Doctoral Thesis

Submicroscopic development and structure of starch granules in cereal endosperms

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Submicroscopic Development
and Structure of Starch Granules in
Cereal Endosperms

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by

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Submicroscopic Development and Structure of Starch Granules in Cereal Endosperms

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In the first part of this study the nature of amyloplasts in cereal endosperms and of the development of starch granules in them has been investigated by electron microscopy. In barley, one granule, and in oats, many granules form in the young proplastids, followed by characteristic growth of simple and compound granules. The small granules of barley (and probably wheat and rye), which have for long been recognized as distinct from large granules in the same cell, are formed in vesicles budded off from the large amyloplasts.

To gain more knowledge of the submicroscopic structure of the cereal starch granule, starch has been treated with amylolytic enzymes and hydrochloric acid, and the effects examined in the electron microscope. The only clear structures appearing are the granule shells. Both reagents reveal a resistant and susceptible portion in each shell. With higher amylpectin content shells are more distinct. Evidence is provided that one shell is formed per day, and that under constant environment none are formed.

Our present knowledge of the starch granule has been recently reviewed by Badenhuizen (3). Starch granules are normally formed in plastids in the higher plant, chloroplasts in green tissues, and leucoplasts in storage tissues, and they are almost invariably found in the proplastids of meristematic cells. Duvick (10) found starch granules in the maize endosperm developing in filaments resembling mitochondria. A further exception to plastid location has been proposed in respect of the small granules of barley, rye, and wheat endosperms, such granules being distinct from large granules which develop in leucoplasts. It has been suggested that these granules form in mitochondria (2), and it is still not known whether they arise simply free in the cytoplasm or in some organized body.

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There is now conclusive evidence that starch granules do not arise as a coacervate by rapid crystallization, but that they grow over an extended period of time (21) by apposition (4, 44). Meyer (24) assumed such a method of growth and used it as a basis to explain the shell structure visible in the light microscope in the form of concentric rings. He proposed that the density of packing of starch molecules would vary from day, with a plentiful supply of carbohydrate substrate, to night, when the supply would fall off, thus resulting in alternating water-poor and water-rich layers in a radial direction. This theory was substantiated by Bakhuyzen (6) who found no rings visible in granules of wheat starch from plants grown in a constant environment. In contrast, however, are the observations made with granules of potato tuber and Pellionia starch from plants grown in a constant environment (7, 12, 19, 23, 33, 34). Such granules have rings normally developed and the results of Bakhuyzen have been thought to be due to a failure to distinguish the closely spaced rings of wheat granules. However, it appears that similar work has not been repeated with seed endosperm starches. As fluctuations in carbohydrate supply do not appear to be responsible for shells, the activity of the starch-synthesizing enzyme phosphorylase over 24-hour periods has been determined (5), but no significant fluctuation could be observed. However, the importance of phosphorylase in starch synthesis is by no means certain (39). The current theory is that some endogenous rhythm may regulate transport of carbohydrate substrate to starch-synthesizing tissue, resulting in fluctuations in density of packing of molecules. That such a fluctuation is diurnal is not certain, but attempts have been made (15) to measure frequency of shell deposition of potato granules, results indicating one to two per day; and a comparison of the total growth period of seed starch granules and the number of shells present at maturity indicates that the formation of one shell per day is probable (21).

Of the structure of the native granule extraordinarily little is known. Polarization optic studies show it to be a spherocrystal with the chains of glucose residues oriented radially, the radial orientation being confirmed by X-ray analysis (18). The visibility of rings, referred to above, has been shown to be due to a regular decrease in refractive index from the inside to the outside of each shell, with a sudden discontinuous rise at the boundary of the next outer shell (13). Distinct knowledge of how the branched and unbranched molecules are distributed and crystallized is lacking. The two types of molecule may be separated or form an intimate mixture. Electron microscopy has been singularly unsuccessful as a tool in providing new information. Ultrathin sections have been examined by several workers (31, 40, 41), but apart from the rudimentary presence of rings representing variations in electron-scattering power, the material appears homogeneous, and any fine granulation seen is almost certainly artifact. Following enzymatic corrosion of maize starch granules a shell structure
has been exposed (27, 31), corresponding to the saw-tooth corrosion pattern observed in the light microscope (36).

Starch granules have a high density of 1.5–1.6, and accordingly it would be expected that their electron-scattering power in the microscope would be of a high order. This, however, is not the case, for normally they are among the most electron-translucent objects in the cell. It may therefore be that immediately on exposure to the electron beam the starch material becomes vaporized, leaving only a very thin residue or, in some cases, an empty space. Frequently, dark bands are seen radiating to the edge of a granule. In one case these have been assumed to be protein membranes (32), but are actually artifacts due to a compression and folding of the starch during sectioning. It is noteworthy that where such areas have three times the thickness of surrounding parts of the section, they have much more than three times the electron-scattering power. Possibly part of the starch is protected from vaporization in such circumstances. Sterling and Spit (38) recognized the modifications undergone by native starch during preparation for electron microscopy and endeavored to overcome these difficulties by using replica techniques on inner surfaces exposed by fracture. They published micrographs showing what appear to be isolated microfibrils, but their occurrence is so rare and dispersed that it is not possible to learn anything of the ultrastructure of the native granule from them.

With the realization that the starch granule must first be pretreated before submicroscopic structures become recognizable in the electron microscope, the present study was initiated to learn of the effects of corroding agents on starch granules. Furthermore, a morphological study was made of the initiation and development of starch granules in cereal endosperms.

MATERIAL AND METHODS

The main material for study was the endosperm of barley (Hordeum distichum L. var. Prior), but the endosperm starch of maize (Zea mays L.) (waxy, normal, and high amylose types), oats (Avena sativa L.), wheat (Triticum vulgare Vill.), and rice (Oryza sativa L.) was also used. Where immature endosperms were used, they were taken from plants grown in the open garden in summer, and also in the case of barley from plants grown in a constant environment cabinet. Details of this artificial environment have been published elsewhere (9).

Pretreatment of granules was based on staining with potassium permanganate, enzyme corrosion during germination or incubation with pure enzyme preparations of $\alpha$-amylase and $\beta$-amylase (1% solutions in acetate buffer at pH 6.2), and treatment with hydrochloric acid. Acid treatment included the so-called lintnerization—exposure to 7.5% hydrochloric acid for a prolonged period of weeks.

1 Samples of waxy maize were supplied by Iowa State College and the Botany School of the University of Cambridge, and high amylose corn by Dr. T. J. Schoch, of Corn Products Company, U.S.A.
In preparation for electron microscopy of developing endosperm cells, small blocks of tissue were cut from the endosperm, as a rule at the center of the "cheeks" midway between base and apex, fixed in buffered potassium permanganate or osmium tetroxide solutions at 2°C, dehydrated, and embedded in methacrylate or araldite according to the usual procedures. In the case of pretreatment, small blocks of endosperm tissue were used for convenience, and after pretreatment these were washed, dehydrated, and embedded in methacrylate.

OBSERVATIONS

STARCH GRANULE DEVELOPMENT IN THE BARLEY ENDOSPERM

Following fertilization, the triploid endosperm nucleus divides to give a polynucleate sac, in which cell walls are then laid down between the nuclei. The endosperm is first readily separated from the surrounding pericarp tissues at about five days after anthesis, at which stage its cells have contents typical of young meristematic cells. The nucleus is prominent, together with numerous mitochondria, proplastids, and elements of the endoplasmic reticulum and of the Golgi apparatus. Proplastids are bounded by the normal double membrane, the inner membrane being invaginated to give tubular processes into the plastid stroma (Fig. 1). At this stage it is difficult to distinguish proplastids from mitochondria if starch granules do not appear in the section, though the tendency of the tubuli to lie respectively either parallel or perpendicular to the bounding membrane serves as a criterion (29). Starch granules form in the young proplastids, but normally only one per plastid, while as a general rule in meristematic tissues several form in each proplastid (28). At this stage the granule occupies only a small volume of the plastid, being embedded apparently free in the stroma, but it grows rapidly until it fills the greater part of the volume (Figs. 3, 4). The plastid and enclosed starch granule increase in size, the latter continuing to lie free in the stroma. The outline of the whole amyloplast structure in section suggests its oblate spheroid shape in three dimensions, as observed in the light microscope. The initiation of starch granules in proplastids as thus described has been observed up to about two weeks after anthesis, those forming toward the end of this time being found more at the periphery of the endosperm where cells are formed later.

The tubular invaginations of the inner plastid membrane persist in modified form

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Fig. 1. Portion of a very young barley endosperm cell. P, proplastid with small starch granule; M, mitochondria. × 38,000.

Fig. 2. Proplastid from young oat endosperm. S, starch. × 33,000.

Fig. 3. Developing barley granule in plastid, showing the location of tubuli at the widest periphery of the flattened granule, and the groove in the granule at this point. × 30,000.

Fig. 4. A section parallel to the flattened plane of a developing barley granule, showing the orientation of tubuli around the periphery. × 9000.
throughout growth of the starch granule. In section they appear as numerous cylinders of varying length, but apparently they lose association with the inner membrane and lie free in the stroma layer (Figs. 3, 4). They will be referred to as tubuli. They are not distributed at random in the stroma, but are oriented tangentially in a limited portion extending right around the starch granule at the widest periphery, or at what may be regarded as the equator. This may be understood from Figs. 3 and 4 which are sections respectively perpendicular to, and parallel to, the flattened plane of the granule. These tubuli appear to have an effect on starch deposition, as invariably in their immediate vicinity there is an indentation in the surface of the granule (Fig. 3). In three dimensions this must represent a furrow or groove extending right around the granule at the equator of the oblate spheroid. Such a formation has been observed up to three weeks after anthesis, but the groove becomes progressively less distinct. At the same time in the maturing amyloplasts the tubuli become few or absent.

Sometimes two or more starch granules are found in a proplastid, but sooner or later such plastids are found to divide to give daughter plastids containing only one granule. Such division may occur when the granules are already several microns in diameter. There are rare cases when several to many starch granules are formed in the young proplastid and persist, each granulum growing independently in the plastid, to result in a compound granule. In this case the whole plastid grows to a size similar to that of a normal plastid containing one granule. A compound granule of this kind may contain up to several hundred granula at maturity, each assuming a polyhedral shape during growth, and it thus resembles a compound granule of the oat endosperm. Such compound granules do not appear to form in any special region of the endosperm, but are abnormalities occasionally appearing.

Owing to the drying out of the endosperm it is almost impossible to obtain good preparations for electron microscopy later than about three weeks after anthesis. Thus, while there is little doubt from the observations that granules grew till three weeks, it could not be learnt from this study if they grew till endosperm maturity at about 30 days after anthesis.

Particular attention was paid to the origin of the small starch granules of barley which are known to make their appearance at about two weeks after anthesis (21, 35).

Fig. 5. At about two weeks after anthesis active evagination of the membrane of large barley amyloplasts takes place, with the formation of small starch granules in the processes. \( \times 15,000 \).
Fig. 6. Development of small starch granules between a large barley granule and the amyloplast membrane at two weeks after anthesis. \( \times 20,000 \).
Fig. 7. Small barley endosperm starch granules enclosed by a double amyloplast membrane after being budded off into the cytoplasm. \( \times 23,000 \).

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1 The term granule is used to refer to the whole starch structure contained within an amyloplast, and the term granulum to the separate constituents of a compound granule.
From this time on, no proplastids forming starch have been found to appear in the endosperm or any other cellular inclusion to store starch. The small granules in fact arise from the existing large amyloplasts in the following manner. At about 12 days after anthesis the amyloplast double membrane is seen to form stroma-filled tubular evaginations into the cytoplasm between the large granules. Small starch granules develop in these evaginations, extending them at these points to spherical shape (Fig. 5). It appears that relatively extensive tubular networks may thus originate from large amyloplasts. Sooner or later the evagination is separated from the parent plastid by constriction, and where more than one granule is present further constriction occurs so that eventually single membrane-enclosed granules lie free in the cytoplasm (Fig. 7). Also from two weeks after anthesis small granules are found to develop in the narrow layer of stroma between a large granule and the plastid membrane. Such an example is shown in Fig. 6, the small granules lying just above the equatorial groove. In this case it appears that the tubuli have migrated to form a layer between the large and the small granules. Where such small granules develop, the plastid membrane protrudes, surrounds the granule, and separates from the parent plastid by constriction. In some cases the tubuli have been seen to form a wall across a protrusion, enclosing the small granules, but it is not known if separation from the parent plastid takes place following this. There also appear in the cytoplasm small double-membraned vesicles (Fig. 7, bottom right) which contain no starch, but which also arise from the large amyloplasts. Possibly they too are capable of building starch granules. The small granules lie free in the plastid stroma, which, however, has not been found to contain tubuli. In shape they are spherical to egg-shaped, in contrast to the large granules, but with further growth toward maturity they become closely packed and assume a polyhedral shape.

Development in Other Endosperms

Maize

The development of starch granules in both normal and waxy types has been followed in the early stages. They are initiated in proplastids, normally one per plastid, and where more than one is formed a membrane grows between them, followed by plastid division, so that in the maturing endosperm one granule per plastid is the

Fig. 8. Mature barley starch granule showing rings towards the periphery after KMnO₄ treatment. × 10,000.
Fig. 9. Maturing compound starch granule from oat endosperm. Individual granula often show rings after KMnO₄ treatment. × 14,000.
Fig. 10. Waxy maize starch granule showing a ring formation and signs of a radial organization. × 25,000.
rule. The shape of granules tends to spherical, though in cases they are bilaterally flattened, thus resembling large granules of the barley endosperm. In the growing amyloplast, tubuli appear to be absent.

**Oats**

The proplastids in the young oat endosperm have long, well-developed invaginations of the inner membrane, and starch granules are initiated at various points in the stroma (Fig. 2). Where a granule is seen to be bounded by a tubular invagination it is usually elongated in the same direction. The separate granules grow rapidly in size, and new ones are initiated so that soon they fill the greater volume of the plastid. The whole structure continues to grow slowly, the individual granula becoming tightly packed so that opposing faces are flat and each granulum becomes polyhedral. At all stages a layer of stroma persists between them, and often tubuli can be seen lying in it. Tubular invaginations around the periphery are well preserved during development (Fig. 9). An adult plastid may contain well over a hundred granula.

**Rice**

The rice endosperm starch granule is also compound, and develops similarly to that of oats. Many small spherical granula are initiated in the proplastid stroma, and by assuming a polyhedral shape with growth soon fill the plastid. Unlike oats, there is often a fusion between granula at some points, so that each may not be completely surrounded by a stroma layer.

**The Structure of the Starch Granule**

**Appearance of Untreated Granules**

In the present study no structural detail has been observed in sections of whole barley granules following direct embedding. After potassium permanganate ($\text{KMnO}_4$) staining, however, concentric rings are often seen clearly, representing the well-known shells of the granule (Fig. 8). They are spaced at about three to four per micron towards the periphery, and extend right around the section. Toward the center of the granule rings are not seen. Up to 12 rings have been found in a section. They are visible as alternating dark bands of about $0.1 \mu$ width and lighter bands of about

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Fig. 11. Starch granule from germinating wheat grain showing the saw-tooth corrosion pattern. The section is perpendicular to the flattened plane of the granule. $\times 14,000$.
Fig. 12. Barley starch granule after $\alpha$-amylase treatment, showing the corrosion pattern. Section parallel to the flattened plane of the granule. $\times 12,000$.
Fig. 13. Rice starch granules after $\alpha$-amylase treatment. A small central portion, and the periphery, are more resistant to enzyme attack. Saw-tooth corrosion is not clearly evident. $\times 21,000$. 
0.2 μ width. No sharp boundary between the bands can be seen, although the transition from one to the other appears to be quite sudden rather than a gradual merging. Signs of rings can also be distinguished in the separate granula of the oat granule (Fig. 9). Each granulum appears therefore to be built up of shells. Apart from often rendering the rings visible in the electron microscope, KMnO₄-treatment appears to some extent to preserve starch in the electron beam. Starch then has a much higher contrast, which, apart from the staining effect of KMnO₄, must indicate a reduced vaporization of the starch. It then shows a distinct spongy texture, but as this becomes more pronounced and coarser with longer treatments it must be concluded to be an artifact. It has been found (25) that with increasing concentrations of KMnO₄ starch gains progressively in contrast in the electron microscope.

**Appearance of Enzyme-Treated Granules**

Amylase attack on the intact barley and wheat granule takes place most readily in the equatorial plane, corroding a channel into the center where the starch is much more susceptible to attack than at the periphery (Figs. 11, 12). This confirms the findings made with the light microscope (36). The enzyme digests only at a surface and there is no loosening of the starch substance behind this surface. Most marked is the well-known saw-tooth pattern left by the enzyme in the corrosion channel. This indicates an alternating relative susceptibility and resistance to enzyme attack of the starch in a radial direction. It is interesting that the susceptibility in the equatorial plane appears generally greater than that of the susceptible part between two "teeth" a little above or below this plane. It seems likely that the "tooth" pattern left after enzyme attack corresponds to the shell structure of the native granule. On this basis a portion of Fig. 11 was enlarged and rings drawn in in the most logical manner, the result being presented in Fig. 14. In most cases the rings can be drawn to follow round the inner edge of each "tooth". When this is done it is seen that there are 12–13 shells in the granule, spaced at approximately four per micron. There is therefore a close correspondence with the rings observed after KMnO₄ treatment. Indeed in some sections of granules treated with KMnO₄ after partial enzyme digestion, it could be seen that a "tooth" was a continuation of a dark band. From Fig. 14 it would appear that the starch within each shell decreases in a radial direction in resistance to enzyme attack, with a sudden discontinuous rise in resistance at the beginning of the next outer shell. This is most clearly demonstrated in the shells midway between granule center and periphery. Near the center a variation is still evident within each shell, but the exposed face is not smooth; and at the periphery the shells are closely spaced and a clear gradation cannot be distinguished.

The enzyme corrosion patterns of maize, rice, and oat granules have been observed. As found by previous workers, maize granules show a pattern very similar to that
of barley or wheat. With rice and oats, the individual granula have a very resistant outer portion while the center is rapidly broken down. With rice there is often a small residual portion at the very center of the granulum, as in Fig. 13. It is seen that with rice there is no clear indication of a saw-tooth corrosion pattern, though its rudimentary presence has been observed in some sections. While the individual granula in a compound rice or oat granule are polyhedral, enzyme attack often clears a more spherical area, and possibly the inner wall exposed follows the outline of a shell, which away from the periphery may be spherical (Fig. 9).

**Appearance After Acid Treatment**

Initially the classical lintnerization treatment with 7.5% hydrochloric acid at room temperature for a prolonged period (two months or more) was used. It was found that similar results were obtained in much shorter time with stronger acid and higher temperatures.

A typical result of acid treatment of barley granules is produced in Fig. 15. In section all granules are clearly ringed, indicating a shell structure in three dimensions. About 8–10 rings, composed of a light and dark band, can usually be distinguished, spaced towards the periphery at about 3–4 per micron. They are therefore comparable with the rings seen in KMnO₄-treated granules and with those indicated by enzyme corrosion patterns, and must correspond to the shells of the native granule. From the study of many preparations it became clear that the dark bands contained particulate matter, while the light bands were empty. The width of the dark bands varies considerably, which may partly be due to angle of sectioning and distortion during sectioning. However, they are on the average found to be about 0.1 μ wide and separated by an empty band of about 0.2 μ wide. These dimensions are again comparable with those of bands sometimes seen in intact granules. It must be concluded
from these observations that acid preferentially hydrolyzes the starch in a certain portion of each shell, the remaining portion being resistant. It is seen that the dark bands are in higher contrast than is normal for starch in the electron microscope, which suggests that the molecules in this region have been modified by acid treatment so that they are more stable in the electron beam. It is interesting that the center of the granule is apparently resistant to acid hydrolysis. As this part is most susceptible to enzyme attack it was thought that it would be readily broken down by acid also. The center is usually not as dark as the peripheral dark bands. There is no doubt that acid was able to penetrate the central region, as it can in many cases be seen to contain one or two somewhat paler bands, indicating acid hydrolysis. At all events it is clear that the central region differs in structure from the peripheral region.

Effect of Phosphorus on Shell Structure

Starch granules from plants grown under phosphorus deficiency (8) were lintnerized and a typical result appears in Fig. 16. While in normal material rings are seldom clearly seen in the small granules, they are extremely prominent in this material, and furthermore, a distinct close spacing of rings is seen at the periphery of large granules. It is noticed that with small granules the outer rings do not necessarily extend right around the granule but may appear only at a corner of the polyhedron. Rings occur here at the rate of about 16 per micron; that is, a light and dark band together have a width of about 600 Å. It is once more noticed that the central region of granules is resistant to acid hydrolysis.

As the appearance of rings in small granules and at the periphery of large granules developed under phosphorus deficiency is not exactly similar to that of normal granules, it could be that phosphorus influences the formation of shells in the developing granule, or that it modifies the structure of the shells in respect of their reaction with acid. Granules obtained from plants grown under nitrogen deficiency were also lintnerized. Results were as for normal granules, though the appearance of rings was more rapid.

Influence of Amylose/Amylopectin Ratio on Shells

In order to study this relationship, the effect of acid treatment on waxy (about 98% amylopectin), normal (about 75% amylopectin), and high amylose (about 40% amylopectin) maize has been observed.

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Fig. 15. Barley starch granules after acid treatment (19% HCl for three weeks). ×4000.
Fig. 16. Barley starch granules from plants grown under phosphorus deficiency, after acid treatment (7.5% HCl for two months). ×19,000.
Fig. 17. Waxy maize starch granules after acid treatment (7.5% HCl for one month). ×17,000.
With waxy maize the process is very rapid and rings are clearly defined after three to four weeks in 7.5% acid at room temperature, as in Fig. 17. Dark bands representing resistant portions of shells are separated by empty spaces. The width of the bands is approximately 0.1 μ, this remaining relatively constant from center with wide spacing to periphery with close spacing, and is similar to the width of bands observed in barley granules. In contrast to the action on barley starch (amylopectin content about 75%) acid breaks down the central region of waxy maize granules. About 17 shells may be counted in this section, but up to 30 have been found.

With normal maize granules the process is much slower, and after a prolonged treatment, while the rings are clearly seen, the space between the dark bands is often not entirely empty. The central region is more resistant as for barley granules.

With high amylose maize the process is still much slower, and rings less clearly defined. Shells, however, correspond in number and size to those of waxy maize granules.

From these observations it can be concluded that the formation of shells is a phenomenon quite independent of amylose/amylopectin content, but that the structure within each shell is greatly modified by differing proportions of the two molecular types.

Rings have also been seen very distinctly in sections of waxy maize granules after KMnO₄ treatment (Fig. 10). The rings in such cases do not appear to consist of two parts, a light and dark band, but are evident because of a sudden transition from darker to lighter material in a radial direction. Within a layer the contrast in the electron microscope appears similar across the whole width, though it may increase slightly towards the outside, and the next outer layer has less contrast throughout. Rings are widely spaced and few, which is probably due to the section being near the surface. Such sections are, however, exceptional, and as a rule alternating light and dark bands are seen, as for barley, and as observed earlier with maize (31).

Frequently in this material, as seen in Fig. 10, a distinct radial orientation is evident. This is due to the higher contrast of needle-like portions of about 250 Å width extending across the layer, thus having lengths of up to 0.5 μ. It is possible that these structures correspond to the rodlets observed by Sterling and Spit (38), and their radial orientation would be in keeping with our knowledge of the spherocrystal nature of the starch granule. However, it is considered equally likely that they are artifacts arising during staining and dehydration treatments. As starch granules are spherocrystals it is understandable that stresses arising during dehydration and other treatments could result in a tangential disorganization of the chain lattice. It is to be noted however that such a radial orientation has been observed only in the case of waxy maize granules consisting almost entirely of branched molecules.

Rings are occasionally seen in granules of normal and high amylose maize starch
after treatment with KMnO₄, and they are similar to those described for barley granules.

With amylase treatment waxy and normal maize granules both behave like barley granules in giving a typical saw-tooth corrosion pattern. High amylose granules show quite a different reaction. While the central region is again the most susceptible to attack, no clear "teeth" are visible and a well-defined corrosion boundary does not exist. It appears almost as if the starch structure is loosened and the enzyme can penetrate, rather than acting strictly at a surface as in the case of barley and higher-amylopectin starch.

The Nature of the Residual Rings

It was necessary to learn if the shells remaining after acid treatment were composed of starch. Accordingly, treated granules were thoroughly washed and incubated with either α-amylase or β-amylase solutions for 24 hours. Results using waxy maize material are shown in Figs. 18 and 19, representing α- and β-amylase treatments respectively. Before enzyme action the granules corresponded to that of Fig. 17. It is seen that little residue remains after α-amylase treatment, so that acid resistant shells must be composed almost entirely of starch, or at any rate of glucose chains. There is an appreciable residue remaining after β-amylase treatment but it is disorganized and clear rings are missing. This enzyme must therefore be capable of hydrolyzing much of the residue from acid treatment, and of breaking up the organization of the remaining molecules. Similar results have been obtained with normal maize and barley material.

Relation of Rings to Environment

If one shell is laid down per day, as Meyer (24) originally proposed, then before a granule is mature it should be composed of as many shells as it is days old. It was therefore of interest to lintnerize granules harvested at various stages between flowering and seed maturity to see if such a correlation could be established. It is realized that a precise measurement cannot be made as the exact age of starch granules could not be known. It is known, however, that the first granules appear in the barley endosperm at about the sixth day after anthesis (21), and it is reasonable to assume that the first-formed granules will be the largest found during development. On this basis then the largest granules found in a preparation may be said to have been initiated at six days after anthesis. Difficulty in counting the number of shells per granule is also encountered, as, if a section does not pass through the center of the granule or close to it, it may not contain all the rings actually present. An extensive and carefully controlled investigation is thus called for to study the relationship of shell number and granule age. However preliminary results have been obtained in
the present study. Fig. 22 shows granules harvested nine days after anthesis, thus probably three days old, and then acid treated. Up to three rings are present. Similarly where granules were harvested at 12 days (6 days old) they were found to have up to 6-7 rings; and at 18 days after anthesis (12 days old) they were found to have up to 10 rings. It is clear that these results are of a preliminary nature, but they do favor the hypothesis that one shell is formed per day.

As Bakhuyzen's (6) work studying the effect of constant environment on shell formation in starch granules has not been repeated with endosperm starch, it was desired to use the techniques of the present study to settle this problem. Granules from plants grown under conditions of constant light and temperature, and practically constant humidity, were treated with α-amylase or lintnerized. In Fig. 20 is a typical result of α-amylase attack on such a granule. Most notable are the smooth exposed faces where enzyme corrosion has occurred, and the complete absence of "teeth". By comparison with normal granules which invariably have well-defined "teeth" after attack, it would seem that shell formation was absent or abnormal in constant environment material. In Fig. 21 is shown the result of acid treatment on such granules. Considerable breakdown of the starch has taken place, but there is no sign of an organized ring structure except at the very periphery. Lastly, intact granules previously stained with KMnO₄ were investigated, but in those sections viewed no ring structure was seen.

These observations show that under constant environmental conditions shells are not formed, or are greatly modified so that they cannot be observed by the methods employed here. The results therefore confirm the findings of Bakhuyzen (6), but conflict with those referred to in the introduction where objects of study have been potato or Pellonia granules. Perhaps the rings observed in the light microscope are different in nature to those observed in the electron microscope, or perhaps the factors controlling shell formation in cereal endosperm granules and potato granules are fundamentally different. This problem calls for further investigation. In the present work a brief examination was made of the effect of acid treatment on potato granules. In contrast to cereal starches, potato granules require extremely long treatments with

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Fig. 18. A lintnerized (see Fig. 17) waxy maize granule after treatment with α-amylase. ×7000.
Fig. 19. A lintnerized waxy maize granule after treatment with β-amylase. ×7000.
Fig. 20. Pattern of α-amylase corrosion of barley starch granules from plants grown in constant environment. Exposed surfaces are smooth. ×7000.
Fig. 21. Barley starch granules from constant environment plants show no ring structure after acid treatment. ×7000.
Fig. 22. Barley starch granules harvested nine days after anthesis (granules approximately three days old) and then acid treated. Up to three rings are present. ×6000.
Fig. 23. Potato starch granules after acid treatment (7.5 % HCl for six months), showing the closely spaced rings. ×10,000.
7.5% acid before a ring structure becomes evident, and then it is not as clearly defined. In Fig. 23 is seen a section from a granule treated for six months, and rings are only just distinguishable. What is notable is that rings here are spaced as for cereal starch at the rate of 3–4 per micron, and it is obvious that these could not be seen in the light microscope. These preliminary results therefore suggest that the question must be opened as to whether rings seen in the light microscope correspond to the shells demonstrated by the techniques of the present study.

DISCUSSION

An interesting observation made with barley endosperm proplastids is that they normally develop only one starch granule, which stands in contrast to those of oats and rice, and to the many published findings made with proplastids of meristematic plant tissues. No evidence was found of any special organized point at which starch deposition first occurs in a proplastid, but rather a granule appears to be initiated at any point in the plastid stroma. The most noticeable difference between barley proplastids, which form one granule, and oat proplastids, which form many, is in respect of the tubular invaginations of the inner membrane. In the case of barley they are numerous but short, while in that of oats they are few but very long and clearly defined. It was seen that with barley the granule lies free in the stroma relatively remote from the invaginations, while with oats a granule may be in very close association with them. It may be that the invaginations play some part in initiation of granules, or it may simply be that granule initiation depends on primer sites, which differ in number between barley and oat proplastids. It has been found that occasionally compound granules are initiated in barley endosperm cells, and it has been observed that the tubular invaginations of the proplastids resembled those of oats rather than those in the normal barley proplastid.

That tubuli persist in the barley amyloplast during granule growth and that starch granula in oat plastids are frequently seen in section to be in close association with invaginations, suggest that these structures also play some part in granule growth. Two suggestions could be made: first, that invaginations are involved in conduction of starch precursors; and second, that they are involved with starch-synthesizing enzymes. If sugar or sugar phosphate molecules met with greater resistance to penetration at the inner membrane than the outer, it could be understood that an increase in area of the inner would increase substrate supply inside the plastid. There seems however no a priori reason for so thinking. Furthermore the tubuli in the developing barley plastid appear to be separated from the inner plastid membrane. The possibility that they are involved with starch-synthesizing enzymes must therefore be considered.
It is still not certain that phosphorylase is an important starch-synthesizing enzyme in vivo (39), but its occurrence in plant tissues and its undoubted activity in starch synthesis in vitro warrants its consideration. The question of phosphorylase location in the plant cell is controversial. The histochemical method of Yin and Sun reveals it to be present only in plastids (43), while after homogenization of plant tissues it has been found active in the final centrifuge supernatant (37). It has therefore been suggested that, while originally confined to plastids, the enzyme is readily released following slight damage to these bodies. Again it can be argued that phosphorylase is free in the cytoplasm, but starch molecules can only be crystallized and build granules inside the plastids. It is difficult to conceive of the large starch molecules passing through a plastid membrane after extra-plastid synthesis. In the present work preparations were found from time to time with a plastid membrane tangentially sectioned, exposing a considerable surface area. No pores could be distinguished, so that any present must have been amicroscopic. It would therefore appear most likely that starch-synthesizing enzymes are located inside the plastid. It is now interesting to consider the possibility of these enzymes being arrayed on the tubular invaginations of the inner membrane, by analogy with the cristae of the mitochondria. If this were so, then in the case of oat proplastids active centers of starch synthesis would be carried into the stroma, and could explain the formation of several to many granula in each plastid. In Fig. 2, where a granule is seen to lie between two invaginations, its growth is elongated in a parallel direction. In barley proplastids, where invaginations do not penetrate far into the stroma, the whole plastid inner surface, as it were, would be the synthesizing center supplying a common granule. In contrast to this theory of a specific location of enzymes stand the theoretical considerations of Frey-Wyssling (14) who proposed an anti-parallel molecular crystallization in the granule, requiring synthesis in situ, and so soluble enzymes in the plastid.

The location of tubuli in the developing plastid provides further evidence of their influence on starch synthesis. It has been described how the tubuli in the large barley plastids derive from membrane invaginations, and how they are oriented around the equatorial region of the oblate spheroid plastid. It can be understood from the shape of the starch granule that it grows approximately twice as rapidly in this equatorial plane as it does toward the poles. Therefore the tubuli are in association with the granule in the region of its most rapid growth. An anomaly is found in the apparent inhibition of starch deposition in the immediate vicinity of the tubuli (Fig. 3). This could be due to space limitations. By contrast the small starch granules in the barley endosperm may be considered. These develop in the same cells as the large granules, and basically in the same plastid, but they are spherical in shape, and the surrounding stroma contains no tubuli. Also maize granules tend to a spherical shape, and no tubuli are present. Then it is remembered that in oat pro-
plastids granule growth occurs most rapidly in the vicinity of a tubular invagination. These facts taken together indicate that the tubuli of the large barley amyloplast play an important role in starch deposition, possibly by being a site of enzyme action.

It has been shown that with large barley and wheat granules enzyme attack occurs most readily in the equatorial plane. A central crack (Medianspalte) was long ago described by Nägeli (30) for such cereal starch granules, and more recently studied by Melchior and Feuerberg (22). It is evident that the starch in this region of the granule has special properties, possibly the molecules being not as tightly packed as in other regions. The presence of an equatorial groove during granule growth shows that starch deposition in this medial region is abnormal, but it cannot be learned if the molecular packing is more open. Possibly non-starch material is entrapped here during starch crystallization, thus causing the structure to be more open and susceptible to splitting and enzyme attack.

The development of the small granules in the barley endosperm at about two weeks after anthesis, and thereafter, poses a very interesting problem. New large-type granules are formed up to this time but not later, while no small-type granules are formed before this time (21). It is likely that initiation of large-type granules in the region of the endosperm from which tissue was normally taken in the present study (see Methods) was completed within the first eight days or so after anthesis, and that initiation of large-type granules up to 14 days takes place in the younger cells towards the endosperm periphery. Thus in the cells under study a certain number of large-type granules grow steadily until about two weeks after anthesis, and then suddenly the plastid forms new granules and the membranes start proliferating into the cytoplasm, budding off vesicles containing small-type granules. May and Buttrose (21) considered the possible factors responsible for small granule initiation and concluded that space limitation was the most likely. Carbohydrates for starch synthesis are present in excess amounts, and presumably enzymes are in ample supply. Now the endosperm has a limited number of cells and they can only grow in volume to a fixed extent. If, furthermore, large-type granules have a definite shape, a time may come when, owing to their rapid growth, space in the cell becomes limiting, and starch molecules are produced more rapidly than they can be deposited on the granule surface. It is indeed seen in the light microscope that at two weeks after anthesis the cells are densely filled with large granules, with little space in between. It could be that under such conditions small granules form under the plastid membrane, which then proliferates to liberate them into the cytoplasm. As all granules continue to grow slowly in size there will occur continually more space which may be occupied by small granules. This will take place as long as carbohydrate substrate supply is in excess. There is good evidence that when this supply is reduced the production of small granules falls off, as when barley plants are shaded.
or grown at low light intensity (9) their number is greatly reduced. The number of large-type granules initiated in proplastids appears however to be genetically fixed, as it does not vary significantly with different shading and mineral deficiency treatments, which affect the total amount of starch laid down, and the number of small-type granules (8).

At this point the number of shells found in granules must be considered. In mature large-type granules approximately 10 distinct shells can be observed, and evidence has been produced that one shell is formed per day. As the granules under study were initiated at about six days after anthesis, it would appear that shells were laid down until 16 days after anthesis, roughly corresponding to the time at which small-type granules first appeared. From a study of the growth of large-type barley granules, May and Buttrose (21) predicted a shell structure based on the assumption of one shell per day, a granule having up to 16 shells. From their diagram it can be seen that shells predicted up to 16 days were spaced roughly as found in the present study by direct observation, while the remaining eight shells were spaced very closely at the periphery at the rate of about eight per micron. During these last eight days of growth it was thus calculated that the granule grew little in diameter, although, owing to its large volume, a considerable amount of starch could be added for little increase in diameter. That these predicted eight shells were not observed may be due to a fault in calculation of the number of days over which the granules grew; or to their closeness not allowing of a differential corrosion becoming visible; or to the starch deposition after 16 days being so reduced in a radial direction that differences giving rise to shells could not exert their effect during such deposition. The prediction of eight closely-spaced outer shells receives support from the phosphorus-deficient material (Fig. 16) which had eight such rings contained in the outer half-micron. As shells at the poles of the barley granule will be spaced closer than at the equator, these rings could correspond almost exactly with those predicted. At all events it appears that large-type granules grow by a regular deposition of one shell per day until about 16 days after anthesis, after which time possible further growth is very slow and any daily shell formation ill-defined.

As shells in the barley granule are deposited one per day and are not found in granules developing in constant environment, Meyer's (24) theory to explain their origin must again be considered. A day-night alternation does not affect phosphorylase activity and so, assuming this enzyme to be important in starch synthesis, it is most likely that variations in carbohydrate supply from day to night give rise to shells. It is generally thought that during the day carbohydrate products of photosynthesis are in high supply and a dense packing of starch molecules results, while during the night the supply becomes low and packing looser. It would be expected that the fall-off in supply at the end of a day would be gradual, and the increase in supply
at the beginning of the next day likewise gradual. On these grounds the concentration of starch precursor would not be expected to change abruptly, nor packing density to show a sudden gradation. However the results of refractive index measurements (13) and of enzyme corrosion patterns (Fig. 14) indicate a sudden discontinuous change in the starch substance at the junction of two shells, and a continuous regular change in nature across each shell. From the refractive index measurements there can be no doubt that the change observed is due to a variation in water content, and thus the density of packing of starch molecules. The reservation must be made, however, that rings in potato granules may not be of the same nature as those representing daily shells in the barley granule. It is considered very unlikely that substrate supply falls in a fashion parallel to refractive index curves, from an initial suddenly-attained high level to a minimum during a 24-hour period, and therefore it must be proposed that factors other than mere substrate supply control density of packing. Possibly a certain concentration of starch molecules must be built up at the beginning of each day before a sudden crystallization takes place, resulting in a discontinuous change in packing. At all events factors responsible must also be dependent on environmental conditions, as when these are constant density of packing is without variation across the barley granule.

It is manifest that shell formation does not depend on differences in location of branched and unbranched molecules, as it is most distinctly developed in waxy maize granules which contain only amylopectin. It has been found (42) that amylose/amylopectin ratio remains constant in starch of wheat plants grown under different day lengths, suggesting that it would also be normal in constant environment when no shells develop. Further, it has been found with maize starch that amylose and amylopectin are produced simultaneously, and not one during the day and the other at night (11). The older theories of mixed crystals of the two molecules are therefore substantiated, though results have been obtained suggesting that little if any amylose occurs in the crystalline regions of native starches (26). However, in the present case we may consider that in non-waxy granules the two molecular types are intimately mixed across a shell. Where branched molecules alone are present, as in waxy maize, acid hydrolyzes a portion of each shell relatively rapidly to soluble products. However with increasing amylose contents the process becomes progressively slower, and the shells less clearly defined, indicating that amylose molecules have a pronounced effect on packing and crystallization of the starch.

The meaning of the acid hydrolysis pattern must now be considered. It has been suggested (7) that when carbohydrate supply is rich, retrogradation of the molecules laid down at this time will be high and attack by acid reduced. Kerr (16) also suggested this, and that in the presence of acid further hydrogen bonds would develop resulting in extremely resistant portions. Kerr (17) obtained evidence that during acid hydro-
lysis amylopectin is preferentially attacked, amylose probably forming stable resistant complexes with additional hydrogen bonds forming and linking these molecules to segments of amylopectin. The degree of polymerization of amylopectin drops very rapidly at the beginning of acid treatment, while that of amylose remains unchanged till much later. He suggests that branched molecules are attacked first at the most vulnerable points, these possibly being the amorphous regions of the granule through which the gigantic branched molecules pass as they extend from one crystallite region to another. The residue remaining after acid treatment is strongly birefringent, approaching that of the native granule in intensity, which means that it is very highly crystallized and that the portions removed during treatment were probably amorphous or not strongly crystallized. It has been found chemically (16) that the residue consists of chains of approximately 25 glucose residues containing little or no branching.

The observation made in the present work that shells are less readily made evident by acid treatment as the amylose content increases, are in favor of the views put forward by Kerr. It could be that with increasing amylose the highly organized crystallite regions extend in size, so that amorphous regions, susceptible to acid hydrolysis, are correspondingly reduced in extent.

The confusing observation that the barley granule center is very susceptible to enzyme but not to acid attack must be mentioned again. It is seen that enzyme attacks most readily in the water-rich or loosely packed regions of the shells, and accordingly it would be suggested that the granule center was composed of loosely packed molecules. There is evidence from appearance in the light microscope (35) and indirect measurement (21) that starch granules increase in density during growth, and as they grow by apposition it is concluded that the central oldest portion is less dense and thus more loosely packed than the outer regions. It would thus be expected that acid could readily penetrate and hydrolyze the central core. That it does not do so may be due to a higher amylose level in the center. It was noted that the center of waxy maize granules was readily dissolved by acid, and while the central core of such granules often contains some amylose (20) it is almost certainly a great deal less than in barley.

If the residue after acid treatment consists of unbranched glucose chains of about 25 residues, it would be expected that \(\beta\)-amylase would be capable of hydrolyzing them completely. This enzyme is apparently able to penetrate and partially hydrolyze the residue but not completely. Alpha amylase, however, effects a complete hydrolysis. These results then, though admittedly somewhat crude, suggest that linkages other than 1:4 may exist in the residue.

The residual shells following acid treatment have a relatively constant width of 0.05–0.1 \(\mu\), while the intervening spaces vary widely in width from granule center
to periphery (Fig. 17). The residue therefore corresponds to the dark bands observed in intact granules after KMnO₄ staining, and thus probably represents the inner portion of a shell. This could mean that at the beginning of each daily period throughout granule development there occurs the deposition of a constant width of starch molecules in a highly crystallized state, followed during the rest of the daily period by a larger or smaller width of molecules in a more amorphous state. No physiological reason can be given for a constancy in width of highly crystallized regions, and at this stage it seems more likely that differences in molecular size or type must be responsible. It is interesting that the width of residual shells corresponds roughly to the length of an amylose molecule, but as they are most clearly seen in amylose-free granules, this molecular type cannot be responsible. That amylopectin can crystallize so well over such a length indicates that mixed crystals with amylose could easily occur.

REFERENCES

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Es konnte ferner gezeigt werden, dass jeden Tag eine neue Lage gebildet wird, während unter konstanten Bedingungen eine Schichtung ausbleibt.
CURRICULUM VITAE

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