastid membrane could protect granules from cytoplasmic amylase.

There is some supporting evidence in the literature dealing with potato tuber starch granules. These break down *in vivo* by reverse apposition (Chandorkar and Badenhuizen 1967), whereas *in vitro* in the presence of amylases breakdown appears different (Leach and Schoch 1961). As a working hypothesis it is suggested that the findings made with grape vine starch granules are applicable to granules in other living tissues.

It has already been noted that phosphorylase is generally regarded as the alternative to amylase as responsible for starch dissolution. However, Stocking (1952) obtained evidence that phosphorylase is located in the cytoplasm itself and not inside plastids. Confirmation of this finding is needed. At the same time it should be remembered that a starch synthetase enzyme system has been shown to be associated with starch granules themselves (Pottinger and Oliver 1962), and is therefore probably located inside plastids either in the stroma or on membranes. Further consideration should be given to the possibility that a reversal in action of such an enzyme system is involved in reverse-apposition breakdown of starch granules.

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