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An experimentally-based predictive model for the separation of amylopectin subunits during starch gelatinization

Neil J. Atkin, Rukmal M. Abeysekera*, Sophia L. Cheng, Anthony W. Robards

Institute for Applied Biology, University of York, Heslington, P.O. Box 373, York YO10 5YW, UK

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Abstract

Starch granules in excess water gelatinize in a predictable manner, where radial and tangential expansion displaces amylopectin units from granular rings as a gradual centrifugal process concluding at the granular surface. Amylopectin appears as ellipsoidal particles in waxy maize starch granules in excess water at ambient temperature and swell to spherical particles on heat application. During swelling, the amylopectin units in waxy starches remain aligned along granular rings prior to displacement. This transient stage is absent in amylose-containing starches, suggesting packing of amylose between amylopectin molecules interferes with intermolecular amylopectin interactions. Loss of granular crystalline order, as determined by loss of birefringence, is a result of displacement of amylopectin units from granular rings. The granular surface encloses the structural amylose and amylopectin and modulates gelatinization endotherms. Chemical modifications of starches were also shown to alter the sequence of molecular events during gelatinization to give predictable changes.

Keywords: Starch; Microscopy; Gelatinization; Amylopectin subunits

1. Introduction

The processing and manipulation of starch is vital for its use in many industries. For most countries starch is a major resource and plays a key role in many economies. Within the EU 6.6 million tons of starch were produced in 1992/93 (Lillford, 1997). The estimated world production totalled 27.5 million tons in the same period illustrating the global importance of starch (Lillford, 1997). Although traditionally thought of mainly as a food ingredient, the use of starch in non-food materials is increasing as more innovative technologies take advantage of the unique properties offered by this renewable resource (Bachelor et al., 1996a).

Biologically, starch is the major form of carbohydrate storage for most higher plants. It is naturally found in the plant as compact, semi-crystalline granules. These granules are used by the plant for both long- and short-term storage of the sugars generated by photosynthesis. For industrial use starch must be extracted from raw material in plant tissue. There are several mechanical procedures for starch extraction, generally specific to the origin of the starch (Bachelor et al., 1996b). After extraction, starches can be chemically modified in order to enhance the properties demanded by industry, such as stability in solution or viscosity levels. Industry would ideally like to obtain starch of consistent, well-defined specification and properties. Several types of modification can be performed such as esterification, cross-linking and oxidation. The effects of these modifications on starch structure are not always clearly understood.

The modified starches are subsequently utilised in various industrial processings. Often a major step in starch processing involves heating in excess water. The heating process (e.g. batch cooking, jet cooking and extrusion) induces many physical changes within the granule giving rise to gelatinization: the process of loss of crystallinity and simultaneous swelling of granules (Blanshard, 1987). In order to understand the physical changes during gelatinization, the molecular events within the granule during processing conditions must be unveiled. There are very few in-depth studies of macromolecular events during starch gelatinization, although a vast amount of information is available on measurements of these changes, as determined by a variety of physical techniques (French, 1984). Nevertheless, the molecular events of gelatinization are still not clearly understood, representing an important gap in knowledge in relation to starch processing. An improved understanding of these events is crucial in order to optimise

* To whom all correspondence should be addressed. Tel. +44 1904 433028; fax: +44 1904 432860; e-mail rma2@york.ac.uk

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processing conditions and reduce costs, both during starch modification as well as during industrial utilisation. The aim of this study is therefore to examine and understand the mechanisms by which these macromolecular changes take place within the starch granule, during gelatinization, using direct visual evidence obtained by a variety of microscopy techniques.

2. Experimental section

2.1. Materials

The starches used in this study, the industrial source and the amylopectin content are given in Fig. 1.

2.2. Methods

2.2.1. Preparation of starch solutions

A 5% (w/v) aqueous solution of each starch source was heated using a water bath to 50, 60, 65, 70, 80, 90 and 100°C with constant agitation using a magnetic stirrer. The solutions were incubated at each temperature setting for 15 min. An aliquot of the hot samples at the set temperatures was examined immediately by light microscopy while a second aliquot was then removed into a glass test tube and stored immediately on ice. The rate of heating between 30 and 70°C was approximately 4°C min^{-1}, between 70 and 90°C was 2°C min^{-1} and 90 and 100°C was 1°C min^{-1}. In addition to the above, Hylon VII starch was prepared in an autoclave at 2×10^5 Pa at 125°C for 1 h. Prior to heating, all starch samples were also examined after 15 min in water at ambient temperature. Deionised water was used for the preparation of all starch solutions.

2.2.2. Light microscopy of fresh starch samples

Cooled samples from each temperature step were placed on glass microscope slides at room temperature. Samples were immediately examined, first unstained between crossed polarised filters to determine the birefriengence and, secondly, after iodine vapour or Lugol's iodine solution staining to localise amylose and amylopectin by bright field or phase contrast microscopy. Hot samples were examined unstained to confirm that cooling does not affect structural changes within the starch granules. All the recorded micrographs in this manuscript are from cooled samples due to the ease of their handling. For light microscopy, a Zeiss photomicroscope and a Nikon Diaphot-TMD inverted microscope were used. Light micrographs were recorded on TMax 100 film.

2.2.3. Scanning electron microscopy

Starch granules were dried in a desiccator and sputter coated with palladium/gold and examined using a Hitachi S-2400 scanning electron microscope. Electron micrographs were recorded on TMax 100 film.

2.2.4. Fixation and embedding of starch samples for light and transmission electron microscopy

Starch samples were prepared as described above and centrifuged at 1000 r.p.m. using an Eppendorf 3200 mini-centrifuge. The pellets of granules were fixed in 2.0% glutaraldehyde in ethanol, dehydrated in ethanol and embedded in LR White resin (London Resin Co.) or Nanoplast resin (R. Bachhuber, W. Germany).

2.2.5. Light microscopy of embedded starch: periodic acid Schiff's (PAS) reaction

Thick 200 nm sections of the embedded starch material were prepared using a Reichert OMU3 ultramicrotome. The sections were reacted with the PAS reagent to reveal the distribution of the total amylose and amylopectin within the granules. In parallel, a control reaction was carried out using 2,4-dinitrophenyl-hydrazine to block the binding of the Schiff's reagent to aldehyde groups in order to determine specificity of the PAS reaction.

2.2.6. Transmission electron microscopy of embedded starch

Thin (60–90 nm) sections were prepared with a Reichert OMU3 ultramicrotome and mounted on copper grids for staining with uranyl acetate and lead citrate (Hayat, 1989). Sections were also mounted on nickel grids for staining with the thiocarbohydrazide/silver proteinate (Roland, 1978) method to visualise the total carbohydrate within the granules. The stained sections were examined using a Jeol 1200EX transmission electron microscope at 80 kV and micrographs were recorded on Kodak 4489 film.

3. Results

3.1. Gelatinization of starches

The process of starch gelatinization involves the loss of crystallinity and the simultaneous swelling of granules. The study presented here is an in-depth examination of the macromolecular events that occur during this process of gelatinization up until the granular swelling is completed. The temperature range of maximum granular swelling in the nine starches examined in this study is given in Fig. 1. The maximum swelling temperatures were determined by visualisation of starch granules during heating in excess water by use of iodine staining on samples cooled from high temperatures. The temperature of maximum swelling was the temperature at which the granular swelling was complete and after which there was granular disruption.

Each starch sample at a specific temperature contained granules exhibiting more than one macromolecular organisation. There are also some granules in which the macromolecular organisation is not visible. However, there is a predominantly progressive pattern

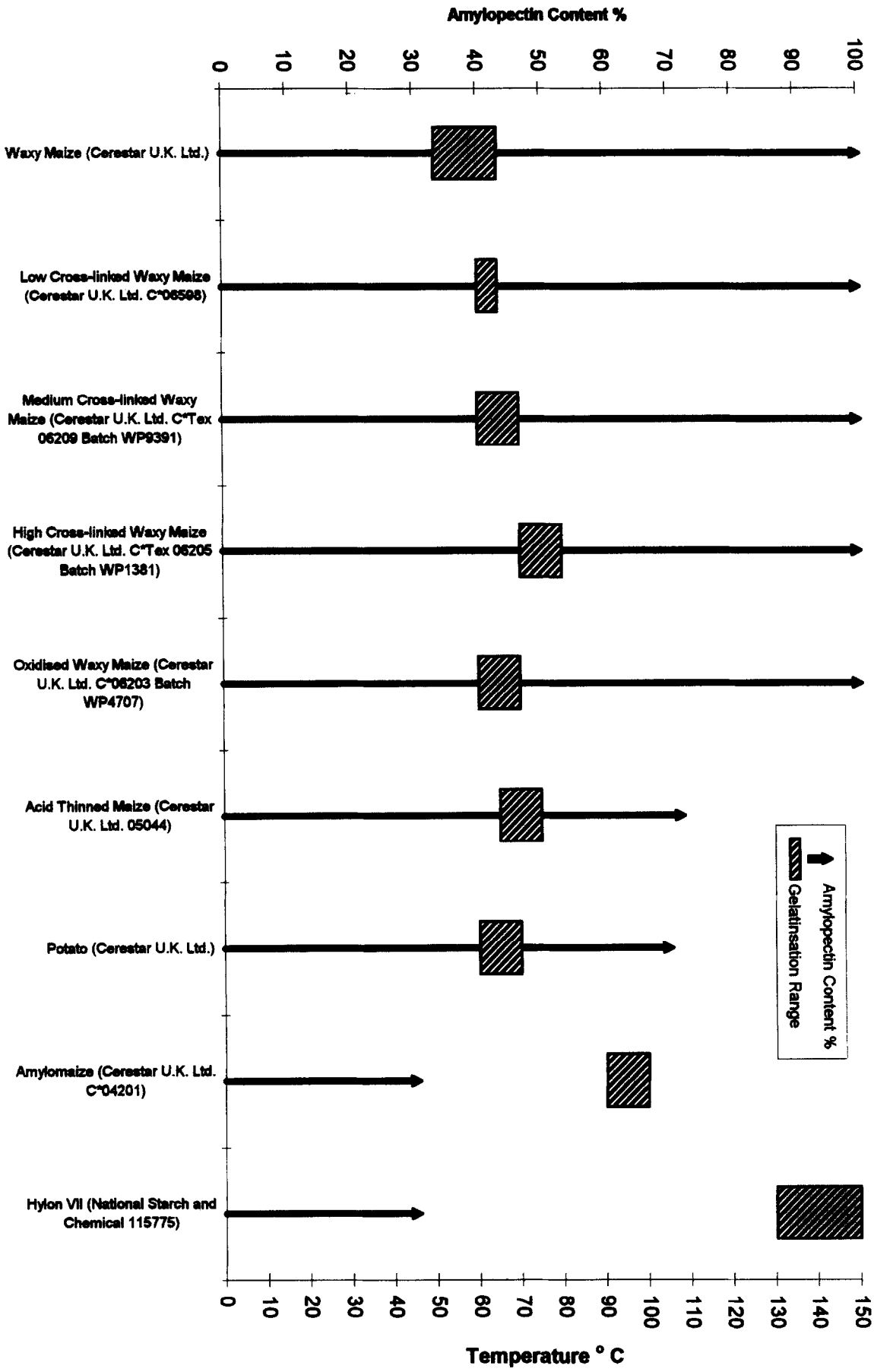


Fig. 1. The starches used in this study, the industrial source, the gelatinisation temperatures and the apparent amylopectin content.

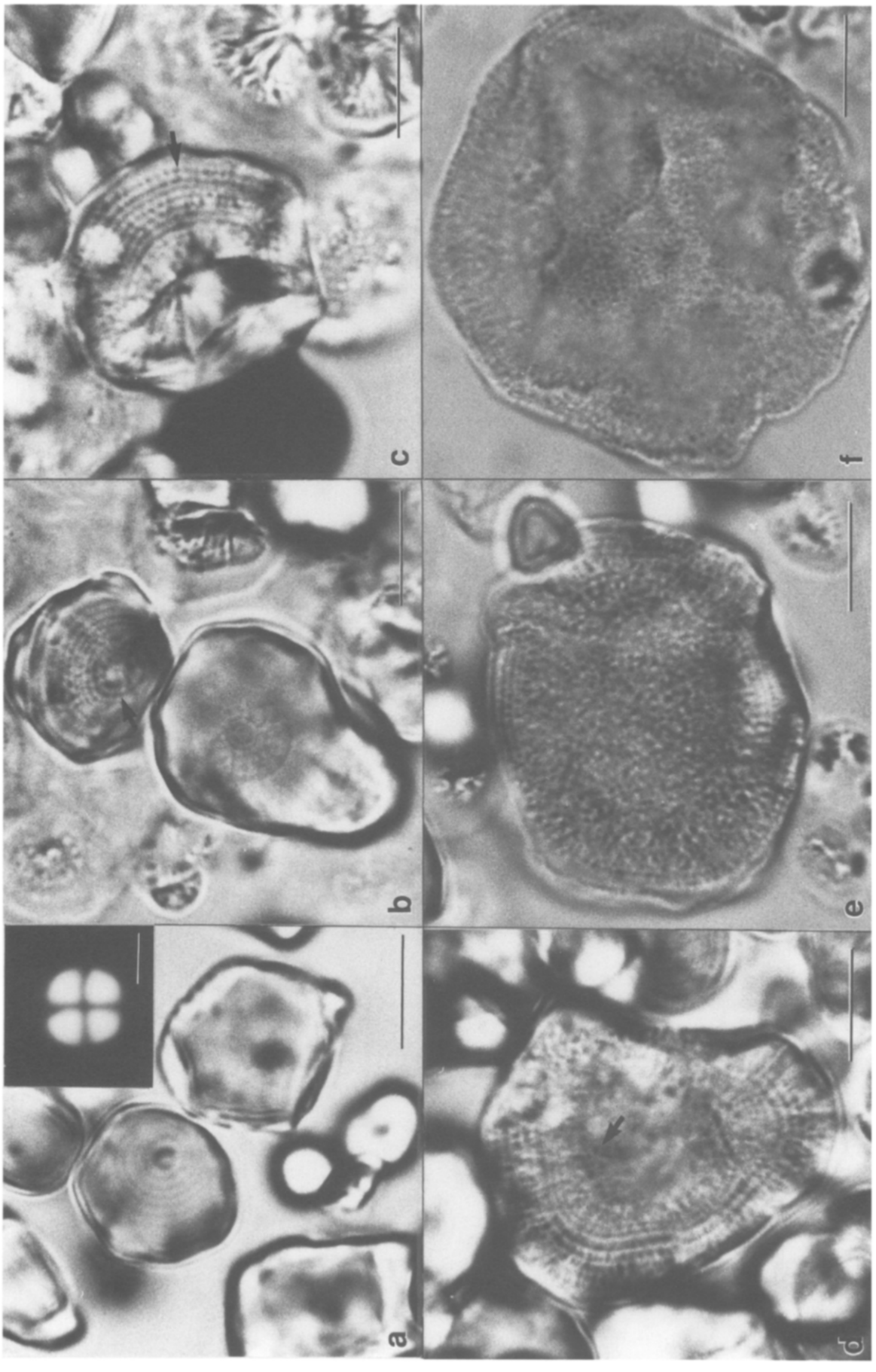


Fig. 2. Macromolecular events during gelatinisation of native waxy maize starch visualised by iodine vapour staining of cooled fresh starch solutions. Bars = 10 μm. (*a*) A starch granule in excess water at ambient temperature illustrating clearly defined rings. The inset is a micrograph of the birefringence of a granule containing defined rings. (*b*) A granule at 60°C, illustrating the break-up of rings to 400 nm particles (arrow). (*c*) A partially swollen granule at 60°C, illustrating further break-up of rings into particles (arrow) where the particles are still aligned at the position of the original rings. (*d*) Further increase in granule swelling at 60°C disrupts the organisation of the 400 nm particles in the centre (arrow) of the granules while those at the periphery remain aligned on rings. (*e*) The majority of the 400 nm particles in the centre of the granule are randomly organised while two to three intact rings are still present at the granular periphery. (*f*) On complete swelling, the intact granule comprises a 'bag' of randomly orientated 400 nm particles.

of macromolecular changes during gelatinization and the observations reported below are representations of the dominant changes that occur during progressive steps of gelatinization.

3.2. Gelatinization of native waxy maize starch

The sequence of macromolecular events that occurs in native waxy maize (*ca.* 100% amylopectin) starch during granular swelling, as observed by light microscopy, is illustrated in Fig. 2 and summarised below. This sequence of events provides a basis against which the other starches examined in this study can be compared.

As the compact dry granule is exposed to excess water at ambient temperature, radial swelling due to hydration increases granule diameter up to 80% (a six-fold volume increase). Rings become visible in granules at this stage (Fig. 2*a*) and birefriengence remains unperturbed as indicated by a clear Maltese cross (Fig. 2*a*, inset). We have used the term 'granular ring' to describe these iodine-positive rings observed in starch granules in favour of 'growth ring' because the evidence present in literature to relate granular rings to growth is limited and inconclusive (Evers, 1979).

A temperature increase up to 65°C causes further granular swelling, increasing the granular size to a maximum of approximately 200% its original diameter (a 25-fold volume increase). During the initial stages of swelling, further radial expansion occurs where the spaces between granular rings continue to expand and granular rings breakdown homogeneously into particles of approximately 400 nm size (Fig. 2*b–f*). The breakdown of rings to 400 nm particles commences from the centre of the granules and progresses towards the surface of the granules. At first appearance, the 400 nm particles remain aligned in same position as the rings from which they were derived.

As tangential swelling begins, the particles are displaced from their original position in the rings and thus appear randomly distributed within the swollen granule (Fig. 2*e, f*). If granule surfaces are intact on full swelling, they are seen as turgid sacs of 400 nm particles. Examined hot solutions of starch also contained the 400 nm particles.

There appears to be a maximum force or a load the granule surface can bear during gelatinization prior to rupture. This corresponds to an approximate granular volume increase of about 25-fold relative to the volume of the dry granule. Application of temperatures above 70°C, plastically deforms the surface of the waxy maize granule, rupturing the surface and releasing the granular contents. Once the contents are released, the granule surface, often called granular ghosts (Obanni and BeMiller, 1996), collapses and frequently remains intact in solution (see Fig. 3).

3.3. Gelatinization of modified maize starches

3.3.1. Cross-linked waxy maize

Three starches with low, medium and high degree of cross-linking between glucan chains were examined. The starches are acetylated distarch adipates commercially produced by Cerestar UK Ltd. All three starch types followed the same pattern of macromolecular changes during the granular swelling process as observed in the native waxy maize: the breakdown of rings, the appearance of 400 nm particles initially aligned on rings, and finally the random dispersal of particles (Fig. 3*a–d*). This activity again proceeded from granular centre towards the periphery. Granular rings at ambient temperatures were, however, not as prominent as in native waxy maize and the extent of swelling was restricted (Fig. 1). The collapsed starch shells remaining after gelatinization (Fig. 3*b, d*) were also more distinctive than the collapsed native waxy maize shells (Fig. 3*d*, inset). The appearance of thick coiled grooves and furrows on the surface of collapsed cross-linked granule shells are a prominent feature of these starches.

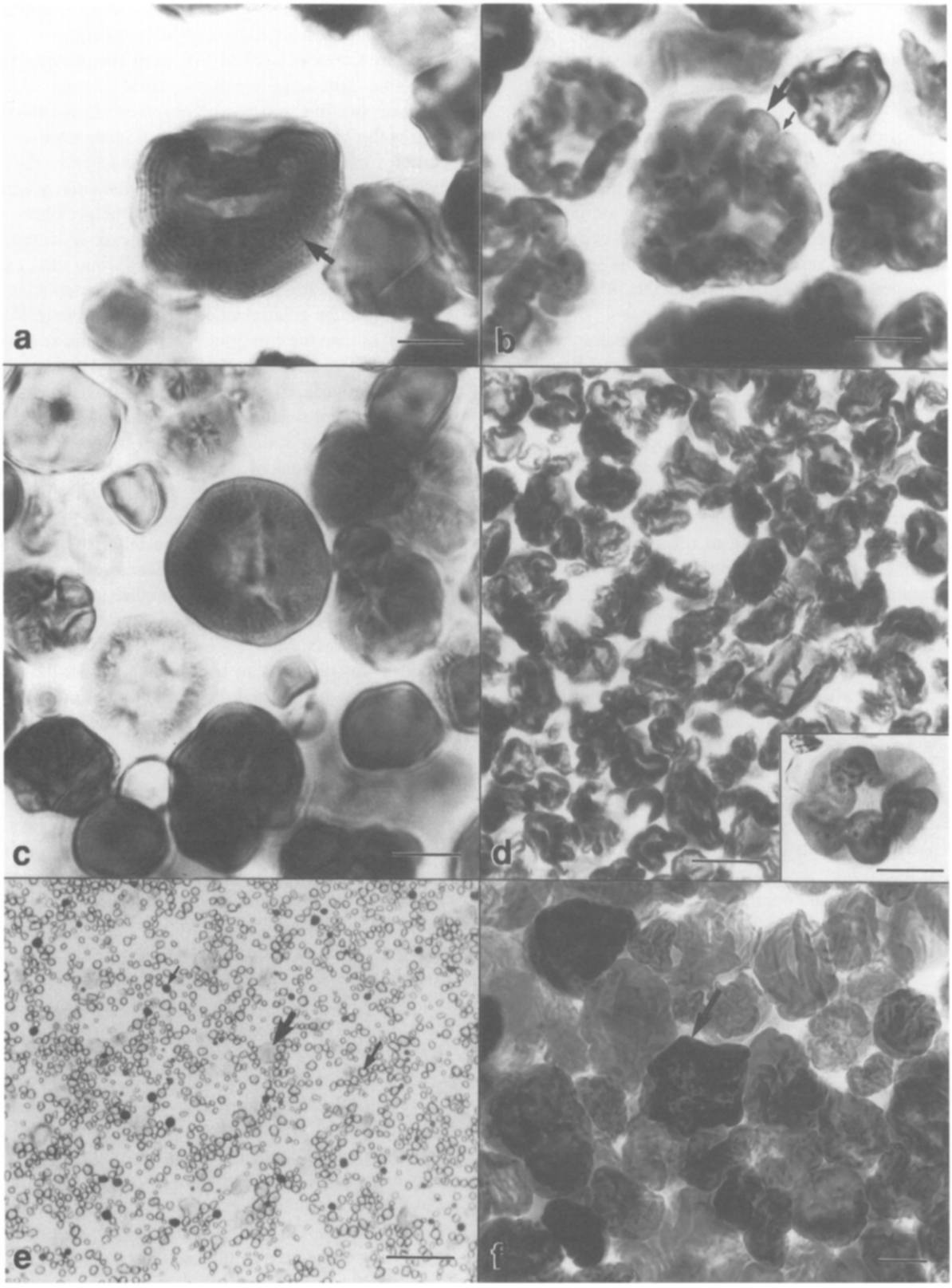
Iodine staining of the cross-linked starches identified two different populations of starches (Fig. 3*e, f*): approximately 10% of the starch granules were stained intensely blue/black while the rest of the granules were stained reddish brown and showed swelling behaviour similar to that described above for native waxy maize. The swelling of the blue starches occurred only at a temperature above 90°C, and at 100°C they appeared as sacs full of 400 nm particles (Fig. 3*f*) stained intensely blue by iodine. This sub-population of starches was observed to be common for all the examined native waxy maize and its derivatives and acid-thinned maize. This was not observed in potato and Hylon VII starches.

3.3.2. Acid-thinned maize

These starches are prepared commercially by treatment in dilute acid. Again, swelling causes breakdown of the granular rings into 400 nm particles (Fig. 4*a*) progressing from the centre of the granule towards the surface. This 400 nm particle displacement from granular rings directly coincides with the loss of birefringence from the centre of the granule towards the surface (Fig. 4*b, c*).

3.3.3. Oxidised waxy maize

The macromolecular events during gelatinization of oxidised waxy maize appeared to take two different paths. Approximately 25% of the population of swollen granules, followed the above described pattern of macromolecular dispersion: breakdown of rings to 400 nm particles progressing from granular centre towards the surface. A second population of granules, approximately 25%, exhibited the reverse pattern of macromolecular dispersion: granular rings were broken down into 400 nm particles at the granular surface first and progressed towards the granular centre (Fig. 5*a–d*). Many different focal planes of granules were examined to confirm that this reversed pattern of macromolecular dispersion was not an artifact of visualisation. The structure of the remaining 50% of the granules could not be visualised.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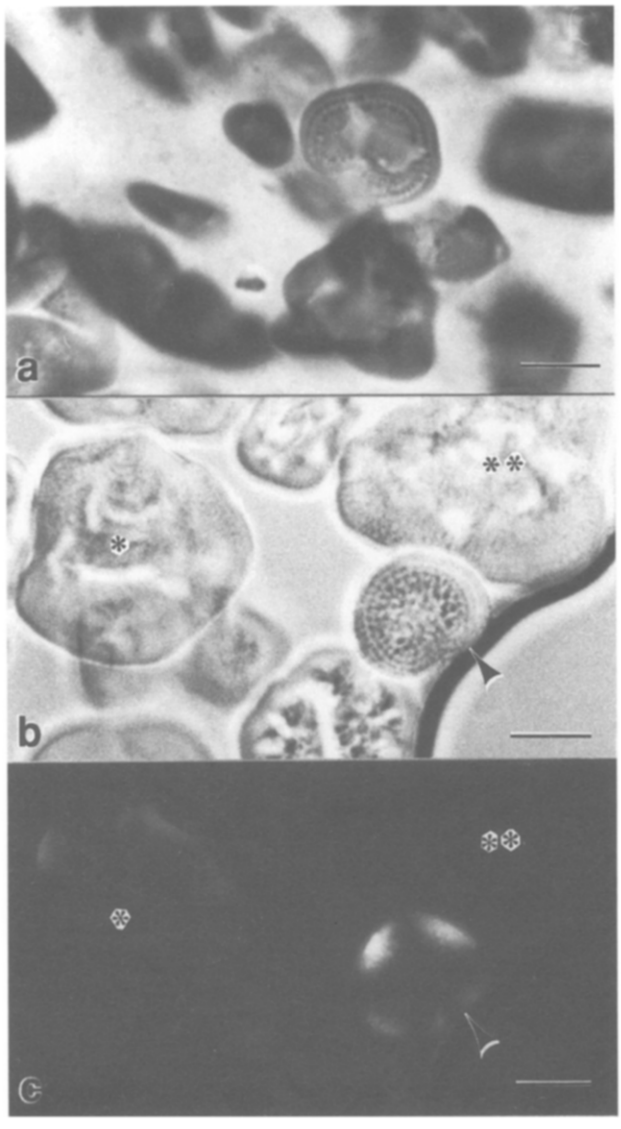


Fig. 4. Acid-thinned maize starch granules during gelatinization. Bars = 100 μm. (*a*) A partially swollen granule iodine vapour stained to illustrate the breakdown of rings into 400 nm particles commencing from granular centre. (*b*) Unstained granules examined by phase contrast optics illustrating various stages of swelling. The arrowhead points to a partially swollen granule with 400 nm particles randomly organised in the granular centre but ordered in rings near the periphery. The (*) marked granule is swollen further with only few ordered particles near the periphery. A fully swollen granule is labelled by (**) where the 400 nm particles are completely randomly arranged within the granular shell. (*c*) Same starch granules as in (*b*) but examined with polarised light. Note that there is birefringence only where the 400 nm particles are distinguishable and organised along the granular rings.

3.4. Gelatinization of potato starch

Commercially available potato starch contain oval shaped granules ranging in size from 10 to 100 μm. The macromolecular events during granular swelling followed the same predominant pattern of granular ring dispersion into 400 nm particles (Fig. 6*a–e*) as shown for waxy maize granules. However, visualisation of the transient stage where the 400 nm particles are organised in rings is difficult in potato starch. The arrow in Fig. 6*b*, points to a rare sighting.

3.5. Gelatinization of amylomaize

Granular rings were present when hydrated at ambient temperatures but, as in potato starch, the 400 nm particles appear in random organisation. No micrographs are presented.

3.6. Gelatinization of Hylon VII starch

Hylon VII starches are irregular in shape. Part of the population is polygonal and the other part are elongated rods, sometimes up to 100 μm in length (Fig. 7*a*). Swelling is limited in this starch. A temperature as high as 130°C in excess water is required before any swelling is observed. Granular rings were not observed. Surrounded by a thick outer sheath, Hylon VII does, however, give rise to the 400 nm particles in the granular centres (Fig. 7*b*, *c*).

3.7. Total carbohydrate distribution in starch granules during gelatinization as determined by light microscopy

The periodic acid Schiff's (PAS) reaction involves the formation of aldehyde groups in glycol rings by oxidation, and the subsequent binding of the Schiff's reagent to the aldehyde groups gives rise to a bright pink coloration. This staining reaction does not discriminate between amylose and amylopectin or between different types of carbohydrate. Fig. 8*a–e* shows micrographs of waxy maize and potato starch during gelatinization, sectioned and stained by the PAS reaction. The microtomed sections are approximately 200 nm thick and can therefore pass through the centres of granules. Images of sectioned native waxy maize (Fig. 8*a*, *b*) stained by PAS reagent support the observations made of the same material when intact and stained by iodine: disruption of granular rings to particles with initial dis-

Fig. 3. Cross-linked waxy maize starch granules stained by Lugol's iodine. (*a*) A partially swollen low cross-linked waxy maize granule at 50°C, illustrating the breakdown of rings into 400 nm particles (arrow). Bar = 10 μm. (*b*) Ruptured and empty 'starch ghosts' of low cross-linked starch at 60°C. Note the collapsed ridges (large arrows) and furrows (small arrows) on the surface. Bar = 10 μm. (*c*) A partially swollen highly cross-linked starch granule at 60°C illustrating particles in the granular centre and rings at the periphery. Bar = 10 μm. (*d*) The 'starch ghosts' of highly cross-linked waxy maize granules at 100°C. Bar = 50 μm. The inset is a micrograph of a native waxy maize granular ghost. Bar = 10 μm. (*e*) Low cross-linked waxy maize granules in excess water at ambient temperature. Note the three different types of granules. Large arrow points to highly swollen lightly red stained granules. Medium arrow points to the dominant form of partially swollen red granules. The small arrow points to dark blue stained small granules. Bar = 100 μm. (*f*) The arrow points to a swollen dark blue highly cross-linked granule at 100°C. Note the intensely stained (dark blue) particles inside the granule. Bar = 20 μm.

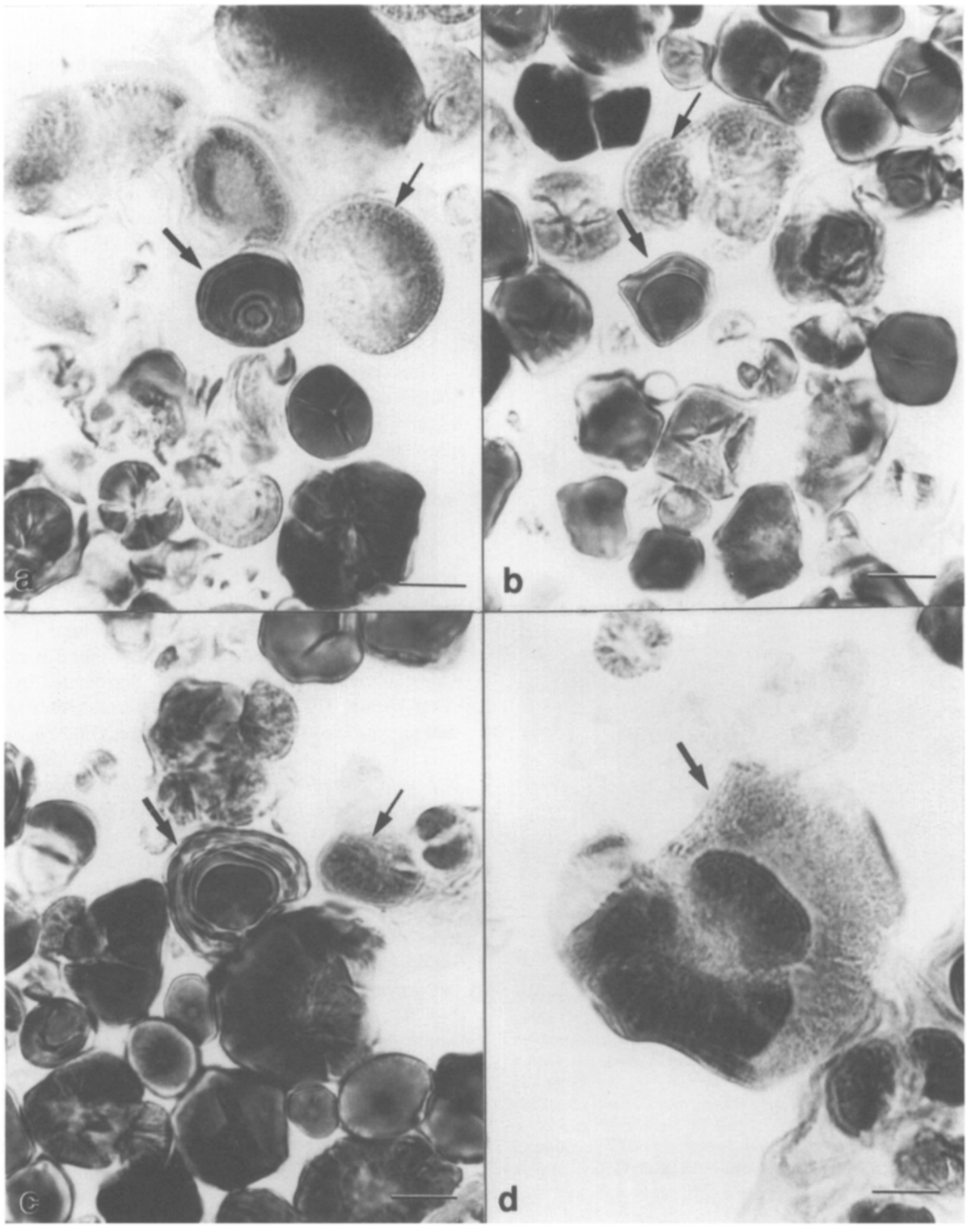


Fig. 5. Oxidised waxy maize starch illustrating the gelatinization path. Small arrows in all micrographs point to examples of granules where swelling results in break down of rings to particles from the centre of granules. Large arrows point to granules where the breakdown of rings into particles commence from the surface of the granules. The description below illustrates this pathway. Bars = 10 μm. (*a*) The large arrow points to a granule displaying intact rings. (*b*) The large arrow points to a granule where the rings near the granular surface are beginning to appear swollen. (*c*) The large arrow again points to a granule where most of the outer rings appear displaced. (*d*) The large arrow points to a swollen granule where the swollen outer rings are broken into 400 nm particles.

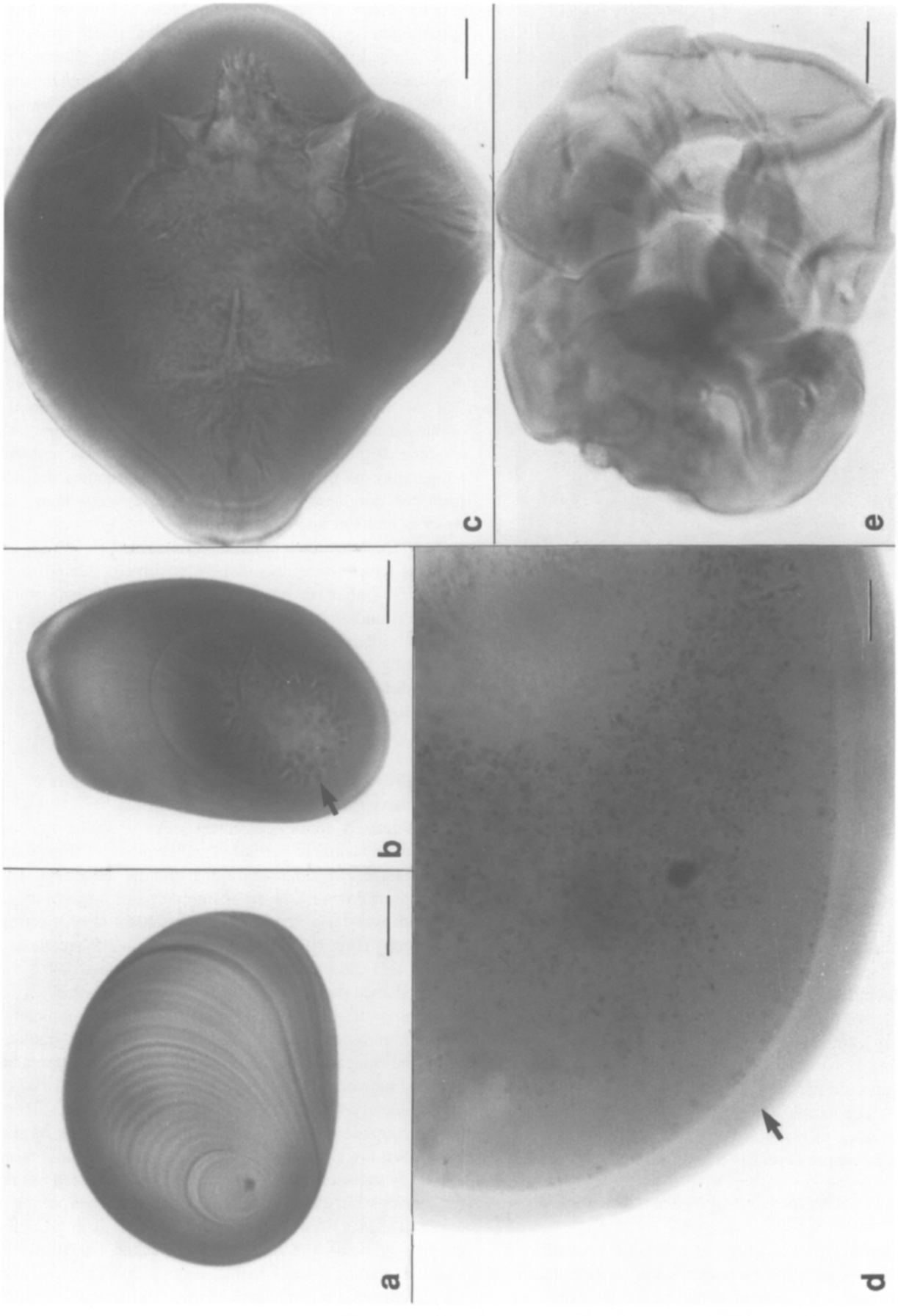


Fig. 6. Gelatinization events of potato starch visualised by iodine vapour staining. Bars = 10 μm. (*a*) A hydrated granule at ambient temperature illustrating granular rings. (*b*) A hydrated heated granule where rings are beginning to breakdown into 400 nm particles from the granular centre. The arrow points to few particles organised along a granular ring. (*c*) A well swollen granule where rings appear only near the periphery of the granule. (*d*) A part of a fully swollen, but intact granule which consists of a thick surface layer (arrow) encasing irregularly organised 400 nm particles. (e) A punctured, collapsed starch granule surface where the contents have been released.

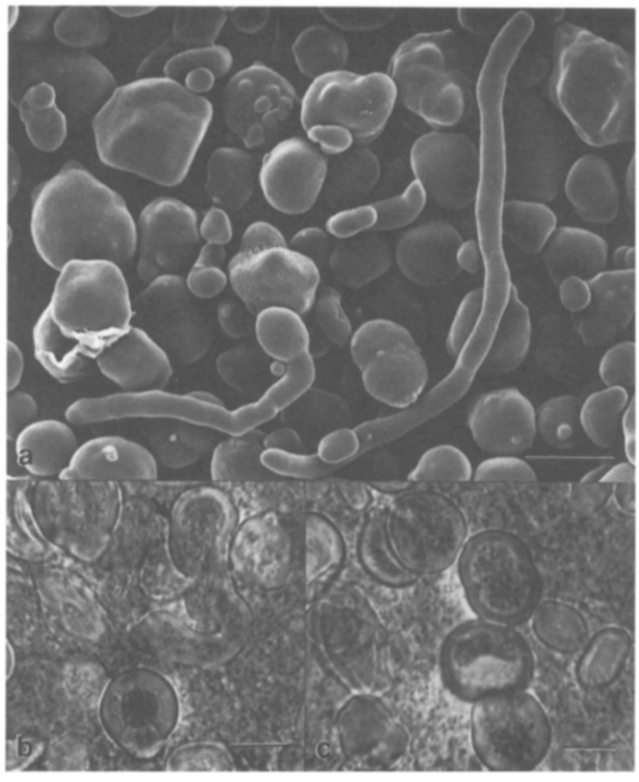


Fig. 7. Hylon VII starch. Bars = 10 μm. (*a*) A scanning electron micrograph of Hylon VII starch granules. The granules are predominantly polygonal in shape but some times also display elongated shapes. (*b*) Hylon VII at 100°C stained by iodine. Both polygonal (small arrow) and elongated (large arrow) partially swollen granules appear to contain a thick unstructured surface layer and randomly distributed particles in the centre of the granules. (*c*) Other granules at 100°C show thinning of this surface layer and the enlargement of the central region containing randomly organised particles.

appearance of ring organisation from the centre of the granule. The same macromolecular organisation during gelatinization was observed by staining sections of potato starch granules by PAS (Fig. 8*c–e*), subsequently leaving behind the collapsed surface of the potato starch granule after granular disruption and content release. The PAS staining of Hylon VII starch is also consistent with the iodine staining observations (results not shown). Sectioning of the granules also allows visualisation of distinct cracks in the centre of some starch granules (see Fig. 8*a*) and granular disintegration, in contrast to complete swelling during gelatinization, of other granules (Fig. 8*b*) presumably previously damaged.

The use of 2,4-dinitrophenyl-hydrazine did not affect the PAS reaction, illustrating that the PAS staining observed above was specific and indicates the total carbohydrate in the granules. The iodine staining (Figs. 2, and 6), however, gave more clarity to granular rings and particle structures than the PAS staining (Fig. 8).

3.8. Molecular events during starch granule swelling

3.8.1. Dry and hydrated starches at ambient temperature

Examination of structure by transmission electron microscopy is necessary to determine the molecular events of gelatinization within starch granules. Thin sections, approximately 60–90 nm, of embedded starch granules were stained with uranyl acetate and lead citrate and examined at high resolution to determine the organisation of starch polymers (amylose and amylopectin) within the granules.

While rings are not clearly defined in dry waxy maize granules (Fig. 9*a*), they become clear in granules immersed in water at ambient temperature (Fig. 9*b–d*). The granular rings are positively stained by uranyl acetate and lead citrate. The width of the rings varies between 200 and 400 nm and is dependent on the extent of deviation of the plane of section from the vertical axis of the rings. The rings appear to be constructed of 'ellipsoidal' units with 'acute-angled ends' approximately 60–70 nm wide but spanning the width of the rings (see Fig. 9*b*, *c*, *d*). While the centre of the granules in dry starches appear structureless (Fig. 9*a*) that of the hydrated starches consisted of randomly organised fine fibrils (Fig. 9*c*, inset). The entire structure of some of the hydrated waxy maize granules at ambient temperature as fibrillar (Fig. 9*e*). These granules did not form the dominant population of native waxy maize in water at ambient temperature.

Amylomaize starch granules immersed in water at ambient temperature also display positively stained rings (Fig. 9*f*). Unlike in native waxy maize, distinct lateral units are undefined along the granular rings, although a radial fibrillar appearance is prominent (Fig. 9*f*).

No ring structures are visible in Hylon VII granules, although fine radial fibrils can be detected homogeneously within the granules (Fig. 9*g*).

3.8.2. Granular swelling above ambient temperature in native waxy maize starch

Well-defined continuous intact rings of radial fibrillar nature were visible in partially swollen granules (Fig. 10*a*). At this stage, the ellipsoidal units become undefined. The lateral breakdown of the rings into particles during further swelling was also detectable by transmission electron microscopy (Fig. 10*b*). The particles in Fig. 10*b* are closely packed and the particle periphery is ill defined. Note the wedge shaped particles in Fig. 10*b*.

Staining of thin sections of most of the completely-swollen granules was difficult. Other swollen granules stain and appear to be filled with a randomly orientated fibrillar network (Fig. 11*a*, *b*) with fibre thickness of approximately 4 nm (Fig. 11*b*) and finer (Fig. 11*a*). Thiocarbohydrazide/silver proteinate staining (Fig. 11*c*, *d* and Fig. 12*a*) for carbohydrate gives structural details very similar to those observed by uranyl acetate and lead citrate for transmission electron microscopy. Intact rings having a compact radial fibrillar appearance (Fig. 11*c*) in partially swollen granules and more loosely packed fine fibrils in swollen granules were visible (Fig. 11*d*). Thin sections also depicted heavily stained but separated particles

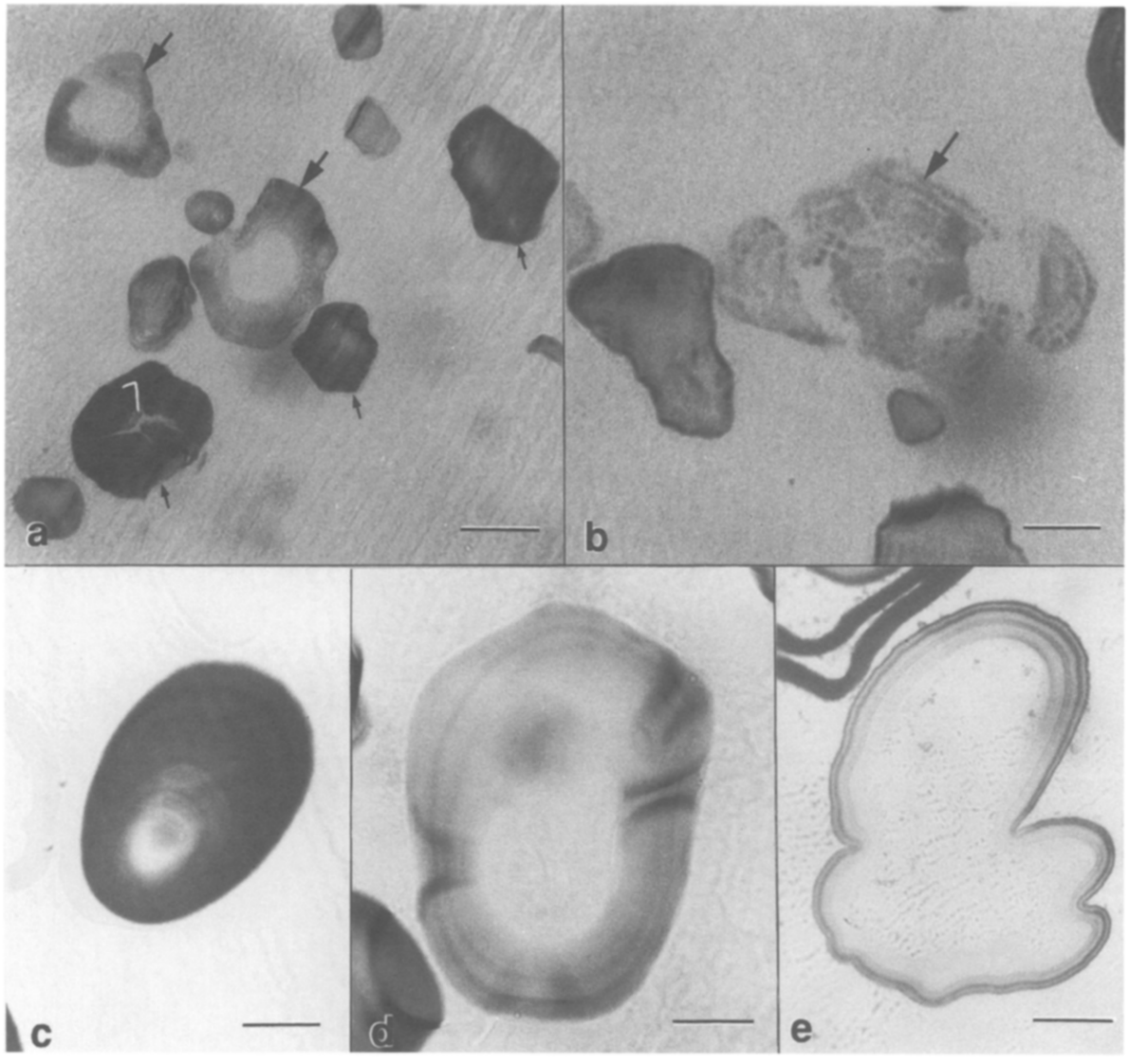


Fig. 8. The periodic acid Schiff's staining reaction on embedded and sectioned waxy maize (*a*, *b*) and potato starch (*c*–*e*) granules during gelatinization. Bars = 10 μm. (*a*) The small arrows point to partially swollen granules intensely stained by PAS. Note the cracks (arrowhead) in a granule centre. The large arrows point to expanded granules containing particles. (*b*) The arrow points to a swollen granule containing 400 nm particles. (*c*) An unswollen potato starch granule. (*d*) A partially swollen potato starch granule where the centre of the granule is void and disintegrated. (*e*) The collapsed potato starch granular surface after rupture and content release.

(Fig. 12*a*) corresponding to the 400 nm particles observed in well swollen granules by light microscopy (Fig. 12*b*).

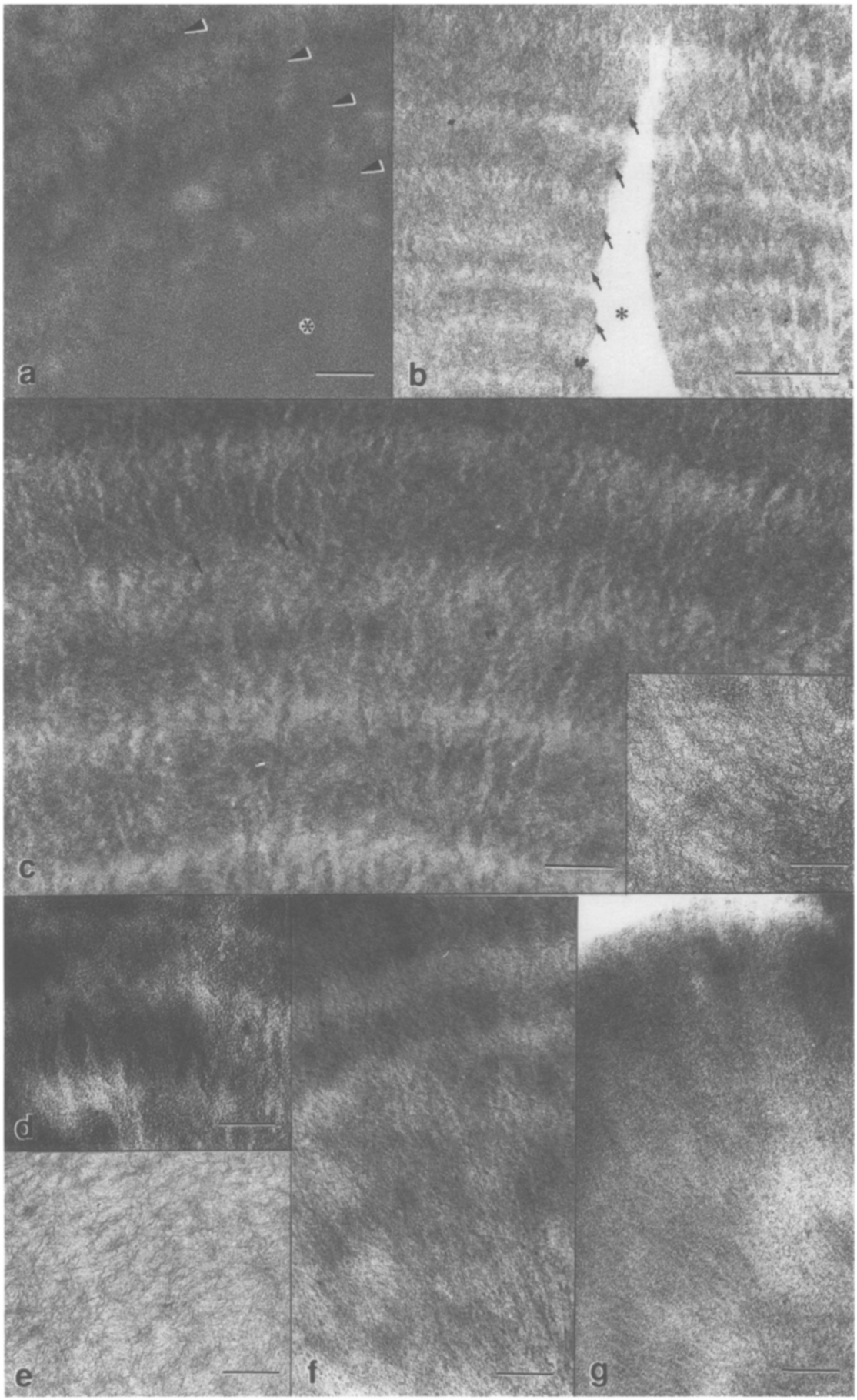
4. Discussion

4.1. Gelatinization of starch

Gelatinization of starch is an entropy-driven phenomenon (Blanshard, 1987). Biphasic endotherms, named G and M, have been commonly observed during starch gelatinization under variable water conditions (see Blanshard, 1987, for an extensive review). These endotherms are believed to represent 'gelatinization' and 'melting' of starch. The G endotherm alone is observed in excess water; the G and M endotherms together at intermediate water levels and the M endotherm alone in limited water. The molecular events underpinning these endotherms during starch gelatinization are still speculative.

Blanshard, 1987 concludes that in excess water, where only the G endotherm is observed, swelling of the amorphous region (space between granular rings) assists disruption of crystallites and dispersion of amylopectin. In limited water, where only the M endotherm is observed, starch gelatinization is believed to proceed via conventional crystallite melting. Both radial and tangential granular expansion (Bowler et al., 1980; Cameron and Donald, 1993) have been implicated in contributing to the loss of granular crystallinity and double helix dissociation during gelatinization.

Our observations, in the study presented here,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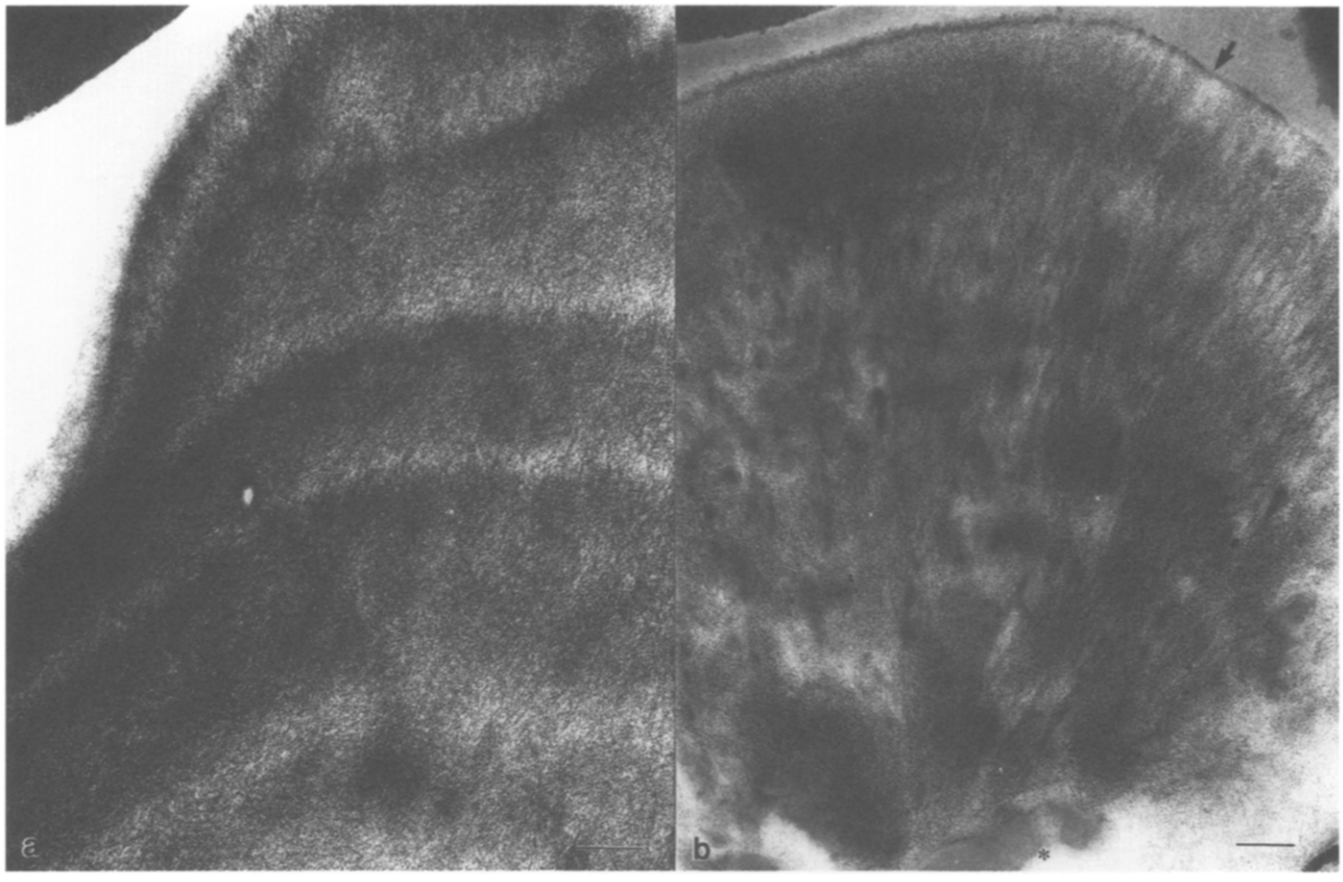

Fig. 10. Transmission electron micrographs of heated waxy maize starch sections stained with uranyl acetate and lead citrate. Bars = 200 nm. (*a*) A partially swollen granule, at 50°C, with well-defined intact granular rings. (*b*) A swollen granule, at 60°C, with particles aligned in close proximity to each other. The (*) labels the granular centre and the arrow points to the granular surface.

provide direct visual evidence of the molecular events of gelatinization as well as providing direct evidence for radial and tangential granular expansion during starch gelatinization in excess water. The molecular events of gelatinization are not as straightforward as indicated by data from physical techniques such as D.S.C. (differential scanning calorimetry), SAXS (small-angle X-ray scattering), WAXS (wide-angle X-ray scattering), light scattering, rheology and N.M.R. (deWilligen, 1976; Blanshard, 1987; Cook and Gidley, 1992; Cameron and Donald, 1993; Liu and Lelievre, 1993; Jenkins and Donald, 1997). These techniques predict a predominant single entropy-driven event of double helix dissociation during starch gelatinization in excess water. Direct observation is a necessity in order to understand fully the process of starch gelatinization. Indirect physical methods alone give an incomplete picture.

A summary of the macromolecular events during starch gelatinization in excess water, deduced from our observations, is presented in Fig. 13. Principally, three types of granular swelling occur in excess water, to different extents, in all the examined starches in this study and are discussed below.

We have described radial expansion occurring where a visible increase in radial dimensions of either the spaces between granular rings or the dimensions of the granular rings themselves have increased. Similarly, tangential expansion is described as occurring where, a clear visible expansion of the granular rings occurred tangentially. While a predominant radial or tangential expansion may occur at any one time, we cannot exclude the possibility of minor contribution from both forces of expansion concurrently.

Fig. 9. Transmission electron micrographs of thin sections of dry (*a*) and hydrated (*b–g*) starch granules at ambient temperature after uranyl acetate and lead citrate staining. (*a*) A dry waxy maize granule. The (*) is the granular centre and the arrowheads point to faint outlines of rings. Bar = 200 nm. (*b*) A hydrated waxy maize granule. The (*) is a granular crack. The arrows point to granular rings positively stained by uranyl acetate and lead citrate. Bar = 500 nm. (*c*) A high magnification view of the waxy maize granular rings containing 'ellipsoidal' units (arrows). The inset is a micrograph from the centre of the waxy maize granule. Note the fine fibrillar structures. Bar = 200 nm. (*d*) Details of waxy maize granular rings. Note the 'ellipsoidal' units along the rings. Bar = 200 nm. (*e*) Fine fibres within some waxy maize granules. Bar = 200 nm. (*f*) Granular rings in amylomaize starch granules. Bar = 200 nm. (*g*) Hylon VII starch structure. Bar = 200 nm.

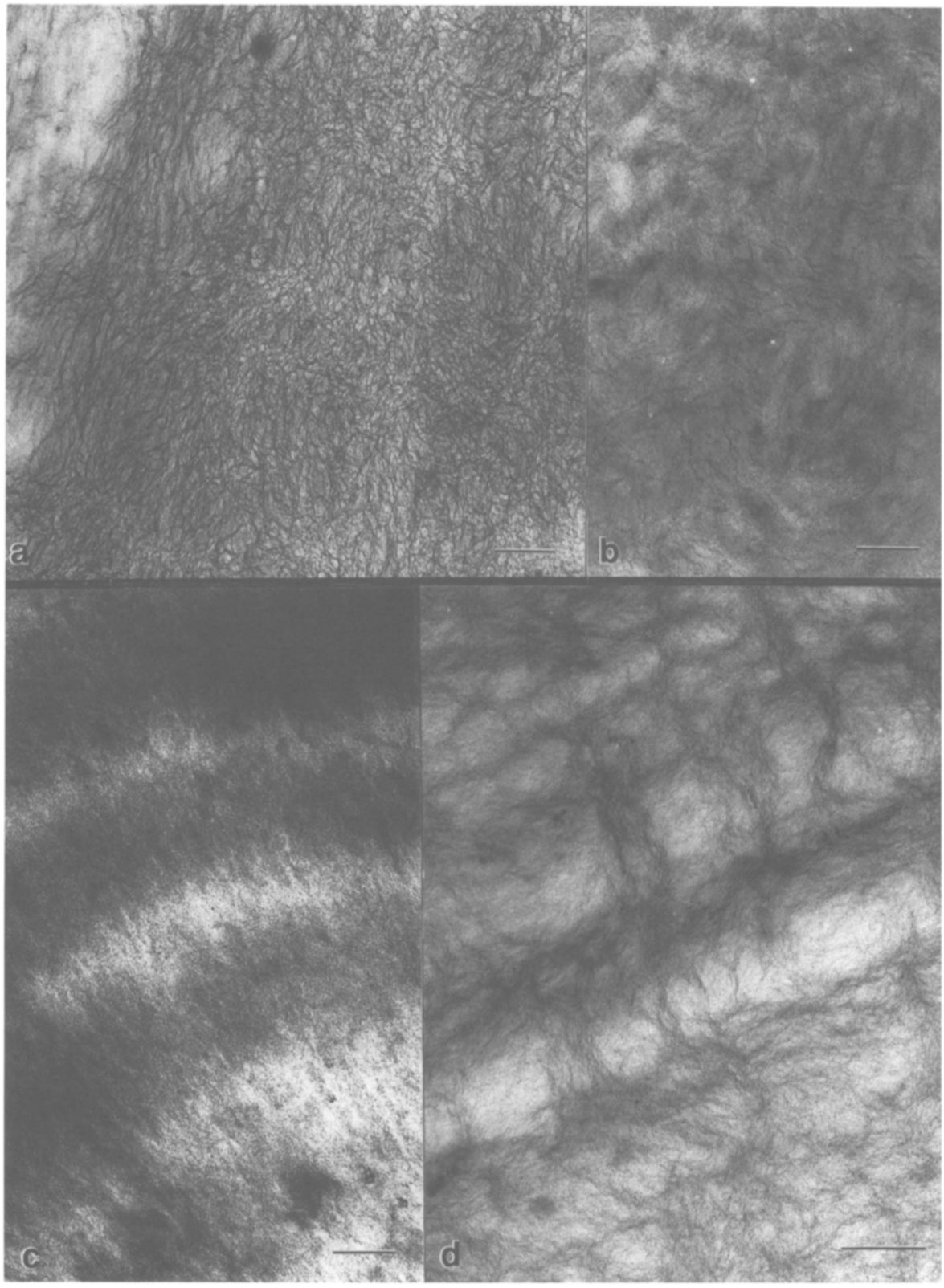

Fig. 11. Transmission electron micrographs of heated (60°C) waxy maize starch granules stained with uranyl acetate and lead citrate (*a*, *b*) or thiocarbohydrazide silver proteinate (*c*, *d*). Bars = 200 nm. (*a*) Note the fine fibrillar network. (*b*) A partially ordered fibrillar network. (*c*) Intact granular rings displaying radial fibrillar nature. (*d*) Fine fibres in ring organisation.

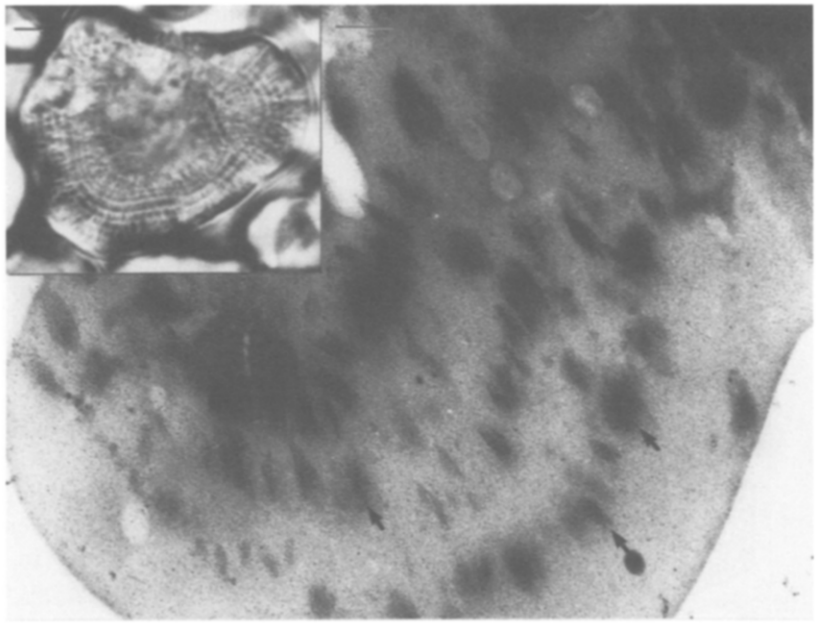

Fig. 12. An electron micrograph of a thin section of a swollen waxy maize granule stained by thiocarbohydrazide silver proteinate. Arrows point to separated particles arranged in rings. Note that the granular background is also lightly stained. Bar = 200 nm. The inset is a light micrograph stained with iodine illustrating the same level of granular swelling. Bar = 2 μm.

4.2. Passive radial expansion at ambient temperature and pressure

Granular swelling first involves radial expansion as a result of water uptake into the granule at ambient temperature and pressure. The spaces between granular rings (the amorphous regions) are filled, widening the ring-to-ring space and enhancing the microscopical ring contrast. This increases the granular diameter to approximately 80%, and the granular volume six-fold relative to its volume in dry native waxy maize, oxidised waxy maize, acid-thinned maize and potato starches. This swelling occurs without any energy input (e.g. heat). Here, closely packed ellipsoidal single entities of amylopectin molecules are visible by transmission electron microscopy in native waxy maize but not in the other starches examined. These entities are 200–400 nm in length, span the width of the granular rings, and are approximately 60 nm wide in waxy maize granules hydrated at ambient temperatures. The granular rings, particularly in waxy maize, must predominantly be composed of amylopectin and therefore the visualised entities by transmission electron microscopy must be units of amylopectin molecules.

4.3. Enthalpic radial expansion

Radial expansion continues at application of low heat since the spaces between granular rings continue to expand. This is also noted by Cameron and Donald (1993). The first visible change of the granular rings occur during this initial heat application where, in waxy starches and acid-thinned maize, spherical particles approximately 400 nm in diameter become clearly visible on granular rings by light microscopy. We describe these particles as amylopectin units derived from the ellipsoidal units observed in hydrated waxy maize starch granules at ambient temperature. The spherical particles were homogeneous in size (*ca.* 400 nm) in all starches examined and therefore cannot be a result of random disruption of granular rings due to swelling. We cannot exclude the possibility that there is some tangential expansion happening at this stage because of the ease of visualisation of these amylopectin units. The particles are clearly visible as separate units by light microscopy but remain closely packed and associated in granular rings. The particle alignment in granular rings was only rarely observed in potato, amylomaize or Hylon VII starches.

The granular ring associated units of amylopectin molecules in waxy starches are crystalline and birefringent. We have demonstrated that birefringence is clearly present if the 400 nm amylopectin units are ordered in rings. Because in both waxy and amylose containing starches the rings remain intact during radial swelling, the actual energy involved in molecular dissociation at this stage must be intramolecular double helical dissociation. In amylose containing starches it is possible that, if amylose is present in spaces between granular rings (the amorphous rings), that amylose would leach out of the granule at this stage as reported in other studies. This amylose will not contribute to the integrity of the amylopectin units in granular rings.

4.4. Enthalpic tangential expansion

Tangential expansion begins at low heat and completes at the end of gelatinization. Tangential expansion begins at the

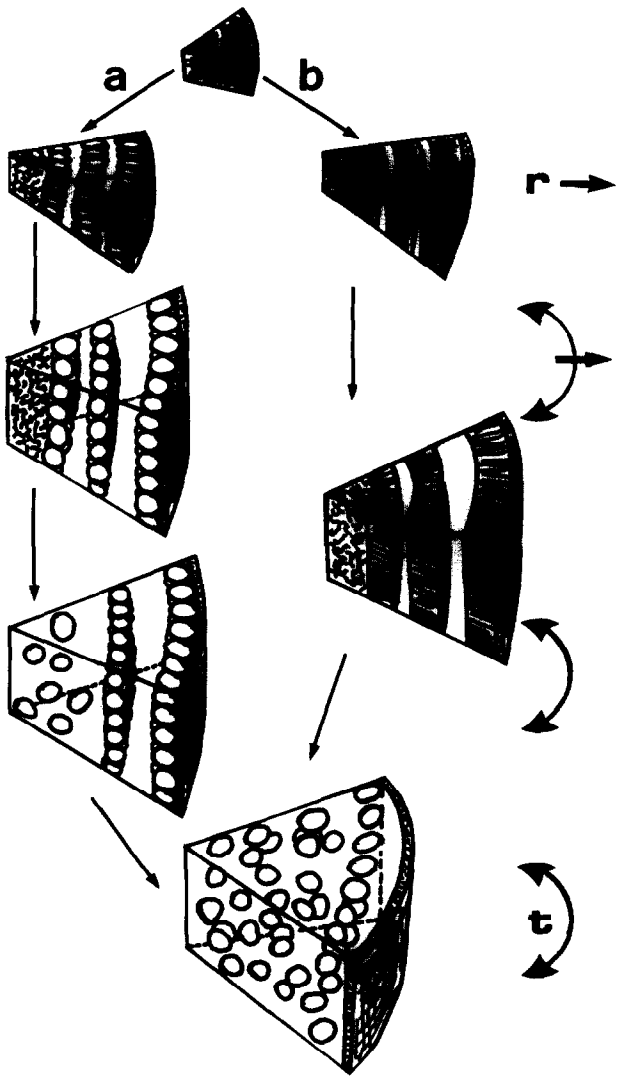

Fig. 13. A diagrammatic description of granular expansion in excess water. The large arrows to the right point to the direction of expansion: the straight arrows to radial expansion (r) and the curved arrows to tangential expansion (t). (*a*) The Molecular dispersion of waxy starches. (*b*) The molecular dispersion of amylose containing starches.

centre of the granule in all the examined starches, except the oxidised waxy maize. Therefore at any one time in any single granule, there is a gradient of tangential expansion, predominantly beginning at the granular centre and completing at the surface. Tangential expansion results in the separation of amylopectin units in granular rings and their subsequent displacement from the granular ring organisation. Granular crystalline order, as observed by birefringence in light microscopy, disappears only during this displacement and must correspond to intermolecular double helical dissociation.

Lelievre (1973), using a hot stage light microscope, examined the birefringence of starch during gelatinization under limited water conditions. But this was shown to be ineffective since birefringence was not easily followed under the conditions of high turbidity. Liu and Lelievre (1993) examined the complete loss of granular birefringence of wheat starch in excess water in relation to the G endotherm and noted that the temperature at which birefringence was completely lost from all granules was much earlier than where the D.S.C. peak ends during gelatinization. Blanshard (1987) reported examination of crystallinity of wheat starch at a stage intermediate between the G and M endotherms. It was reported that while the granular birefringence, as determined by polarised light microscopy and small-angle light scattering, was largely lost the X-ray crystallinity remained largely undisturbed, as examined by X-ray diffraction. The lack of simultaneous disappearance of granular birefringence, X-ray crystallinity and the coincidence of the endotherm peak again reflects the complexity of molecular events during gelatinization.

We were successful in observing the effect of structural changes arising from displacement of amylopectin units associated with granular order. In contrast to observations of gross changes, we studied single granules and the ensuing changes that display different stages of gelatinization. We have clearly illustrated that the loss of granular birefringence is due to the complete displacement of amylopectin units from granular rings; presumably by dissociation of intermolecular amylopectin interactions. Birefringence remains if the amylopectin units are aligned along granular rings, even when visible as separate entities (the 400 nm particles). From this we can hypothesise that birefringence reflects granular order due to intermolecular interactions between amylopectin units. Displacement of these intermolecular bonds occurs during gelatinization as a gradual centrifugal process culminating at the granular surface.

4.5. The effect of amylose on granular expansion

Waxy maize starch which contains almost pure amylopectin has a transition stage of expansion where distinct 400 nm particles appear on granular rings before their displacement from the rings. This transition stage is not frequently visible in the amylose containing starches (except acid-thinned maize starch) where the 400 nm particles are never observed to remain aligned at rings. We interpret this observation to indicate that, in waxy maize, amylopectin units form a larger number of intermolecular interactions (double helices) and therefore during granular expansion their separation is slow, leading to the ease of visibility of amylopectin unit alignment in rings. Amylose interferes with this amylopectin intermolecular interaction by isolating the amylopectin units. This may be a result of simple space filling between amylopectin molecules or by double helix formation with amylopectin molecules (Jane et al., 1992). Therefore, the formation of the 400 nm particles (separation of amylopectin units) is instantaneous in amylose containing starches and occurs at the onset of tangential swelling.

Hylon VII starch (70% amylose) contains a thick surface coating that is not disrupted even at 130°C. There is

evidence in other studies that this coating may be a complex of lysophospholipid with amylose (Seguchi, 1995), distinct from the amylose in the centre of these granules where the amylopectin/amylose organisation is similar to those in the low amylose starches studied here. In such starches an additional endotherm approximately between 110 and 120°C has been reported (Kugimiya and Donovan, 1981).

4.6. The effect of water on granular expansion

All the starches in this study were examined in excess water (5%, w/v). According to D.S.C. studies, we should only observe a single endotherm, the G endotherm, during gelatinization. In all the starches examined, the peak of this endotherm must then occur during tangential expansion where the 400 nm particles are displaced from the granular rings concurrently with loss of granular birefringence terminating at maximum granular surface expansion. This predominantly involves tangential expansion and not the initial radial expansion.

In limited water, radial expansion will be diminished and as a result also limit tangential expansion. A 'melt' or dissociation of intermolecular double helices without granular expansion at higher temperatures should be envisaged (Blanshard, 1987). At intermediate water levels, expanded and non-expanded granules with dissociated amylopectin molecules should be visible (Blanshard, 1987).

4.7. The effect of the granular surface on granular expansion

A feature that is almost completely ignored by the starch chemist is the granular surface in relation to granular expansion. This is a distinct entity that appears to be entirely independent of the rest of the granule and, unless damaged, controls both radial and tangential granular expansion and envelopes the structural amylose and amylopectin within the granule. In fact, at peak gelatinization, an unruptured granule is composed of an envelope encasing separated amylopectin units that are in motion inside the granule: a 'boil-in-the-bag'! Only a further increase of temperature will rupture this, presumably by breakdown of strong molecular cross-links that holds the granule surface together. Liu and Lelievre (1993) reported that the D.S.C. peak of starch in excess water ended at a temperature much higher than at the temperature where granular birefringence was lost completely in wheat starch. The descending slope of the D.S.C. peak, we believe, is predominantly a reflection of the energy required to rupture the granular surface which is able to remain intact after a 25-fold granular volume increase.

4.8. The effect of chemical modification on granular expansion

The tangential swelling of a proportion of starch granules in oxidised waxy maize commences at the granular surface and proceeds toward the granular centre. Oxidation introduces carbonyl and carboxyl groups into starch polymers as well as introducing depolymerisation. We interpret the reversed path of tangential swelling in oxidised waxy maize starch to indicate that the introduced anionic groups predominate at the surface of these granules at the expense of intermolecular cross-linking between amylopectin molecules, subsequently enhancing the tangential separation of amylopectin molecules from near the granular surface during gelatinization.

The radial and tangential swelling of cross-linked starches was diminished by the introduction of cross-linking between the starch chains. The introduced cross-links are probably predominantly intermolecular, particularly near the granular surface, bridging neighbouring amylopectin molecules and diminishing their expansion and separation during gelatinization.

The breakdown of starch chains to low molecular weight fragments introduced by acid thinning appears not to affect directly the radial and tangential swelling of the granular contents during gelatinization. However, very few starch shells remain after full gelatinization, perhaps again suggesting an acid effect dominating near the granular surface.

4.9. Sub-populations of starch distinguished by iodine staining

Two different populations of starch samples were distinguished by iodine staining coloration in the examined native waxy maize, waxy maize derivatives and acid-thinned maize. Approximately 10% of these starches were stained intensely blue. This population showed tangential swelling only at 90–100°C and remained stained blue during gelatinization. The blue coloration is believed to arise due to the formation of an inclusion complex by iodine in the amylose single helices. It is perplexing how entire whole granules in waxy starches could contain single helical amylose. It may be these granules that are visualised by transmission electron microscopy to be entirely filled with fine fibrils.

4.10. Use of microscopy for understanding starch gelatinization

Microscopy allows examination of single starch granules while D.S.C., N.M.R., SAXS, WAXS, light scattering, X-ray diffraction and rheology provide average data from a large number of granules. We have observed by microscopy that, at any one temperature, different granules contain different stages of expansion. While radial expansion, with or without heat, appears to reflect homogeneously throughout a single granule, tangential expansion exhibit a gradient of expansion progressing from the granular centre towards the surface during any one point of gelatinization. The slopes in the G endotherm on D.S.C. traces probably reflect all these activities.

4.11. The 400 nm particles as amylopectin units

The 400 nm particles were visualised most clearly by examination of unembedded starch granules by light microscopy without staining as well as after iodine staining. They are not retrograded starch, as the particles were present in hot solutions examined immediately as well as after cooling. The particles were visible when embedded granules were sectioned and examined by light microscopy, illustrating that they are inside the granules and not granular surface structures. The shape of the particles in swollen granules by light microscopy is spherical while that seen by transmission electron microscopy is irregular. This is because, while a particle is 400 nm, a section for transmission electron microscope is *ca.* 60–90 nm thick. Therefore depending on the plane of the section, the structure of the particle may appear irregular. The granular rings of hydrated waxy maize at ambient temperature are made of compact ellipsoidal units, 60 nm wide and spanning the granular ring and visible only by transmission electron microscopy. We interpret these units as amylopectin molecules containing undisrupted intramolecular double helices. When heat is applied, the units swell tangentially becoming spherical in shape, and increase the width from 60 to 400 nm while the height remained at 400 nm. Here the intramolecular double helices are probably disrupted but, because they remain packed in rings, intermolecular double helices must be undisturbed. The structure between these closely packed particles was unresolvable by transmission electron microscopy. All our observations suggest that these particles must be units of amylopectin molecules. The particles are discontinuous radially between granular rings as clearly visualised by light microscopy. They are ellipsoidal in shape in compact partially swollen granules and spherical in fully swollen granules. If at the point of granular rupture, units of amylopectin molecules exist as 400 nm particles, then the implications for post-gelatinization processing of starch must be re-examined carefully.

Particles in starch granules have been reported as long ago as 1957 (Nikuni and Hizukuri, 1957) and recently reviewed by Gallant et al. (1997). However, there has been no in-depth microscopical examination to understand the origin and the nature of these particles. Ellipsoidal starch granule fragments, *ca.* 800 nm long and 150 nm wide, in potato starch have been reported (Oostergetel and van Bruggen, 1993). These were obtained after treatment in 2.2 N hydrochloric acid treatment at 35°C for 16 h followed by washing and wet mashing. These fragments were described as superhelical composites of continuous left-handed helical segments of amylopectin. The ellipsoidal particles that we observed in hydrated waxy maize at ambient temperature appear discontinuous between granular rings. On heat application, they become spherical in shape.

Both the PAS staining for light microscopy and the thiocarbohydrazide/silver proteinate for transmission electron microscopy, for total carbohydrate staining, was less discriminating of the particles than iodine staining. This is not surprising since while iodine will bind to ordered amylose and amylopectin giving contrast to distinct structures, PAS and TCS will stain both ordered and disordered molecules indiscriminately. The appearance of granules in which macromolecular organisation was not visible by iodine staining can be due to the range of macromolecular order and disorder present in granules at a specific temperature.

To our knowledge, this study is the first report where untreated starch granules have been successfully examined by transmission electron microscopy. In past studies, lintnerisation where starch granules were extensively treated in acid from 24 h to 8 months (Frey-Wyssling and Buttrose, 1961; Buttrose, 1963; Gallant et al., 1972; Yamaguchi et al., 1979) wet mashing (Yamaguchi et al., 1979), dimethylsulphoxide treatment (Yamaguchi et al., 1979), sodium periodate and thiocarbohydrazide/silver proteinate (Kassenbeck, 1975, 1979), α-amylase (Gallant et al., 1972) treatment prior to fixation and embedding for transmission electron microscopy were carried out in order to obtain structural visualisation. We have used both a low viscosity acrylic resin (LR White) and a water-soluble melamine resin (Nanoplast) for embedding of starch granules only fixed in glutaraldehyde without any prior harsh treatment. We believe that the better granular structural preservation with minimum pre-treatment reported here is due to the improved infiltration of granules by the embedding resin.

The 7–10 nm periodic structures observed in chemically treated and wet mashed starch granules examined by transmission electron microscopy (Frey-Wyssling and Buttrose, 1961; Buttrose, 1963; Gallant et al., 1972; Kassenbeck, 1975, 1978; Yamaguchi et al., 1979; Oostergetel and van Bruggen, 1993) and by small-angle X-ray scattering (Jenkins et al., 1993) and wide-angle X-ray scattering (Imberty and Pérez, 1989) of native granules were not resolved in our transmission electron micrographs of intact granules. However, a minority of all granules during different stages of gelatinization contained numerous fibres, 4 nm wide and finer. These were unequivocally granular fibres and not of plant cell wall origin and may be related to the 7–10 nm periodicities observed in other studies.

If the surfaces of starch granules are intact at the time of full gelatinization, a granule can be thought of as a turgid sac containing predominantly separated mobile amylopectin units (a 'boil-in-the-bag'). The conformation of the amylopectin molecules within the fully swollen granule will depend on the rate and temperature of heating, the amount of water available, the applied pressure and the nature of industrial pre-treatments to the granule. The interpretation of the structure of an amylopectin molecule is currently based on the cluster model (Kainuma and French, 1972). The 400 nm particles observed in our study are single units of amylopectin molecules that span the width of the granular ring and possess a non-fibrillar molecular conformation. These particles may be hypothesised as single units containing composites of amylopecitn clusters.

The fibrillar structures reported here in some waxy maize starch granules could be of amylopectin in a different molecular conformational order. As mentioned earlier, the blue coloured sub-population of granules identified by iodine staining may, alternatively, be the granules containing the fibrils. Industrial contamination of an amylose starch or a grazing section through the granular surface is also possible. Fibrillar structures are also prominent in granular centres in all stages of gelatinization in waxy as well as amylose starches, again suggesting that they could be amylopectin molecules of different conformation.

5. Conclusions

Microscopic techniques have been used in the study of the gelatinization of starch granules creating a greatly improved understanding of the substantial array of published physical data. A model has been proposed for the sequence of events observed during the gelatinization of starch granules. This model illustrates the effect of the different mechanical forces of radial and tangential swelling on molecular structure of starch granules. The granule contents are surrounded by a surface layer which acts as a 'bag', initially retaining the starch structures as they breakdown. The breakdown was shown to initiate from the central hilum of the granules, continuing out towards the periphery. This breakdown results in the separation of independent 400 nm amylopectin units from the granular rings. Heating granules at rates of 1–4°C min^{-1} in ambient pressures in excess water releases these amylopectin units in all the examined starches. These particles are randomly distributed within the 'bag' created by the surface layer until the surface ruptures releasing the amylopectin units into the surrounding solution.

We have illustrated how the granular expansion is affected by several factors. The amylose content of the granule appear to decrease the intermolecular interactions between amylopectin units thus increasing the rate of breakdown of granular rings during gelatinization. The concentration of water influences the 'energy barriers' which control gelatinization. The granular surface modulates the expansion of granules, where the strength of this layer is of great importance during the later stages of gelatinization. Chemically modified granules were examined and compared with native granules. Chemical modifications give rise to predictable changes in the molecular events of gelatinization.

Industrially, gelatinization of starch is of great significance in many different processes. The importance of our model, which begins to explain in a predictive manner the macromolecular changes occurring within a single granule, is clear. A more precise understanding of the physical data can now be used to create industrial processes which have greater efficiency. The separation of granular rings into independent amylopectin units and their subsequent release has wide implications for the uses of starch as a material component.

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