

HYDROLYSIS OF NATIVE WHEAT AND CORN STARCH GRANULES BY
GLUCOAMYLASES FROM ASPERGILLUS NIGER AND RHIZOPUS NIVEUS

by

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INTRODUCTION

The significance of amylolytic enzymes in industrial and research applications is immense, with the most important industrial use being the production of glucose and fermentable sugars from starches of various sources (1).

An important group of amylases, isolated primarily from various molds, yet also found in animal tissues, is the glucose-producing glucoamylases (α -1, 4-glucan glucohydrolase, E.C. 3.2.1.3.). It has been assigned many trivial names including amyloglucosidase, glucoamylase, γ -amylase, maltase and limit dextrinase. Glucoamylase will be used in this thesis.

As with all amylases, interest has developed regarding the extent and mode of degradation of native¹ starch granules by glucoamylase. Hydrolysis of starch granules has been studied by light microscopy (2), transmission electron microscopy (3) and scanning electron microscopy (4). Although many studies, using microscopy, have been made on the action of glucoamylases on native starch, there still remains many deficiencies in our understanding of this phenomenon.

We studied hydrolysis of native starch granules using glucoamylases isolated from two fungi, Aspergillus niger and Rhizopus niveus. Two common starches, wheat and white corn, were used as substrates.

The two glucoamylase isoenzymes from A. niger (glucoamylase I and II) were separated and were studied individually to determine their action on

¹Native starch designates starch that is undamaged by mechanical, thermal, chemical or enzymatic means.

native starch. The isoenzymes were then recombined in the original ratio to observe whether any differences in action on starch occurred.

This study was done for essentially three reasons: (1) to determine the extent of hydrolysis of wheat and corn starch by purified glucoamylases, (2) to determine the nature of attack on native starch granules by glucoamylases using scanning electron microscopy to observe the pattern of attack and to determine whether there were differences in attack by glucoamylases from different origins, and (3) to compare hydrolysis patterns of two different starches by glucoamylases.

LITERATURE REVIEW

Starch Granule Structure

The starch granule is composed of two molecular entities: amylose, linear molecules of α -D-glucopyranosyl residues joined by 1,4-linkages, and amylopectin, a high molecular-weight branched component consisting of linear chains of α -D-glucopyranosyl residues joined by 1,4-linkages occasionally branched by α -1,6-bonds forming a tree-type structure (5). There is some evidence that a third component, a low molecular weight amylopectin with limited amounts of branching, is contained in starch (5,6).

It is these two (or three) components in some combination that gives the starch granule its unique properties. However, it appears that amylose is not necessary for general starch granule structure since "waxy-starches," which may contain as little as one percent amylose, still exhibit crystallinity and form starch granules (7,9).

Starch is characteristically found in plants as discrete granules whose size and shape depend on the botanical source. Starch granules may be from one to one hundred microns in diameter with shapes varying from simple, rounded, small wheat granules to complex, multilobed, wrinkled-seeded pea granules (9). Of the many kinds of polysaccharides produced by plants, only starch is laid down unmixed with other polysaccharides.

Several physical properties have been used to characterize starch, yet none of the properties, in itself, reveals the molecular arrangement in the starch granule. One of these properties, gelatinization (the irreversible swelling at high temperature and humidity resulting in destruction of the granule) has received considerable attention. Some work indicates that

the gelatinization temperature is a function of the amylose to amylopectin ratio, but this has been shown true only in a few cases. Studies by Bathgate and Palmer (10) on small and large granules of wheat and barley starches show considerable variance in gelatinization temperatures, yet the amylose content is relatively constant. This points out that the main differences in gelatinization temperatures between various starches are probably more a function of the degree of crystallinity and ease of disruption of these forces, rather than amounts of the amylose and amylopectin components.

The birefringence properties of starch granules have been interpreted to indicate a certain amount of crystallinity. Birefringence patterns in undamaged granules, generally, are oriented in a radial direction away from the hilum. This has been explained by some as due to a radial arrangement within the starch granules (11). Mechanical, heat or enzymatic damage, results in a loss of birefringence (12).

There are two distinct levels to be considered when discussing the structure of the intact starch granule. On the molecular level, the individual structures of amylose and amylopectin isolated from the disrupted granule must be considered. Both of these molecules have been fairly well characterized, yet there is still much disagreement as to the fine structure of the amylopectin component (5,13).

On the granular level the organization of the two components, amylose and amylopectin, has received considerable attention. Many granules, when viewed under the light microscope, exhibit concentric rings centered around a generally eccentrically located spot, called the hilum. The nature of these rings has been the subject of much controversy. It has been suggested that the rings are formed during synthesis of the starch granule, the

alternating nature being caused by naturally-occurring alternating light and dark photoperiods (4). When wheat and barley were grown in constant light, no layering was seen; however, in potatoes, the rings are formed even in the presence of continuous light. The absence or presence of rings in continuous light has been explained as being controlled by the supply of amylose and amylopectin precursors. Apparently light and dark periods affect the precursors in some plants but not in others (14).

Since the shells are accentuated by acid treatment of granules (3,14), it has been proposed that the outer layer of the shell is composed of an acid-labile amylose and the inner shell of a denser, crystalline amylopectin (12). However, Buttrose (14) concluded from acid hydrolysis studies that the shells were merely due to packing density of mixed amylose and amylopectin, and were not due to molecular-type gradients. Meyer (9) proposed that amylopectin is arranged in granules in such a way that the outer chains form crystalline regions similar to amylose crystallites. Reversible swelling of granules would allow some amylopectin crystals to expand but, at the same time, highly crystalline regions would not be able to do so. On the other hand, a shortage of hydrating water would lead to optimal formation of hydrogen bonds between hydroxyl groups on adjacent molecules, resulting in a tighter crystallite (15).

Amylopectin, in the amorphous regions, is preferentially degraded during acid treatment indicating that these regions contain both amylose and amylopectin. Later studies conclude that the only difference between amorphous regions and the shells is water content (which indirectly relates to the amount of crystallinity) (16). However, studies by Robin *et al.* (17) on lintnerized potato starch components indicated amylose as the more

susceptible of the two to acid hydrolysis with amylopectin being more resistant.

Basically two models have been proposed to explain the orientation and organization of amylose and amylopectin in starch granules (12). The blocklet concept visualizes the granule as composed of isodiametric micelles containing crystalline, folded molecules of amylose and amylopectin. The core consists of crystalline micelles of amylopectin interlinked via the nonreducing ends by Van der Waals' forces (12,18,19). One model has amylose included in both the core and jacket, yet amylose and amylopectin are not packaged in the same micelle (18).

A more promising model of the starch granule arrangement, the fibrillar concept, is that proposed by Meyer (20) and others (12). The starch granule is viewed as a composite of spherulites (round aggregates of radiating crystals). This concept would explain the granule's birefringence radiating from the hilum. Amylopectin was suggested as the backbone with the outside branches of several molecules forming a "fringe micelle." The resulting overall picture then is a radiating paracrystal formed from amylopectin molecules with amylose in the interstices (21). A recent study, using scanning electron microscopy, of slowly gelatinized potato starch granules agrees with the fibrillar concept. On rupture of the granules, radial fibrillar structure was quite apparent, yet no tangential weakness, as would be expected in the blocklet concept, was observed (22). Transmission electron microscopy of unmodified and acid-modified corn starch (granules) revealed small amounts of acid-labile materials running in the radial direction, away from the hilum, in addition to the typical concentric rings (23). Nordin et al. (24) in addition to providing evidence for the radial

arrangement, suggest that the nonreducing ends of amylose and amylopectin molecules are located at the surface. This would be compatible with the fibrillar concept.

Some researchers have argued for an outer membrane on the starch granule with different composition and chemical properties than the interior portions (7). Most of these assumptions were based on swelling properties of granules and, in some cases, were later retracted (16). Digestion of starch granules by amylases did not change the amylose to amylopectin ratio even at 50% degradation (25). This was interpreted as evidence against an outer membrane since, as such a membrane is digested away, one would expect a change in the amylose to amylopectin ratio provided the outer portion of the granule did differ in composition (12). It appears there is some difference at the surface of the granule which is perhaps better explained by Whistler (26) who reasoned that drying processes cause a surface "case" or hardened shell.

Although the amylose and amylopectin in the intact starch granule are fairly well characterized, there is still much to be defined concerning the finer points of granule structure. The fact that the granule is composed of essentially two types of molecular entities, both containing different arrangements of α -D-glucopyranose hasn't speeded the elucidation of the structure of the granule. There is no doubt that further investigations into the starch granule structure are warranted.

The Glucoamylases of *Aspergillus* and *Rhizopus* Species

The occurrence of amylolytic enzymes (α -amylases and glucoamylases) in molds has been known for many years. The Japanese were among the first to study the glucoamylases in detail since these enzymes are widely used in

the fermentation industries. Several researchers have suggested classifying the glucoamylases into two or more groups based on their ability to hydrolyze starch (solubilized) or on their activity towards inter-chain linkages (α -1,6-linkages).

There has been some confusion in earlier works concerning the nature (or actual existence) of the amylases isolated from various mold species. Since the molds produce α -amylase in addition to glucoamylases some workers confused the two enzymes (2).

Purification by Pazur and coworkers allowed for classification of the glucoamylases isolated from Aspergillus niger (27-29) and Rhizopus delemar (30). Pazur and Kleppe (27) found that the glucoamylase from A. niger had the ability to hydrolyze α -D-1,6- and α -D-1,3-linkages but at rates much slower than α -D-1,4-linkages. A. niger produced two isoenzymes, termed glucoamylase I and II on the basis of their electrophoretic mobility. The Rhizopus species appear to contain only a single glucoamylase (1,30). The glucoamylase from Rhizopus niveus has been characterized and is now commercially available in highly purified, crystalline form (31,32).

Although glucoamylases from Aspergillus species have been known for some time, it was only recently that studies comparing the isoenzymes have been performed. Lineback et al. (33) found the two isoenzymes from A. niger differed in electrophoretic mobility, molecular weight, isoelectric point and temperature stability. The two glucoamylases had similar patterns of action on malto-oligosaccharides. Andrzejcuk-Hybel and Kaczkowski (34,35), working with Aspergillus oryzae, found isoenzymes similar to those described for A. niger (33). Although no extensive studies were done with the glucoamylases isolated from A. oryzae, they differed considerably in mobility on columns

of DEAE-cellulose and DEAE-Sephadex.

Smiley et al. (36) did extensive kinetic studies, using various substrates, with glucoamylases I and II isolated from two different varieties of Aspergillus niger and one variety of Aspergillus awamori. The chromatographic behavior of the three sets of enzymes, on columns of DEAE-cellulose, was essentially the same with the only differences being the relative amounts of I and II obtained. Two glucoamylases were found in all three cases. When combined, the comparable isoenzymes from A. niger and A. awamori were found to have the same chromatographic mobility on DEAE-cellulose. The isoenzymes used in this study gave almost quantitative yields of D-glucose from soluble starch, yet the reaction rates on some substrates differed considerably. The velocity of glucose formation of glucoamylase I and II was similar on α -1,4-oligosaccharides with various degrees of polymerization and on a 16-D.E. dextrin. However, the rate of glucose release by glucoamylase I from soluble starch (corn, waxy maize, potato and rice) was three to four times greater than for glucoamylase II. A similar situation existed using glycogen as substrate, with the rate for glucoamylase I being about six times greater.

Ueda et al. (37) found a similar situation using the glucoamylases (I and II) from Aspergillus awamori (var. kawachii), although the activities were defined somewhat differently. The debranching activity² of glucoamylase I was about four times that of glucoamylase II. This low debranching activity of glucoamylase II was overcome by adding a large excess of isoamylase to the

²Debranching activity is defined as the ratio of the hydrolysis rate of glycogen β -limit dextrin to the hydrolysis rate of soluble starch under identical substrate concentrations and conditions.

reaction mixture. More interesting, yet confusing, was the digestion of native waxy corn starch by glucoamylases I and II. After 135 hours digestion glucoamylase I and glucoamylase II produced only 20% and 2% hydrolysis, respectively. Adding α -amylase and/or isoamylase increased the extent of hydrolysis to about 100% for glucoamylase I; added α -amylase has little effect on the hydrolysis rate of glucoamylase II. These results agree to some degree with data presented by Marshall and Whelan (38) using soluble starches, glycogens, and β -limit dextrans from various sources as substrates. It is the opinion of Marshall (38,39) that complete hydrolysis of starches (soluble) is never attained unless small amounts of α -amylase are present in the glucoamylase preparation. The significance of α -amylases presence during glucoamylase hydrolysis of native starch (raw starch) is mostly undetermined with the exception of work reported by Ueda et al. (37).

Scanning Electron Microscopy of Starch Granules - Native and Hydrolyzed

It wasn't until about seven or eight years ago that scanning electron microscopy (SEM) was introduced to investigations of starch chemistry. This is not surprising since the first commercial SEM was not placed on the market until 1965 (40). Before the advent of SEM, most, if not all, of the various starches were well characterized using the familiar optical light microscope (41). Conventional electron microscopy (CEM) (also called transmission electron microscopy) was used more for internal studies of acid- or enzyme-hydrolyzed starch granules (3,14).

Use of SEM to observe starches has overcome several problems encountered when utilizing light or conventional electron microscopes (42). The light microscope, besides having low resolution (2,000 - 3,000 Å), presents

the image in only two dimensions due to a very limited depth of field. It is only at approximately 200 diameters or below that the depth of field is sufficient to perceive the sample in its true shape and depth. Since starch granules are transparent spherocrystals, it is easy to confuse internal and external structure. CEM has a much greater resolution (maximum of about 2 \AA), but there are still problems with the depth of field being poor. Another major drawback of CEM is the necessity of very thin samples. This requires ultrathin sections of specimens involving fixing and staining so that fine details may be seen.

SEM, although having a lower resolution (about 100-200 \AA) than CEM, provides an excellent depth of field. In going from 15 to 20,000 diameters, the depth of field stays essentially constant. The resulting image is three dimensional, unlimited by the size of the sample. The only preparation required for specimens, such as starch granules, is the application of a thin coat of metal to the entire surface. A coating of metal is required on all specimens that do not conduct a current. More elaborate procedures are required of soft tissues (43), yet they are not necessitated in the case of starch granules. SEM only reveals surface details of specimens, but starch granules can be fractured to reveal details of internal structure (44). This is not as limiting as would first appear. It has been established that enzyme attack and oxidation (by chlorine) (45) of the starch granule occurs at the surface. Even processes such as gelatinization can be followed with SEM (46).

One of the first studies of the microscopic structure of starch using SEM pointed out the usefulness of this technique in characterizing starches (47). Since then common cereal starches such as barley, corn, oats, rice, sorghum and wheat have been well characterized, using SEM, as to size and

shape (48). Hall and Sayre (49) compared light micrographs with scanning electron micrographs for a number of less common starches, e.g. peanut, pea, sago, bean, and pineapple.

More recently, use has been made of SEM to observe degradation of various starches by amylases. A study by Evers and McDermott (50) showed extensive degradation of wheat starch granules by α -amylase from sprouted wheat. While they observed random pitting on the surface of the granule, it appeared that the preferential site of attack was along an equatorial region of the large wheat granules (termed the equatorial groove). By fracturing the granules along the equatorial groove, they were able to observe the interior. This technique revealed concentric rings similar to those seen in acid-hydrolyzed granules and also indicated that once the enzyme entered the granule it digested the inner core. This suggested a weakness along the equatorial groove as compared to other areas of the granule.

In agreement with these results, several other studies have shown the presence of an equatorial groove which appears susceptible to attack by α -amylases. Dronzek et al. (51), in SEM observation of sprouted wheat, found substantial numbers of granules exhibiting attack along the equatorial groove. The extent of attack correlated with α -amylase production in the wheat kernel. Palmer (52) in a similar study, using malted barley and wheat (8), found that the number of granules with radial channels (equatorial grooves) increased relative to the amount of α -amylase produced. An equatorial groove was seen in large granules of wheat and barley starch, yet was

not observed in small granules of either.³ The attack of small granules involved a more general surface corrosion. Small wheat starch granules and large barley starch granules were the more resistant to amylase degradation of the four types of granules. The existence of the equatorial groove was not apparent until the samples were malted. In a later study by Bathgate *et al.* (53) on isolated granules of barley and wheat starch, it was found that small granules of both starches were resistant to α -amylase degradation. The modes of α -amylase attack on all granules were the same as observed by Palmer (52).

In studies by Kiribuchi and Nakamura (54,55) on starch granules from germinated barley, the equatorial groove was again found to be the site of preferential attack. In some cases corrosion of the large granules resulted in the granules cleaving into two halves along the equatorial groove. SEM observation of these granules revealed extensive corrosion of the interior portions. Some pitting of the granules was observed even in ungerminated barley suggesting that the ripening process of the seed involves some degradation of starch.

The process of glucoamylase digestion of native starch granules is not nearly as well characterized as that of α -amylases. Until recently digestion of native starch by glucoamylase either was not considered (8) or was considered to be nonexistent (1). Manner (56) has reported that glucoamylase has some action on native starch but that it was very limited.

³Starch granules of wheat consist primarily of two types: small round granules 2-10 μ in diameter and large lenticular granules of 25-35 μ in diameter. In barley starch, the large round granules average 20-25 μ in diameter and the small round granules have a mean diameter of about 5 μ . However, it should be noted that classifying granules into two sizes is an oversimplification since there is a gradation of granule size.

Evers et al. (4), using a crude preparation of the glucoamylases of Aspergillus niger, illustrated extensive digestion by this enzyme. Characteristic "disc-like depressions" generally distributed over the entire surface of the granule (the large granule of wheat starch) were observed with SEM. Some attack in the region of the equatorial groove was noted, yet this was true in only some of the granules. Tunneling into interior portions of the granule, with subsequent digestion there, did not seem to occur as it did with α -amylases. Evers attributed this difference in attack patterns to the distribution of α -1,4-bonds at the surface of the granule in the case of α -amylolysis. It may be that there are only certain regions, i.e., equatorial groove, in which the molecular arrangements are favorable for α -amylase attack. In the case of glucoamylases one might expect uniform attack since the enzyme degrades amylose and amylopectin from the nonreducing end. Evidence for nonreducing ends being at the surface of the granule (24) agrees with the observed patterns.

From the work of Rasper et al. (57) it readily becomes apparent that the extents and patterns of degradation of granular starch are functions of the source of the starch and type of amylase. Using three enzyme systems (bacterial α -amylase, fungal glucoamylase, and malt diastase) on ten different starches, no apparent differences in action of the various enzymes with the exception of wheat starch, were seen. An equatorial groove-type attack was observed only with wheat starch. In most of the starches, the extent of degradation by α -amylase and glucoamylase was about the same, with malt diastase giving the lowest amounts in all cases. Cassava and cocoyam starches digested by α -amylase exhibited a spongy-type surface while sorghum and millet starches showed extensive general surface pitting.

The action of α -amylase and glucoamylase on cassava and cocoyam starches was the same when viewed by SEM.

Shetty et al. (58) studied the rate of glucose release from wheat starch degraded by the glucoamylases from Aspergillus niger and a highly purified preparation from Rhizopus niveus. Interestingly enough the extent of enzyme attack by the A. niger preparation approached three times that of the R. niveus preparation (at 64 hours digestion time). SEM observation of the large wheat starch granules at various times of digestion revealed two apparently different attack patterns. A. niger glucoamylase, which has the higher degree of conversion of starch to glucose, exhibited some degree of attack along the equatorial groove (dubbed "tunneling" by the authors). However attack wasn't limited to this area. Extensive general pitting was noted over the entire surface of the granules such as that observed by Evers (4). Digestion by R. niveus glucoamylase, while still exposing an equatorial groove, never developed the large pitting seen with A. niger glucoamylase. The attack was confined to small deep holes in the granules, resulting in a spongy-type appearance over the surface.

MATERIALS AND METHODS

Materials

A commercial fungal (A. niger) glucoamylase preparation obtained from Miles Laboratories Inc., Elkart, Indiana was used as the source of the crude enzyme. Two different preparations were used. Diazyme concentrate was supplied as a tan powder and Diazyme 160 (a more recent product) was supplied as an off-white powder. Glucoamylase (2X-crystallized) from R. niveus was also obtained from Miles Laboratories Inc.

DEAE-cellulose (DE 32, microgranular form, 1 meq/g) was purchased from Whatman Biochemicals Ltd., Clifton, New Jersey. Bovine serum albumin (lyophilized) and research grade Triton X-100 were purchased from Sigma Chemicals, St. Louis, Missouri. "Glucostat X4" reagent sets for enzymatic determination of D-glucose were obtained from Worthington Biochemical Corp., Freehold, New Jersey. Folin-phenol reagent (about 2 N in acid) was obtained from Fisher Scientific Co., Fair Lawn, New Jersey. Soluble starch (Lintner), analyzed reagent grade, was supplied by J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Amylopectin (waxy maize) and amylose (Omar wheat) were generous gifts of Dr. Cheng-yi Lii. β -limit dextrin (waxy milo) was kindly furnished by Dr. Phillip Nordin.

Methods

Isolation of Wheat Starch from Flour. A procedure similar to that described by Wolf (54) was used to isolate starch from wheat flour (Scout-70). A dough ball was prepared by mixing 300 g of flour with sufficient water

(150 ml) to form a stiff dough. The dough ball was covered with a wet cheese cloth and was aged for 2 hr at room temperature.

Starch was washed from the dough ball by kneading the dough under a steadily dripping stream of distilled water, washing the starch through a piece of nylon bolting (16XX, 70-72 μ openings) stretched over a large beaker. Cellular debris retained by the bolting was discarded and the starch-water suspension was again screened through the bolting cloth. After settling, the supernatant solution was removed by suction. This procedure was repeated several times. The settled starch was finally resuspended in about 2 liters of distilled water and centrifuged at 1400 X G for 20 min in 250 ml wide mouth centrifuge bottles from which the top had been removed. The thin layer of light-brown colored tailings covering the prime starch in the centrifuge tubes was scraped away with a bent spoon. The prime starch was resuspended in the same volume of distilled water and was centrifuged again at 1400 X G for 20 min. The tailings layer was scraped away as before. This process was repeated until no colored layer was present on top of the prime starch after centrifuging.

Prime starch was removed from the centrifuge bottles, broken into smaller pieces on a piece of Whatman filter paper, and vacuum dried at 35°C for 24 hr. During the early stages of drying, any large clumps were broken so they would not dry into masses. Recovery was 100 g, representing a yield of 33%. The starch contained 5.1% moisture, 0.6% protein, and 0.11% ash. Fat content was too low to be detected.

Isolation of Wheat Starch from Whole Wheat. Whole wheat (200 g, Scout-73) was soaked at 5°C in excess distilled water for 24 hr with several changes of distilled water during this period. At the end of 24 hr, the

water was drained and the kernels were washed in distilled water and then covered with a minimum of cold distilled water. The kernels were ground in a Waring blender at high speed for 1.5 min and at low speed for 1.5 min. The resulting slurry was filtered through several folds of cheese cloth and then through nylon bolting (16XX). Any starch remaining on the bolting was washed through with distilled water.

Gluten was removed by suspending the starch in distilled water, allowing it to settle, and removing the supernatant solution by suction. This process was repeated about ten times, after which the starch was centrifuged and the tailings removed as described in the preceding procedure.

The isolated starch was vacuum dried at 35°C for 24 hr as previously described. Recovery was 28 g of starch containing 4.1% moisture, and 0.2% protein.

Isolation of Corn Starch from Whole Corn (55). Clean, whole, white corn (Pride of Saline, 600 g) was added to 1.5 of 0.0156 M potassium bisulfite and allowed to set for 24 hr at room temperature. The supernatant solution was decanted (pH of steepwater was 5.5) and the corn was washed with distilled water until free of odor.

Aliquots (250 ml) of corn were ground in a Waring blender with 250 ml of distilled water for three min at medium speed. The resulting slurry was screened through nylon bolting (16XX) using gentle rubbing by hand applied to the bolting screen while the residue was washed with distilled water.⁴ The filtrate was screened a second time and the resulting starch suspension

⁴This was necessary to force the starch through the screen. It also resulted in some cell debris passing through the screen but this was removed by a second screening.

was allowed to settle. The supernatant solution was removed by suction and the starch was suspended in 4 l of distilled water with constant stirring (magnetic).

In order to separate starch and gluten, a starch table was assembled. A 15.3 X 244 cm piece of stainless steel I-beam was positioned on a laboratory bench. The table was supported at one end to achieve an angle of about 4-5°. The stirred starch suspension was tabled by slowly pouring 50-ml aliquots onto the raised end of the table. When all the starch suspension had been tabled about 300 ml of distilled water was washed down the table. The starch was scraped from the table and was resuspended in 4 l of distilled water. The tabling process was repeated two additional times. The starch suspension was finally filtered through a Buchner funnel and dried in a vacuum oven at 35°C for 24 hr. Starch recovery was 183 g representing a yield of 31%. The starch contained 9.6% moisture, 0.6% protein, and 0.11% ash.

Analysis of Starch.

Moisture: Moisture was determined according to A.A.C.C. method 44-15A (61) which involves heating a 2-g sample at 130°C for one hr.

Protein: Nitrogen was determined by the Kjeldahl procedure using A.A.C.C. method 46-11 (61). For wheat, protein was calculated as $N \times 5.7$. In the case of corn starch, protein was calculated as $N \times 6.25$.

Ash: Ash was determined by A.A.C.C. method 08-01 (61).

Fat: Fat was determined by petroleum ether extraction using A.A.C.C. method 30-25 (61).

Total Carbohydrate: Total carbohydrate, for use in determining percent hydrolysis of various starches by glucoamylases, was determined by the phenol-sulfuric acid assay (62) after acid hydrolysis (63). Acid hydrolysis was

performed, previous to the phenol-sulfuric acid assay, to insure a uniform solution required for subsequent dilutions.

Starch (200 mg) was weighed and transferred to a 100 ml volumetric flask to which was added 30-35 ml of 2.0 N sulfuric acid. The flask was heated at 95°C for four hr, cooled, and the contents diluted to 100 ml with distilled water.

After appropriate dilution, 2 ml of the hydrolyzate, containing between 20 and 120 ug of carbohydrate, was mixed with 1 ml of 5% phenol. To this solution 5 ml of concentrated sulfuric acid was quickly added, using an automatic pipette. The sulfuric acid was allowed to splash directly into the solution in order to generate enough heat for the color production.

After cooling at least 30 min at room temperature, the absorbance was measured at 490 nm using a Beckman DU spectrophotometer with an appropriate reagent blank. A standard curve was prepared using D-glucose (16-128 µg). Total carbohydrate was expressed as D-glucose. Each starch sample was assayed in triplicate and each point on the standard curve was determined in duplicate.

Determination of D-glucose. The routine method used to measure D-glucose released from enzymatic hydrolysis of soluble starch or native starch involved the procedures described by Shetty *et al.* (58). This is a modified glucose oxidase-peroxidase procedure using the commercially available "Glucostat" reagent set which provides both enzymes and a chromogen (3,3'-dimethoxybenzidine dihydrochloride) (64).

Glucose Standard: D-glucose was dried 24 hr at 60°C under vacuum prior to use. The standard glucose solution consisted of 1.0 g D-glucose and 2.0 g of benzoic acid diluted to a final volume of 100 ml. This solution is stable indefinitely.

The working standard was made by diluting 4 ml of the standard solution to 250 ml, giving a glucose concentration of 160 $\mu\text{g}/\text{ml}$. This solution was either frozen for later use or used within one week if stored at 5°C.

Tris-(hydroxymethyl)-aminomethane (60.57 g) was dissolved in 80 ml of 5 M hydrochloric acid. This solution was diluted to slightly less than one l, heated to a temperature of 37°C, and the pH adjusted to 7.00 using a pH-meter. This step is critical since the pH of Tris buffer solutions is extremely temperature dependent (65). The cooled buffer was then diluted to one l with distilled water.

Glucose Reagent: One vial of "chromogen" was mixed with 4.0 ml of a 20% ethanolic solution of Triton X-100.⁵ This is then added to 375 ml of 0.5 M Tris buffer which contained the contents of one vial of "Glucostat X 4." The "chromogen" vial was rinsed several times with buffer and the final volume of combined reagents (Glucostat reagent) was adjusted to 400 ml. This reagent was stable for 5 days when stored at 5°C.

Glucose Analysis: For solutions containing 40-160 μg glucose/ml, method A (58) is employed. One ml of the unknown is incubated at $37^\circ \pm 0.1^\circ\text{C}$ with 10.0 ml of "Glucostat" reagent which had been prewarmed to 37°C. After one hr the reaction is terminated by addition of 0.5 ml of 4 M hydrochloric acid. Absorbance of the cooled solution was measured at 400 nm in 1-cm cells using a Beckman DU Spectrophotometer. Analyses were done in triplicate as were the D-glucose standards also.

Method B (58) is utilized for solutions containing 6-18 μg glucose/ml.

⁵The ethanolic solution of Triton X-100 should be injected into the "Chromogen" vial before it is opened. The chromogen, 3,3'-dimethoxybenzidine, is a reported carcinogen with toxicity indicated when the powder is inhaled over a long period of time (64).

Three ml of the unknown are incubated with 3.0 ml of "Glucostat" reagent at $37^{\circ} \pm 0.1^{\circ}\text{C}$ for one hr. The reaction is terminated by the addition of 0.15 ml of 4 M hydrochloric acid. The rest of the procedure is identical to that of Method A.

Determination of Enzyme Activity. Glucoamylase solutions were diluted to approximately 0.02-0.04 units/ml with 0.05 M acetate buffer, pH 4.8, or with 0.05 M citrate buffer, pH 5.0.⁶ Enzyme activity was determined in the manner described by Lineback *et al.* (33). Enzyme solution (0.5 ml) was incubated with 1.5 ml of a 4% soluble starch solution in one of the above stated buffers for 1 hr at $30^{\circ} \pm 0.1^{\circ}\text{C}$, unless otherwise designated.⁶ The reaction was stopped by addition of 5.0 ml of absolute ethanol. The mixture was cooled (to about 5°C) and then centrifuged at 1000 X G for 10 min to remove precipitated protein and starch. If necessary, the supernatant was diluted with 70% ethanol to achieve a concentration of 40-160 μg glucose/ml. Glucose was determined in the supernatant or diluted aliquot using the "Glucostat" reagent as previously described. One unit of enzyme activity is defined as the amount of enzyme required to release one umole of glucose per minute under the conditions of the assay.⁷ Specific activity is expressed as units of enzyme activity per mg of protein.

Determination of Enzyme Protein. Protein in glucoamylase solutions was determined by the Lowry method (66) as modified by Miller (67).

⁶In the initial stages of this research 0.05 M acetate buffer, pH 4.8, was used. This was later changed to 0.05 M citrate buffer, pH 5.0. Since the pH optimum for all the glucoamylases used is 4.8-5.0 and the buffers themselves have no effect, the conditions, for all practical purposes, are the same.

⁷Some activities were determined at $37^{\circ} \pm 0.1^{\circ}\text{C}$ with all other conditions being the same as for activities determined at $30^{\circ} \pm 0.1^{\circ}\text{C}$.

Reagents: Reagent A is 10% (w/v) sodium carbonate in 0.5 N sodium hydroxide. Reagent B1 is 1% (w/v) aqueous copper sulfate pentahydrate. Reagent B2 is 2% (w/v) aqueous potassium sodium tartrate. The solutions are prepared in distilled water and have an indefinite shelf life. Reagent B is made fresh daily by mixing one part of reagent B1 with one part of reagent B2. The alkaline copper reagent, which is also made fresh daily, is formulated by mixing ten parts of reagent A with one part of reagent B. The working stock Folin-phenol reagent is made by diluting (1:11 v/v) commercial Folin-phenol reagent (2 N in acid) with distilled water.

A stock solution of bovine serum albumin was made by dissolving 100 mg of BSA in 90 ml of cold, distilled water. This solution was allowed to warm to room temperature and then diluted to 100 ml with distilled water. The exact concentration of BSA in the stock solution was determined by measuring the absorbance at 280 nm and the concentration of BSA was calculated using $E_{1\text{cm}}^{1\%} = 6.6$. The stock solution was diluted to 160 μg BSA/ml with distilled water. These BSA solutions were stored at 5°C and were stable for at least 9 months.

Protein Determination: One ml of the enzyme solution containing 40-160 μg protein/ml was mixed with 1.0 ml of the alkaline copper reagent and allowed to set for 10 min at room temperature. Folin-phenol reagent (3.0 ml) was quickly added with an automatic pipette and thoroughly mixed. The solution was allowed to set for 45 min at room temperature and the absorbance was then measured at 650 nm.⁸ Protein concentration was determined using a

⁸Bonitati et al. (68) recommend against heating the reaction system to 50°C as suggested by Miller (67) due to possible interference by carbohydrates.

Purification of the Glucoamylases from *A. niger* by DEAE-cellulose

Chromatography. A column (2.6 x 70 cm, bed volume 370 ml) previously packed with approximately 120 g of pre-cycled DEAE-cellulose (69), fines removed, was equilibrated by passing 4 bed volumes of starting buffer⁹ (0.025 M citrate-phosphate, pH 8) through the column at 5°C.

Crude glucoamylases from *A. niger* (Diazyme 160 or Diazyme concentrate) were purified on a column of DEAE-cellulose using a previously reported stepwise elution method (29,33).

Commercial enzyme (5 g) was dissolved in about 75 ml of distilled water, filtered to remove inert carrier material, mixed with 15 ml of 0.04 M calcium acetate, and then mixed with 100 ml of starting buffer (0.025 M citrate-phosphate, pH 8.0). This mixture was then centrifuged and the supernatant dialyzed against running water for 24 hr. After dialysis the volume of enzyme solution was adjusted to 250 ml with 0.05 M citrate-phosphate buffer, pH 8.0, and introduced onto the column of DEAE-cellulose. The enzyme was eluted from the column by successive additions of 500 ml of 0.025 M citrate-phosphate buffer, pH 8.0; 500 ml of 0.05 M citrate-phosphate buffer, pH 8.0; 750 ml of 0.05 M citrate-phosphate buffer, pH 6.0, and 1200 ml of 0.05 M citrate-phosphate buffer, pH 4.0. A constant flow rate of 1 ml/min was maintained by a solution metering pump. Fractions (15 ml) were collected automatically. Protein components were located automatically by scanning the effluent at 280 nm using a constant-flow U.V. monitor and recorder (Uvicord II, LKB Instruments Inc., Rockville, Md.). Starch-hydrolyzing activity

⁹Buffers used for chromatography are the stated molarity in disodium phosphate; e.g., 0.025 M citrate-phosphate, pH 8, is 0.025 M in disodium phosphate with sufficient 1 M citric acid to achieve pH 8.0.

(glucose release) was measured as previously stated and was reported as units/ml.

Glucoamylase Digestion of Native Wheat and Corn Starch. Solutions of glucoamylases I and II, obtained by pooling fractions from the DEAE-cellulose chromatography of Diazyme 160 or Diazyme concentrate, were adjusted to pH 5.0 with 1 M citric acid using a pH-meter. They were then dialyzed for 24 hr against two changes of 0.05 citrate buffer, pH 5.0, in a system containing about 50 ml enzyme solution in a dialysis bag placed in about 2 l of buffer. After dialysis the pH was checked with a pH-meter and adjusted again to pH 5.0 if necessary. The solution was diluted to 10 ± 0.5 units/ml with 0.05 M citrate buffer, pH 5.0.

One enzyme system was composed of 60% glucoamylase II and 40% glucoamylase I. This was made by combining 30 ml of glucoamylase II solution (10 ± 0.5 units/ml in 0.05 M citrate buffer, pH 5.0) with 20 ml of glucoamylase I solution (10 ± 0.5 units/ml in 0.05 M citrate buffer, pH 5.0). This reconstituted system approximates the relative amounts of glucoamylase I and II in the crude enzyme as shown by DEAE-cellulose chromatography.

A solution of the glucoamylase (2X crystallized) from R. niveus was prepared by dissolving 25 mg of the white amorphous powder in 50 ml of 0.05 M citrate buffer, pH 5.0.

Digestion of native starch by glucoamylase was accomplished in a manner similar to that described by Shetty et al. (58). Native starch (100 mg) was incubated with 50 ml of glucoamylase solution (10 ± 0.5 units/ml) in 0.05 M citrate buffer, pH 5.0, at $37^\circ \pm 0.1^\circ\text{C}$. The reaction was gently stirred with a magnetic stirring bar. Toluene (0.5 ml) was added as a preservative. A control was included with each incubation and contained only 100 mg of

starch and 50 ml of 0.05 M citrate buffer, pH 5.0.¹⁰ Aliquots (5.0 ml) were removed periodically (after 0.5, 1, 2, 4, 8, 16, 32, 64 and, in some cases, 100 hr incubation) from the reaction mixture and control. The reaction was stopped by adding 10.0 ml of absolute ethanol. The mixture was centrifuged and the supernatant analyzed for D-glucose with the "Glucostat" reagent using either method A or method B depending on the glucose concentration. Percent hydrolysis was determined by comparing the glucose liberated enzymatically with the theoretical amount of glucose determined by acid hydrolysis as previously described.

Scanning Electron Microscopy of Digested Starch Granules. The sediment obtained from the ethanol precipitation described above was washed three times with distilled water and once with ethanol. It was then vacuum dried several hours at room temperature. A sample was then mounted on specimen stubs using double-backed adhesive tape. The sample was coated in a vacuum with about 60 Å of carbon and about 100 Å of gold/palladium metal. The samples were then viewed in an ETEC auto-scanning electron microscope operating at 20-kv accelerating voltage. Images were photographed on Polaroid film, type 55P/N.

Glucoamylase Activity with Amylose, Amylopectin and -Limit Dextrin as Substrates. Solutions of the various enzymes (10 units/ml) were diluted (0.5 ml to a total volume of 25 ml) using 0.05 M citrate buffer, pH 5.0. The diluted solution (0.35 ml) was reacted for one hr at 30°C with 1.65 mls

¹⁰One incubation was done with Glucoamylases I and II with the reaction being mixed by shaking rather than stirring. This was discontinued for several reasons as noted in Results and Discussion.

of substrate (10 mg/ml) dissolved in the same buffer. Analysis of D-glucose and other conditions were the same as those used in determining enzyme activity. Activity was defined as previously described.

RESULTS AND DISCUSSION

Starch Isolation

The isolation of wheat starch from flour (Scout-70) resulted in considerable physical damage to starch granules. This was apparent in scanning electron micrographs of control and enzyme-hydrolyzed samples (Figure 1a and b). Figure 1a is representative of attack by the glucoamylase from A. niger. It is our opinion that enzyme attack was most prevalent on damaged granules with little attack on undamaged ones. Thus, the use of wheat starch isolated from flour was discontinued. The physical damage to starch granules probably occurred during roller milling to produce the flour.

This sample (Figure 1a) was also mixed by shaking rather than stirring, yet starch granules sedimented to the bottom of the reaction vessel. Shaking was rejected as a method of mixing and replaced by stirring the reaction system in order to expose every granule to enzyme attack.

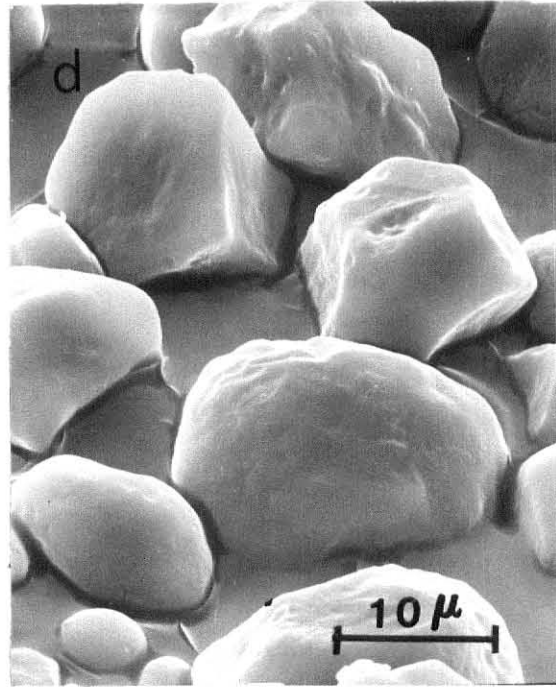
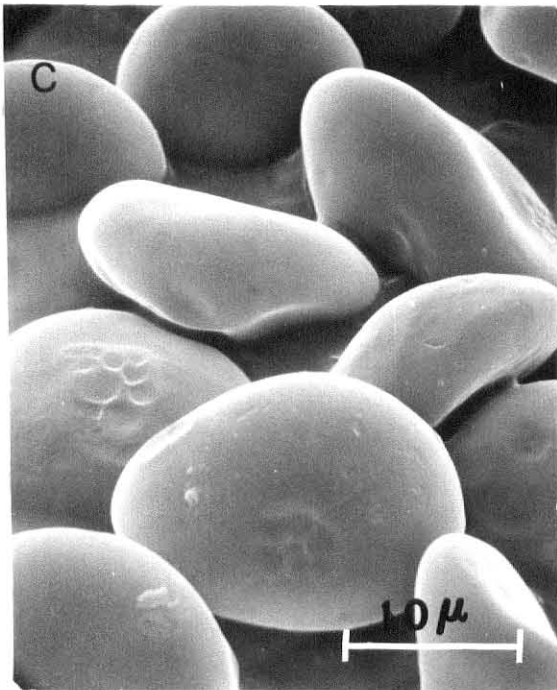
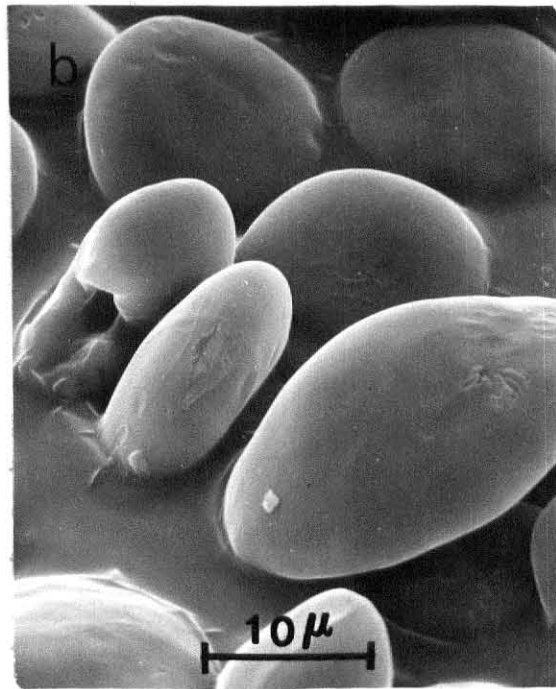
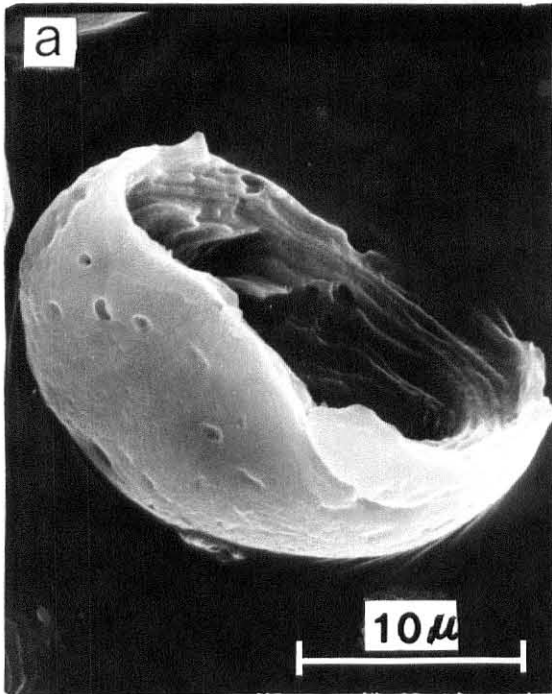
SEM observation of corn starch (Pride of Saline) and wheat starch (Scout-73) isolated by wet milling revealed almost no mechanically damaged granules. Thus, it was assumed that granule hydrolysis upon exposure to glucoamylases (as followed by release of D-glucose and SEM) resulted from enzyme attack of native granules. The term "native" is used with caution since the very act of isolating granules from seed endosperm could possibly alter the granules.

It should also be noted, as revealed by SEM (Figure 1c), that small wheat starch granules were not isolated. We attribute this to the low speed

FIGURE 1. Action of glucoamylase on wheat and corn starch: a. 16 hour digestion by glucoamylase I from A. niger on starch from Scout-70 flour (see Table III); b. Control for wheat starch from Scout-70 flour; c. Control for wheat starch from Scout-73 whole wheat; d. Control for white corn starch.

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used during centrifugation of the starch, which probably resulted in the small granules being removed with the tailings fraction. Thus our studies on wheat starch are concentrated on the large granules.

Total Carbohydrate Used in Calculating Percent Enzymatic Hydrolysis

It is a common procedure for researchers to determine the percent hydrolysis of starch based on the relationship:

$$\text{Actual \% Starch} = 100 - (\% \text{ Moisture} + \% \text{ Protein} + \% \text{ Fat} + \% \text{ Ash}).$$

The theoretical amount of glucose released on complete hydrolysis is then calculated from the amount of starch used. We chose the phenol-sulfuric acid assay (62) to determine total carbohydrate because of its high sensitivity and accuracy. Using this procedure a 100-mg sample of starch released the following amounts of glucose: Scout-70, 102,000 μmoles ; Scout-73, 105,042 μmoles ; and white corn 99,375 μmoles . When the formula cited was used, comparable data were obtained.

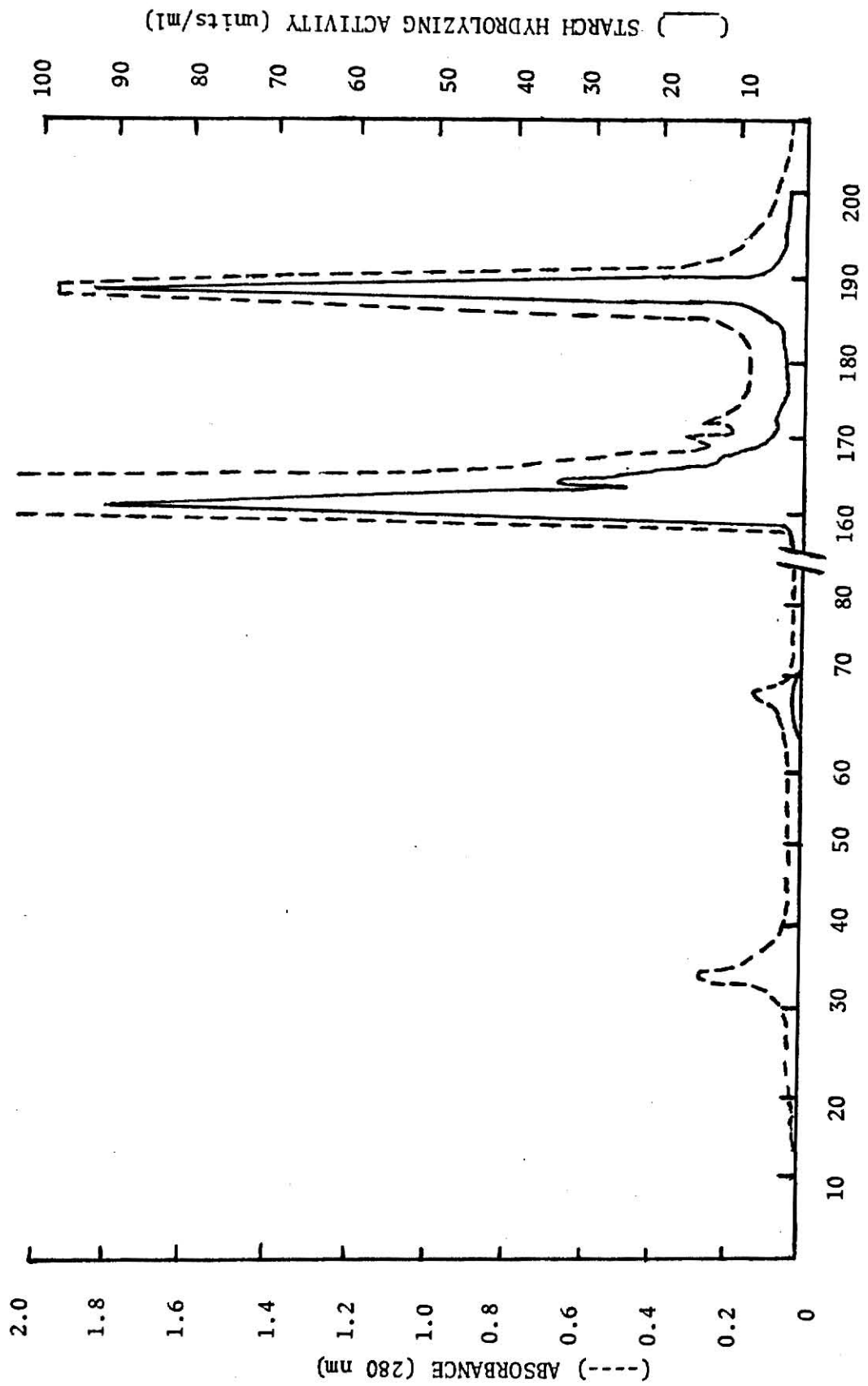
Enzyme Purification

Figure 2 is a representative elution profile obtained using commercial Diazyme 160. The first peak has been identified as glucosyl transferase (33). Glucoamylase II is the peak centered around tube number 164 and glucoamylase I is the peak centered around tube number 188 (33). All elution profiles were essentially the same with the exception of Diazyme concentrate (only one purification was performed) which contained about twice as much protein in the first peak (tube number 33) as Diazyme 160. The efficiency of the purification procedure at each step is indicated in Table I using values from a typical purification procedure. The values obtained are in agreement with those previously reported (33).

FIGURE 2. Purification of glucoamylase from A. niger by chromatography on DEAE-cellulose.

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Tube Number

FIGURE 2

TABLE I
Purification of Glucoamylase from Aspergillus niger
(Diazyme 160)

	MI	Activity (units/ml)	Total units	Protein (mg/ml)	Specific Activity (units/mg)	Yield (%)	Purification
Crude enzyme before dialysis	250	47	11,800	3.6	13.2		
Crude enzyme after dialysis	247	39	9,633	2.9	13.4	100	1.0
Chromatography (DEAE-cellulose)							
Glucoamylase I	54	48	2,600	4.1	11.7 ^a	27	1.1 ^b
Glucoamylase II	147	32	4,730	2.0	16.0 ^a	49	

^aFor several different purifications the values were 12-14 units/mg for glucoamylase I and 16-18 units/mg for glucoamylase II. Values for purified enzymes from Diazyme concentrate were 15 units/mg for glucoamylase I and 16 units/mg for glucoamylase II.

^bBased on combination of I and II.

Hydrolysis of Native Starches as Observed by Release of D-glucose

Hydrolysis of wheat and corn starches, as indicated by release of D-glucose, was found to be extensive with all four enzyme systems used. The kinetics of the four enzyme systems were very similar on both corn and wheat starches, with corn starch showing the most extensive degradation of the two.

Using wheat starch, the glucoamylase from Rhizopus niveus unquestionably showed the most extensive degradation. The extent of hydrolysis was much greater initially (0.5 hr) than with the glucoamylases from A. niger (Table II and Figure 3) and increased to about 32 hours at which time it started to level off. These data show that the presence of an endo-enzyme (α -amylase) is not a necessity for extensive hydrolysis, although it has been reported to be required for complete hydrolysis of starch by glucoamylase (38). Shetty et al. (58), using the same enzyme under practically identical conditions, only achieved 32% hydrolysis after 64 hours compared to 89% hydrolysis at 64 hours in our study (Table II). These two sets of data are in gross disagreement, yet no differences in method could be found. Enzyme activities in our study were the same as those reported by Shetty et al. (58).

During the initial stages of hydrolysis (up to 32 hr), glucoamylase I from A. niger hydrolyzed wheat starch at about twice the rate of glucoamylase II (Figure 3). However when the two enzymes were mixed in the proportions present in the crude enzyme, the rate of hydrolysis, by the reconstituted enzyme, surpassed that of either of the individual isoenzymes. The difference in rates between glucoamylase I and II was also observed on the damaged starch (Table III).

TABLE II

Hydrolysis (%) of Wheat Starch^a to D-Glucose by Glucoamylase

Enzyme	Incubation (hr) at 37°C							
	0.5	1	2	4	8	16	32	64 ^b
<u>A. niger</u>								
Glucoamylase I ^c	0.7	1.2	2.8	6.2	9.3	21.3	41.9	66.3
Glucoamylase II ^c	0.2	0.2	0.4	0.8	2.4	5.9	16.6	38.0
Reconstituted ^c	0.9	1.6	3.9	7.0	12.5	23.8	44.9	77.8
<u>Rhizopus niveus</u>								
Glucoamylase ^d	2.7	5.3	9.5	15.7	26.4	42.9	73.0	88.5

^aWheat starch (Scout-73) was isolated from whole wheat by wet milling. A. niger enzymes were purified from Diazyme 160.

^bControl showed 0.5% hydrolysis at 64 hr.

^cActivities at 37°C were: Glucoamylase I, 17 units/ml; Glucoamylase II, 16 units/ml; Reconstituted glucoamylase I and II, 15 units/ml. Specific activities (30°) were: Glucoamylase I, 13 units/mg; Glucoamylase II, 17 units/mg; Reconstituted, 15 units/mg.

^dActivity was 16 units/ml at 37°C. Specific activities were 20 units/mg at 30°C and 33 units/mg at 37°C.

FIGURE 3. Hydrolysis of wheat starch (Scout-73) by glucoamylases at 37°C in 0.05 M citrate buffer, pH 5.0. ○ glucoamylase I (from Diazyme concentrate; specific activity, 15 units/mg); ● glucoamylase II (from Diazyme concentrate; specific activity 16 units/mg); △ reconstituted glucoamylase I and II (from Diazyme 160; specific activity, 15 units/mg; see Table II); ▲ glucoamylase from Rhizopus niveus (specific activity, 20 units/mg; see Table II).

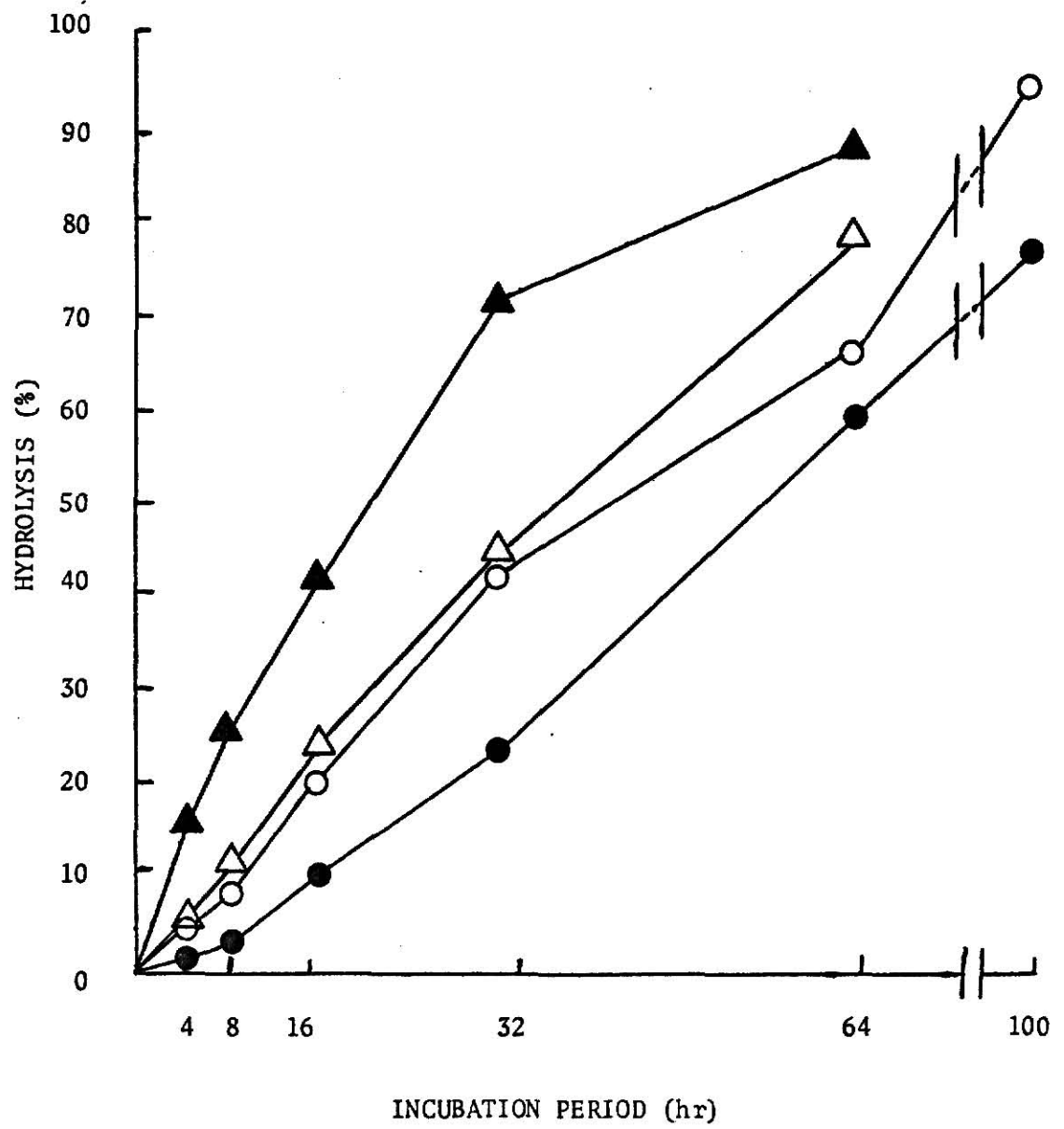


FIGURE 3

TABLE III

Hydrolysis (%) of Wheat Starch^a to D-Glucose by Glucoamylase

Enzyme	Incubation (hr) at 30°C							
	0.5	1	2	4	8	16	32	64 ^b
<u>A. niger</u>								
Glucoamylase I ^c	0.7	1.4	2.1	3.5	5.2	10.5	13.6	17.2
Glucoamylase II ^c	---	---	---	1.5	2.3	2.3	3.5	3.5

^aWheat starch (Scout-70) isolated from flour. Reaction in 0.05 M citrate buffer, pH 4.8, mixed by shaking.

^bControl showed 0.05% hydrolysis at 64 hr.

^cGlucoamylase from A. niger purified from Diazyme 160. Specific activities (at 30°C) of I and II are 12 units/mg and 16 units/mg, respectively.

Comparison of the data for wheat and corn starches reveals similarities, the primary difference being the extent of hydrolysis. As with wheat starch the glucoamylase from R. niveus had the highest initial rate and produced the largest extent of hydrolysis. At 64 hours reconstituted glucoamylases I and II showed greater hydrolysis of corn starch than either of the separated glucoamylases (Table IV and Figure 4). It might be expected that the rate and extent of hydrolysis by the reconstituted enzyme (60% glucoamylase II and 40% glucoamylase I) would be an average of the two isolated isoenzymes. However data for hydrolysis of both wheat and corn starches (Figures 3 and 4) indicates that this is not so.

In the case of R. niveus glucoamylase, results were quite reproducible varying by as little as 1% hydrolysis at 64 hours. However using A. niger glucoamylases purified from different commercial samples, Diazyme concentrate and Diazyme 160, some variation in extents of hydrolysis were observed. This is easily noted for corn starch by comparing the data for preparations 1 and 2 for each A. niger glucoamylase in Table IV. Although the overall extent of hydrolysis was not the same when comparing the enzymes from the two A. niger preparations, the patterns were similar. Reconstituted glucoamylase I and II showed the highest hydrolysis rate while glucoamylase I produced a greater rate and extent of hydrolysis than glucoamylase II.

These results can be directly compared only with the work of Shetty et al. (58). The work of Ueda et al. (37) can be compared generally, but the enzyme to starch ratio used in that report limits finer scrutiny. We used about 5 units/mg of starch at 37°C while Ueda et al. (37) used about 0.02 units/mg of starch at 40°C. Using glucoamylase I and II purified from A. awamori, it was clearly evident that glucoamylase I gave much greater

TABLE IV
Hydrolysis (%) of Corn Starch^a to D-Glucose by Glucoamylase

Enzyme	Incubation (hr) at 37°C								
	0.5	1	2	4	8	16	32	64 ^b	
<u>A. niger</u>									
Glucoamylase I	1 ^c	0.6	1.2	2.0	7.6	17.3	36.5	63.3	86.5
	2 ^d	0.5	0.9	2.3	8.1	16.9	15.0	47.6	78.5
Glucoamylase II	1 ^c	0.2	0.3	0.3	0.5	1.4	4.3	14.2	40.4
	2 ^d	0.2	0.2	0.6	1.4	2.8	5.8	25.2	57.6
Reconstituted I and II	1 ^c	1.0	1.9	5.3	10.2	20.2	39.5	66.7	92.4
	2 ^d	0.5	1.1	5.5	9.0	19.8	30.0	60.1	81.0
<u>Rhizopus niveus</u>									
Glucoamylase ^e	1.4	2.6	10.3	18.9	37.2	64.4	82.9	91.7	

^aIsolated from white corn (Pride of Saline) by wet milling.

^bControl showed 0.3% hydrolysis at 64 hours.

^cSame preparation as in Table II.

^dPurified from Diazyme concentrate. Activities at 37°C were: glucoamylase I, 16 units/ml; glucoamylase II, 17 units/ml, and reconstituted glucoamylase I and II, 16 units/ml. Specific activities (30°C) were: glucoamylase I, 15 units/mg; glucoamylase II, 16 units/mg; and reconstituted glucoamylase I and II, 15 units/mg.

^eSame preparation as in Table II.

FIGURE 4. Hydrolysis of corn starch by glucoamylase at 37°C in 0.05 M citrate buffer, pH 5.0. ○ glucoamylase I (preparation 2, Table IV); ● glucoamylase II (preparation 2, Table IV); △ reconstituted glucoamylase I and II (preparation 2, Table IV); ▲ glucoamylase from Rhizopus niveus (Table IV).

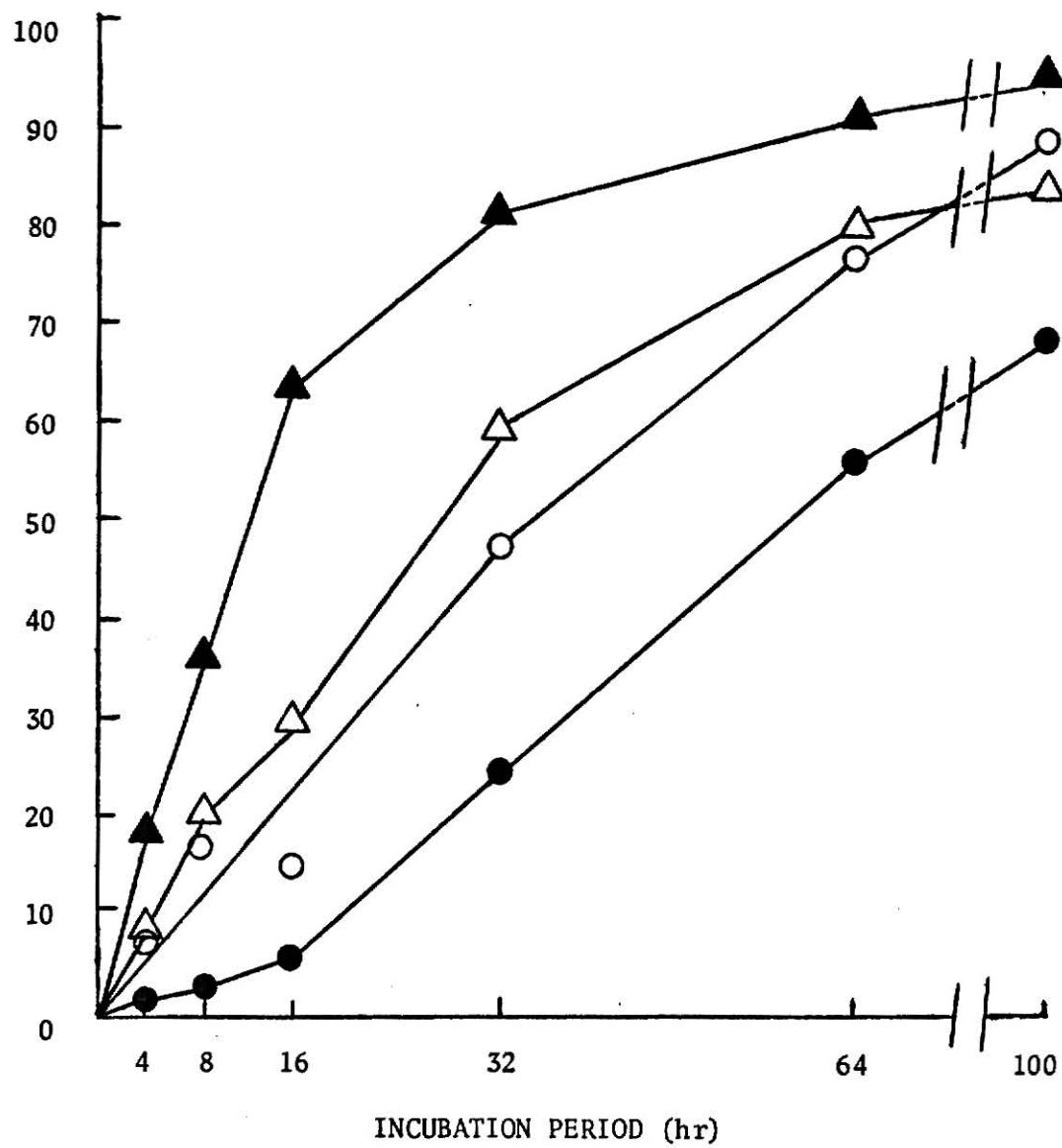


FIGURE 4

hydrolysis than glucoamylase II on both waxy and regular corn starches (37). The rates of hydrolysis by both glucoamylase I and II were much lower on regular corn starch (containing amylose) than on waxy corn starch. Another striking difference between the two glucoamylases was the ability to debranch glycogen -limit dextrin. Glucoamylase I had about 3.5 times greater debranching activity than glucoamylase II. The varying rates on the two starches by glucoamylase I and II were interpreted by the authors to be an effect of enzyme inhibition by amylose present in the regular starch.

The activities of the glucoamylases on various substrates used in our study are shown in Table V. If an experimental variation of ± 0.5 units/ml is placed on the data, the data indicate that very little difference in substrate susceptibility is observed, with the exception of the glucoamylase from R. niveus. The lower activity of the glucoamylase from R. niveus on amylose, amylopectin, and waxy sorghum β -limit dextrin was unexpected since this enzyme had the highest native starch degrading capacity of the enzymes used. This may indicate that attempts to correlate data from isolated starch components to data from intact starch granules may be an exercise in futility.

Scanning Electron Microscopy of Digestion of Wheat and Corn Starch Granules by Glucoamylases

In viewing enzymatic attack of starch granules, a few precautions were taken. First, and foremost, was the problem of insuring that the area photographed was representative of the whole spectrum of attacked granules. This problem becomes greater when working at high magnifications (2000-3000 diameters). To overcome this, large areas of each stub were scanned and then an area selected as representative of the whole was photographed.

TABLE V

Activity^a of Glucoamylase on Various Substrates Measured by
D-Glucose Release

Enzyme ^c	Substrate ^b			
	Starch	Amylose	Amylopectin	Waxy Sorghum β -limit Dextrin
<u>A. niger</u>				
Glucoamylase I ^d	9.8	9.7	9.7	8.5
Glucoamylase II ^d	10.0	8.4	9.1	7.9
Glucoamylase II ^e	10.3	8.7	10.5	8.8
Reconstituted ^d	9.9	7.8	9.7	8.4
<u>Rhizopus niveus</u>				
Glucoamylase	10.3	6.7	6.7	6.4

^aOne unit is the amount of enzyme required to release 1 μ mole of D-glucose per minute at 30°C in 0.05 M citrate buffer, pH 5.0. Activities are given as units per ml of enzyme solution.

^bStarch concentration was 40 mg/ml. All other substrates were 10 mg/ml.

^cSame enzyme dilutions and preparations were used for all substrates.

^dPurified from Diazyme concentrate.

^ePurified from Diazyme 160.

Correlating release of D-glucose with extent of granule degradation, determined by SEM, was attempted in only a general sense.

It was also our conclusion that better resolution resulted from an accelerating voltage of 20-kv (many workers use 10-kv). Viewing the stub at an angle (45°) increased contrast, thus allowing visualization of finer surface details such as the small pitting found in early stages of enzymatic degradation (See Figure 52).

Enzymatic attack of large wheat starch granules by glucoamylase I (Table II) from A. niger is illustrated in Figure 5. Attack starts on the surface of the granule as small pits and along the equatorial region of the starch granules in what begins to appear as an equatorial groove (Figure 5a and b). This equatorial groove seldom appears in granules which have not been exposed to enzyme (Figure 1c). At later stages attack develops more extensively on the surface of the granule rather than at the equatorial groove (Figure 5c and d). At 64 hours incubation, most of the attack is seen as large pits on the granule surface penetrating through several layers of the granule. The granule located in the center of Figure 5e has been degraded extensively into the inner portions of the granule.

Attack by glucoamylase II, as depicted in Figure 6, shows a much different mode than that for glucoamylase I. Extensive pitting of the granule surface is not apparent with glucoamylase II; rather, attack appears to be directed in a radiating manner away from the center of the granule surface producing furrows. These radial furrows (as opposed to an equatorial groove) appear to be the major attacking mode with only minor pitting of the surface (Figure 6d). Even granules with a small amount of surface pitting seem to have it arranged in a radial manner. This is evident in the large

FIGURE 5. Action of glucoamylase I from A. niger on wheat starch (Scout-73); see Table II). Incubation periods: a. 2 hours; b. 16 hours; c. 32 hours; d. 64 hours.

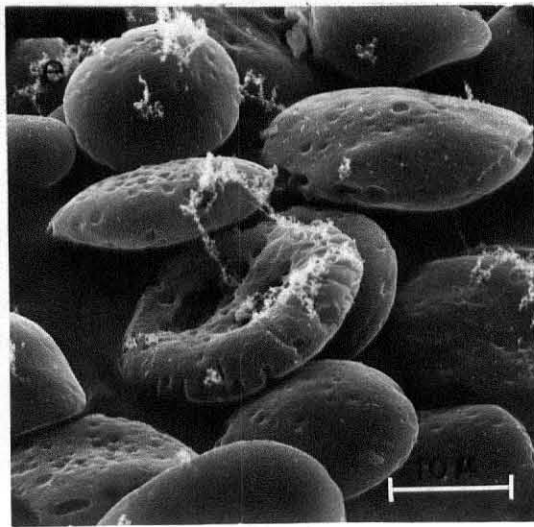
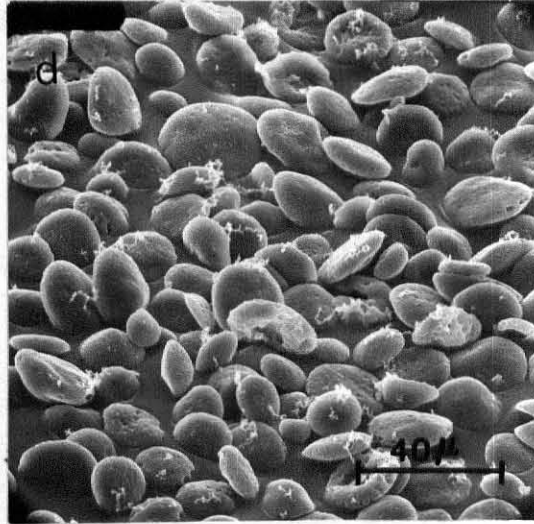
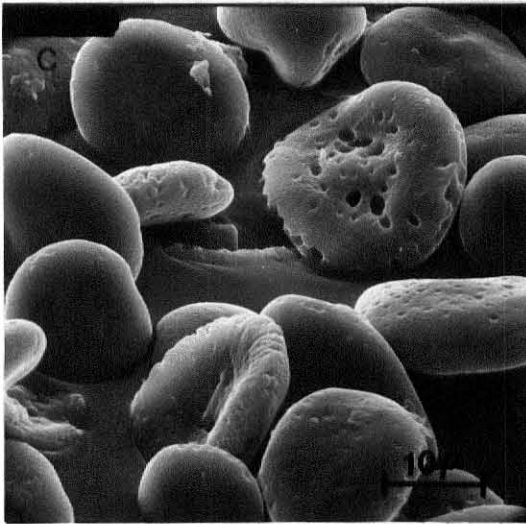
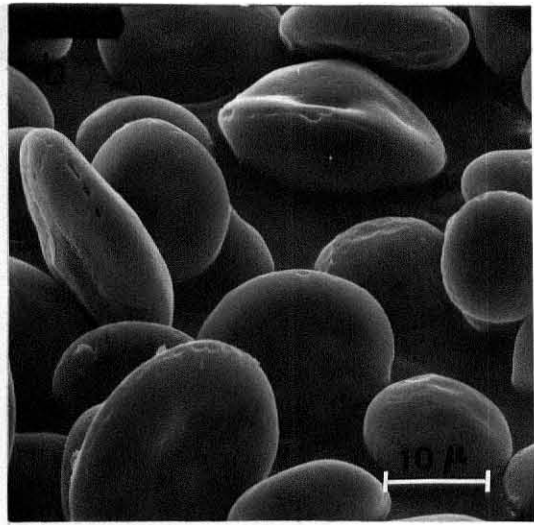
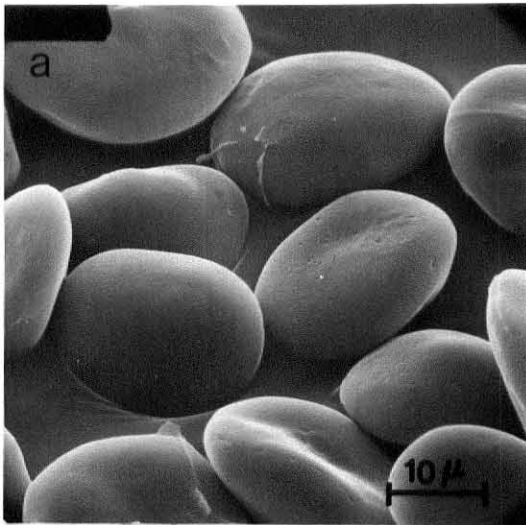
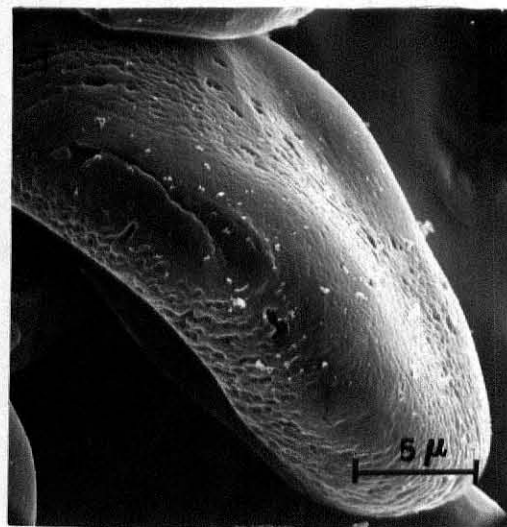
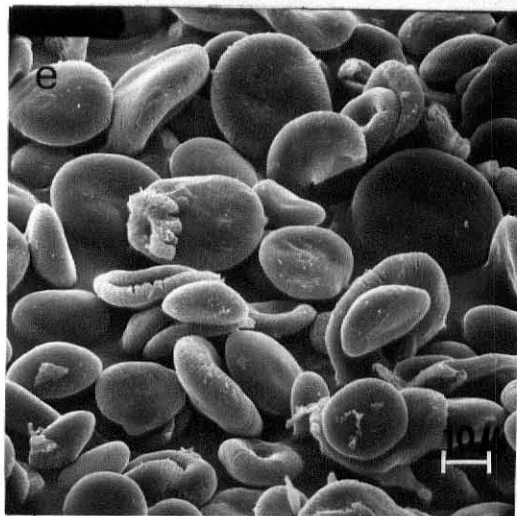
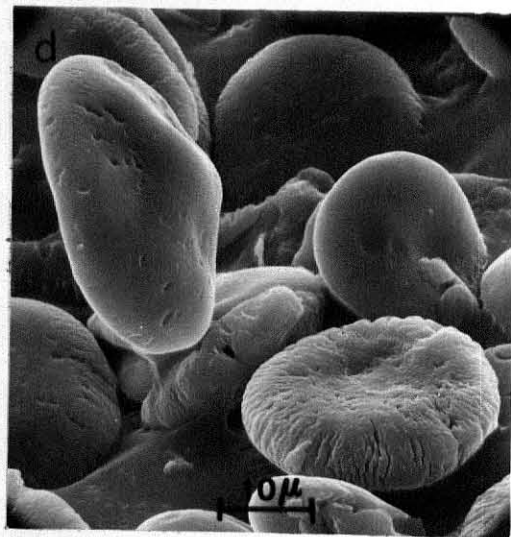
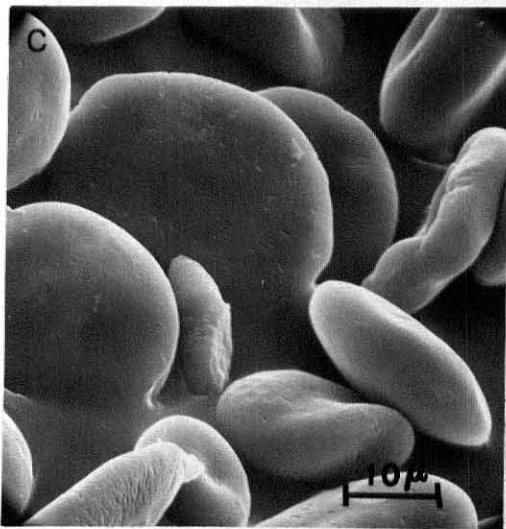
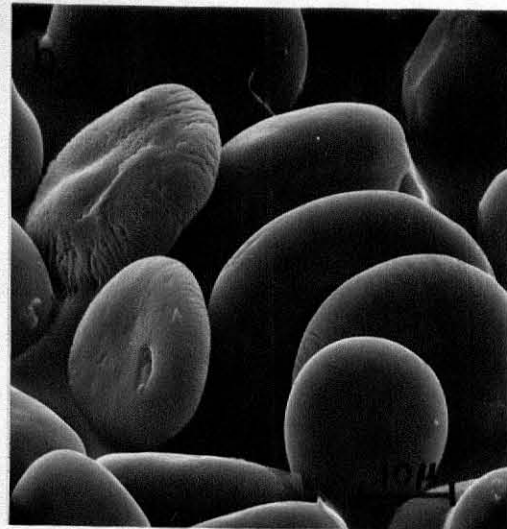
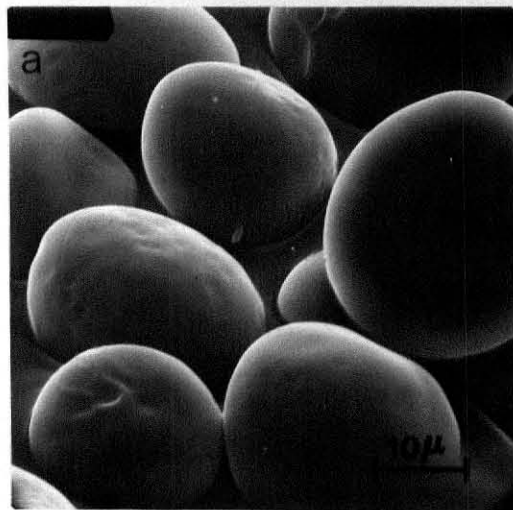


FIGURE 6. Action of glucoamylase II from A. niger on wheat starch (Scout-73; see Table II). Incubation periods: a. 2 hours; b. 8 hours; c. 16 hours; d. 32 hours; e. 64 hours; f. 64 hours.



lenticular granule in Figure 6c. The presence of many granules with collapsed centers (Figure 6e) is an artifact resulting from sample preparation (i.e. vacuum drying the sample). Granules which have not been attacked by enzyme or attacked to only a small extent don't behave this way (Figure 6a, b and c); therefore, it is our contention that the interior of collapsed granules have either been digested or severely disrupted allowing collapse to occur under reduced pressure.

Attacks of wheat starch granules with reconstituted glucoamylases I and II from A. niger resulted in hydrolysis patterns similar to those obtained with glucoamylase I alone. During the early stages of attack (Figure 7a, b and c) the equatorial groove is again accentuated by enzymatic attack. However there is also extensive attack over the entire surface of the granule resulting in the pitting seen with glucoamylase I. Radial channels observed with glucoamylase II are only apparent on a few granules (Figure 7c and d). The presence of radial furrows is not noted at later digestion times (Figure 7e and f). At 64 hours erosion of the granules indicate a more or less uniform attack (Figure 7f). This pattern of attack at the longer incubation times is very similar to that reported by Evers et al. (4) using a crude glucoamylase system from A. niger.

Enzymatic attack of large wheat starch granules by the glucoamylase from R. niveus (Figure 8) showed some development along an equatorial groove (Figure 8a, b and c). In some granules (Figure 8c) enzymatic attack extended along the equatorial groove. The small surface pits seen during earlier stages of hydrolysis apparently expand resulting in large pits after longer incubation times (Figure 8e). Shetty et al. (58) using the same enzyme

FIGURE 7. Action of reconstituted glucoamylase I and II from A. niger on wheat starch (Scout-73; see Table II). Incubation periods: a. 4 hours; b. 8 hours; c. 16 hours; d. 32 hours; e. 32 hours; f. 64 hours.

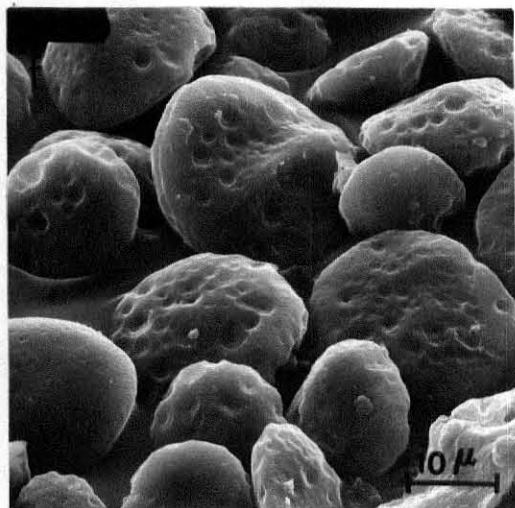
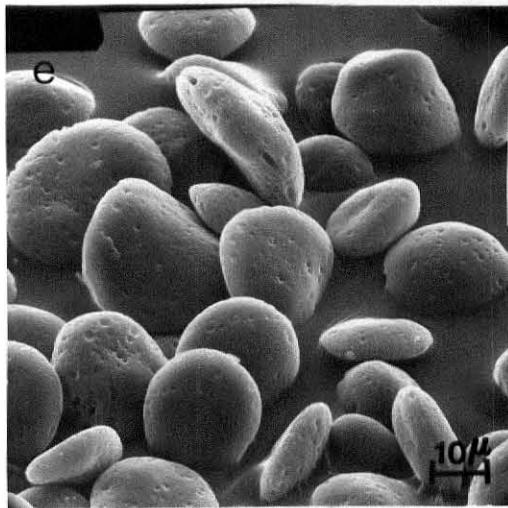
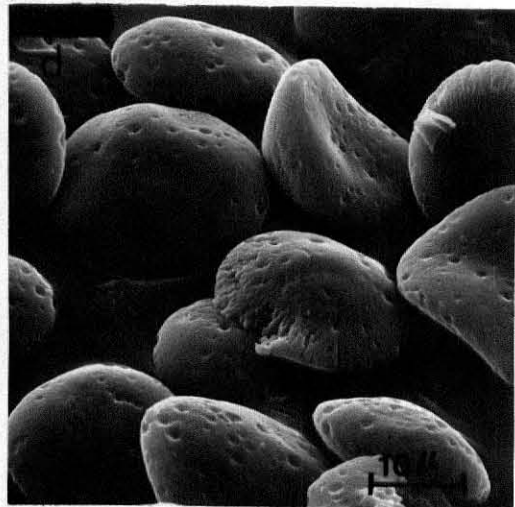
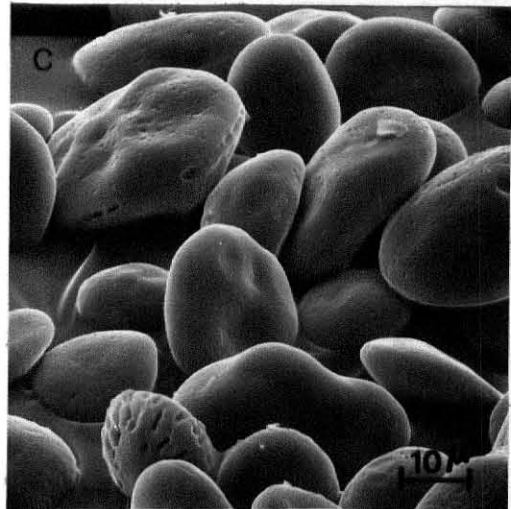
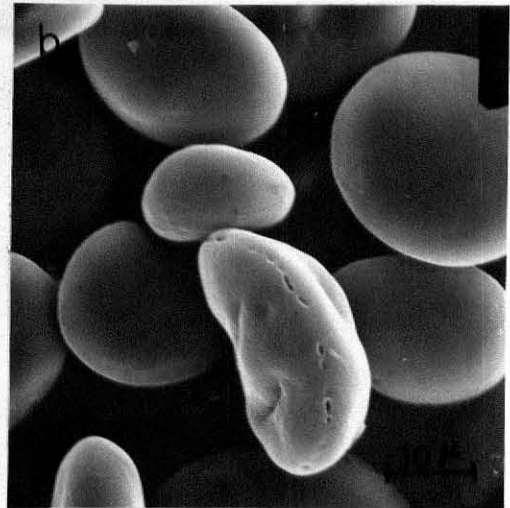
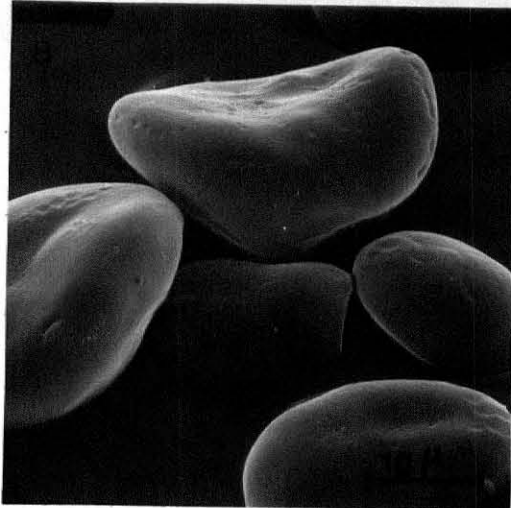
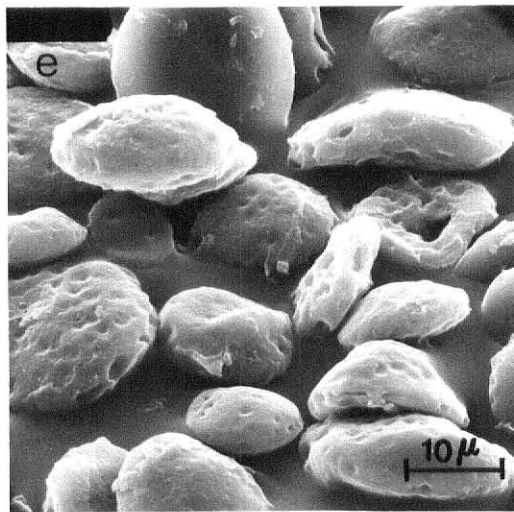
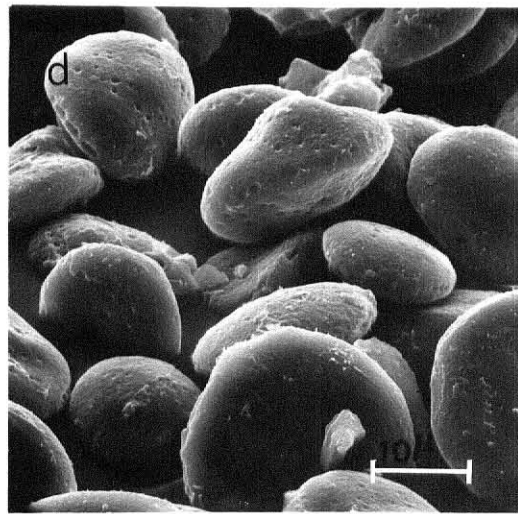
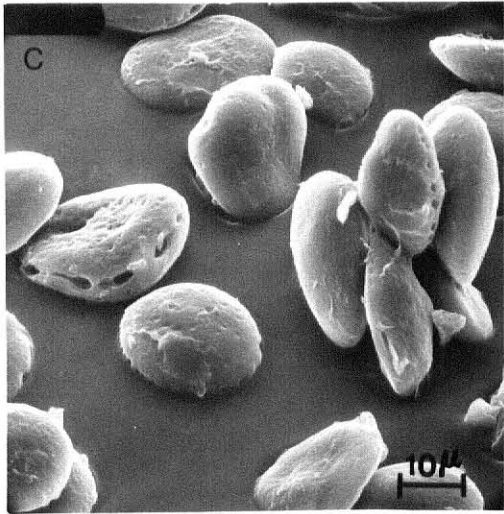
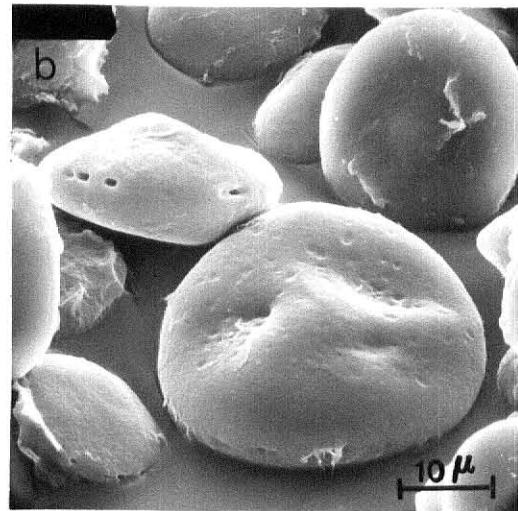
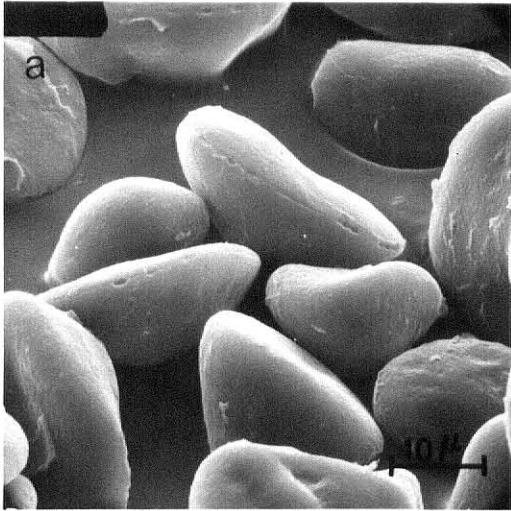


FIGURE 8. Action of the glucoamylase from R. niveus on wheat starch (Scout-73; see Table II). Incubation periods: a. 2 hours; b. 4 hours; c. 8 hours; d. 16 hours; e. 32 hours.



observed extensive "pin-holing" quite different than what we observed.¹¹ The hydrolysis rates we obtained are much higher (Table II) than those of Shetty *et al.* (58), thus direct comparison of the results may not be possible.

Examination of the attack of corn starch granules by the four enzyme systems (Figures 9-12) by scanning electron microscopy surprisingly revealed very similar patterns. No erosion of corn starch granules was observed along an equatorial groove. This observation agrees with a report that corn starch granules do not contain equatorial grooves (48).

The action of glucoamylase I from *A. niger* on corn starch granules (Figure 9) is very similar to that seen for attack of wheat starch granules by the same enzyme (Figure 5) except for the absence of attack along an equatorial groove. Extensive attack is seen at earlier times in corn starch but this is expected since corn starch is hydrolyzed more extensively than wheat starch (Compare Tables II and IV). A slightly different phenomenon observed in the attack of corn starch involves "pin-holing." These are small, deep pits in which the enzyme penetrates the granule, apparently hydrolyzing the starch without greatly expanding the diameter of the pits as penetration into the granule occurs. At longer digestion times the granule has developed large pits extending to the center of the granule (Figure 9e). When magnification of the SEM was three-fold that of Figure 9e, the center of the granule was seen to be hollow.

Glucoamylase II (*A. niger*) shows radial furrows (Figure 10) on corn starch very much like that seen with wheat starch. The furrows develop early

¹¹Shetty *et al.* (58) used a soft red winter wheat starch while we used a hard red winter wheat starch. Since hardness is related to adhesion between starch and protein in the endosperm (70), not in the starch granules, not much difference would be expected between the two starches.

FIGURE 9. Action of glucoamylase I from A. niger on corn starch (see Table IV). Incubation periods: a. 2 hours; b. 4 hours; c. 8 hours; d. 16 hours; e. 32 hours.

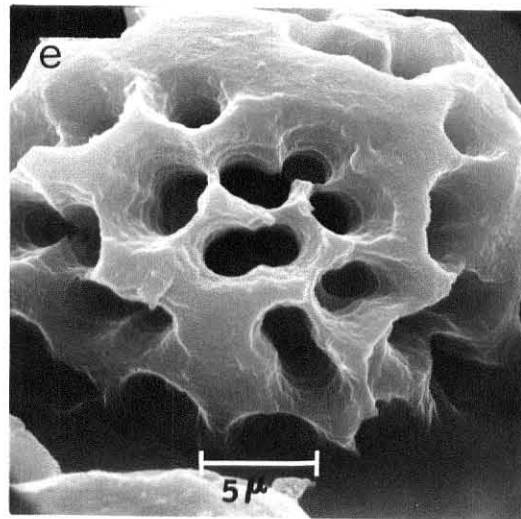
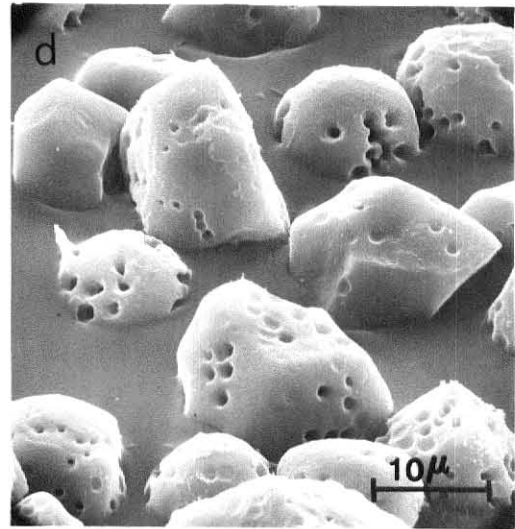
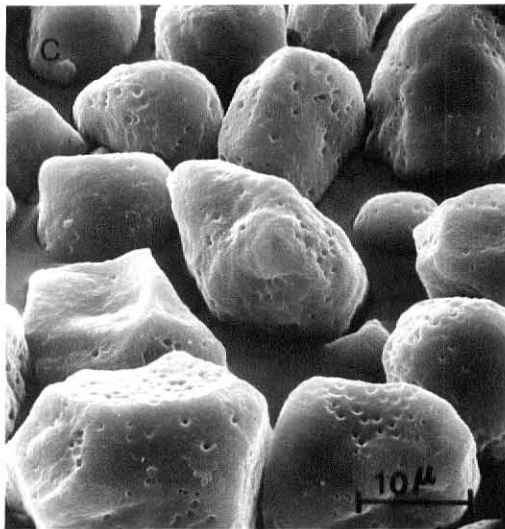
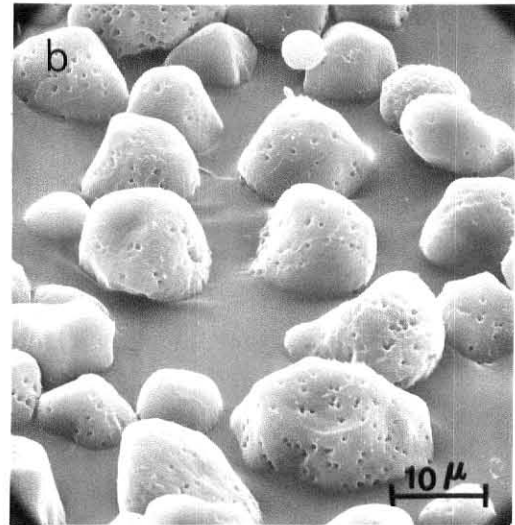
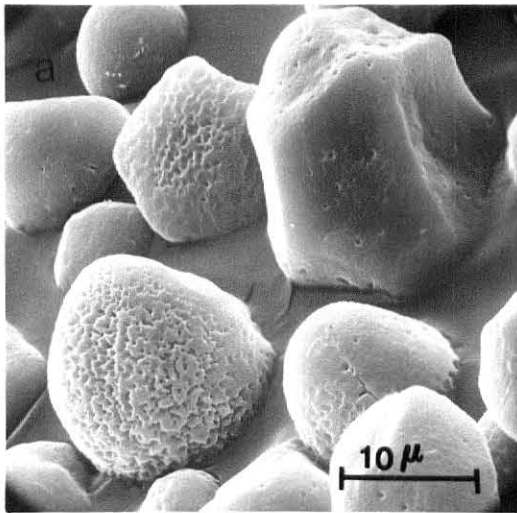
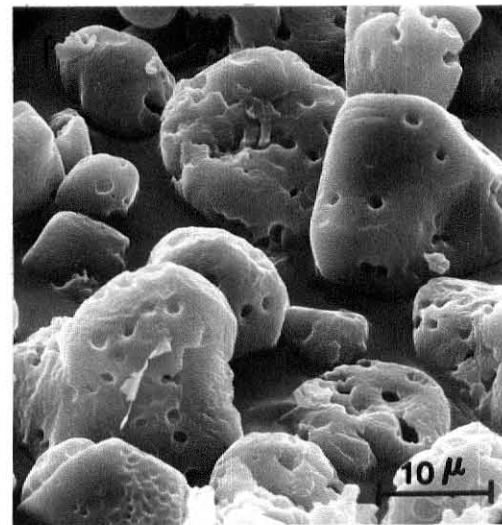
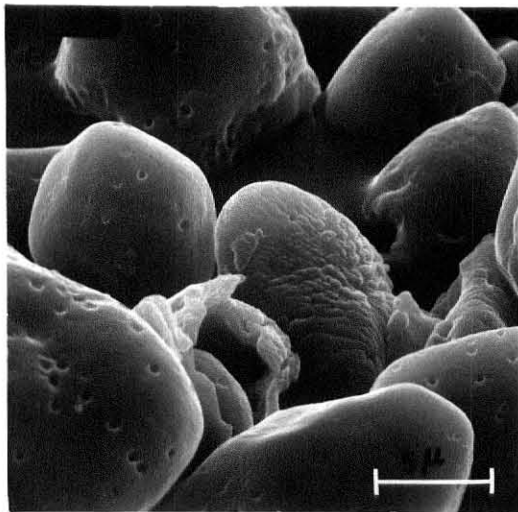
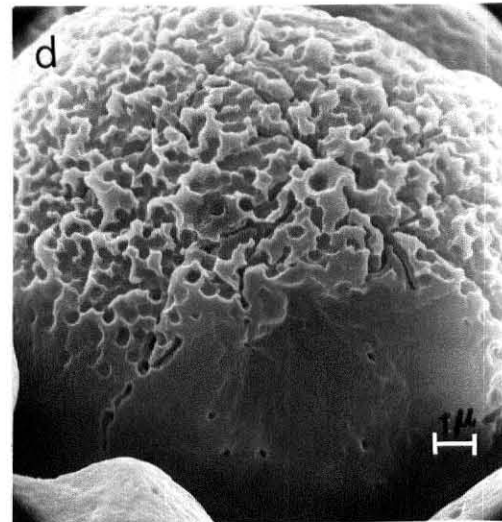
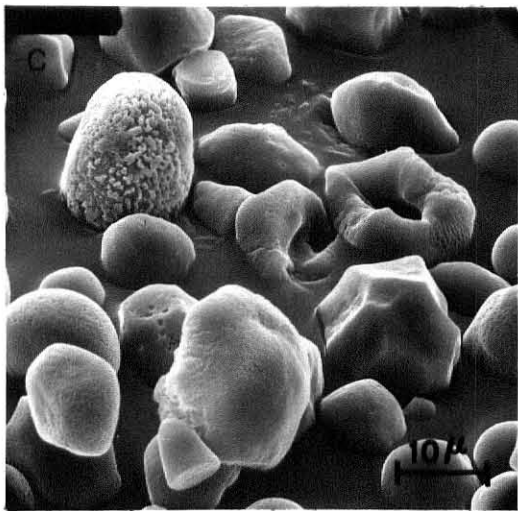
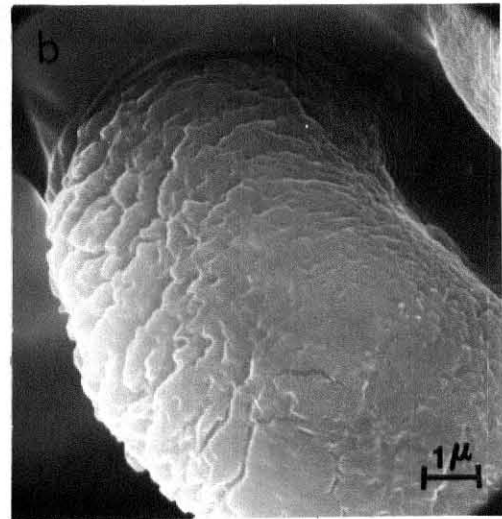
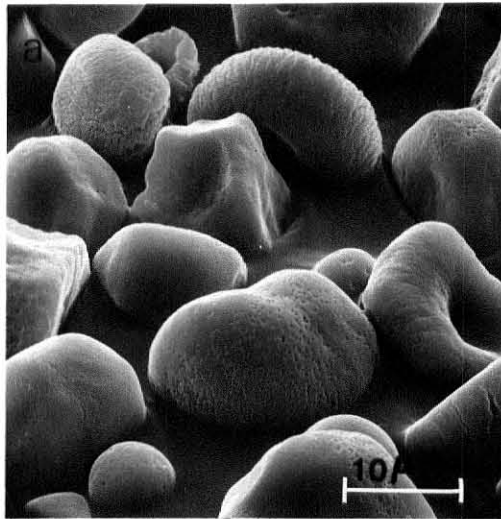


FIGURE 10. Action of glucoamylase II from A. niger on corn starch (see Table IV). Incubation periods: a. 8 hours; b. 8 hours; c. 16 hours; d. 16 hours; e. 32 hours; f. 100 hours.



and are evident throughout the incubation period. Figure 10c and 10d show a different type of "pin-holing." The enzyme has apparently hydrolyzed many parts of the granule surface but has penetrated deeper in only a few areas of the corn starch granule.

When reconstituted glucoamylase I and II was used on corn starch granules (Figure 11) a reduction in radial furrows is apparent. Radial furrows were seen in very few cases (see the granule in the top center of Figure 11c). "Pin-holing" was extensive initially (Figure 11b) and eventually developed into large pits (Figure 11e). After 32 hours incubation (Figure 11e) many granules were extensively damaged and only pieces of granules were visible in some cases.

Digestion of corn starch granules with the glucoamylase from R. niveus (Figure 12) follows a pattern similar to that of wheat starch (Figure 8). The initial development of small "pin-holes" (Figure 12a), eventually enlarging into deep pits (Figure 12c, d and e) was observed. The presence of concentric rings in the interior of the granules is evident in some fractured granules (Figure 12d).

Since the modes of attack by various glucoamylases on wheat and corn starches, with exception of the equatorial groove, are very similar, generalizations can be made. Attack along the equatorial groove of wheat starch granules raises some doubts about the existence of a groove in nondegraded, large, wheat starch granules. Figure 5a and 5b shows some indication of an equatorial groove in certain granules, but it is only partially defined. There are pits along the "equator" of the granule but the groove is not yet a distinct morphological entity. One has to extrapolate between pits to see a groove defined. The very obvious equatorial grooves described by

FIGURE 11. Action of reconstituted glucoamylases I and II from A. niger on corn starch (see Table IV). Incubation periods: a. 4 hours; b. 4 hours; c. 8 hours; d. 16 hours; e. 32 hours.

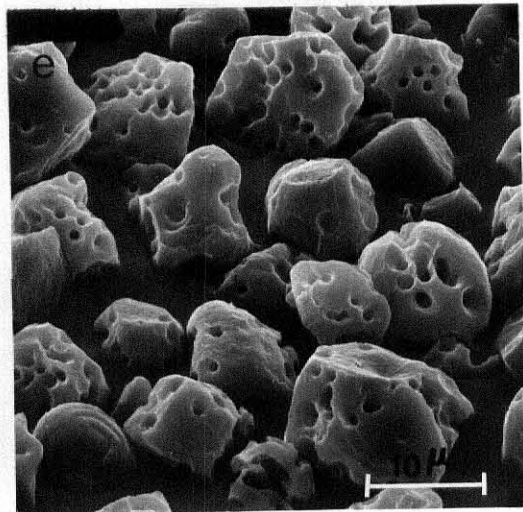
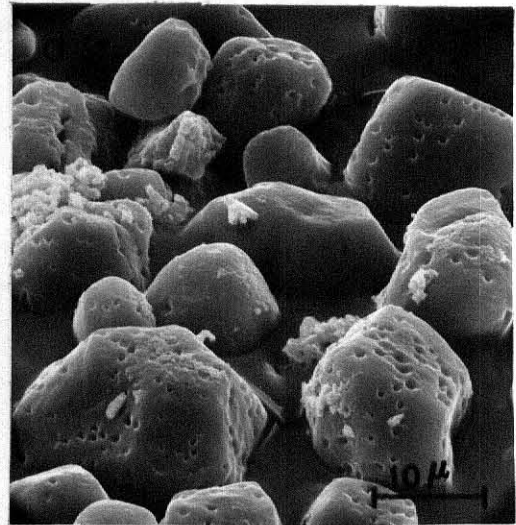
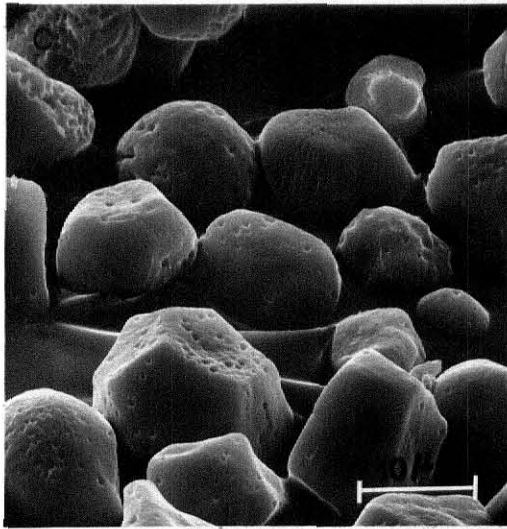
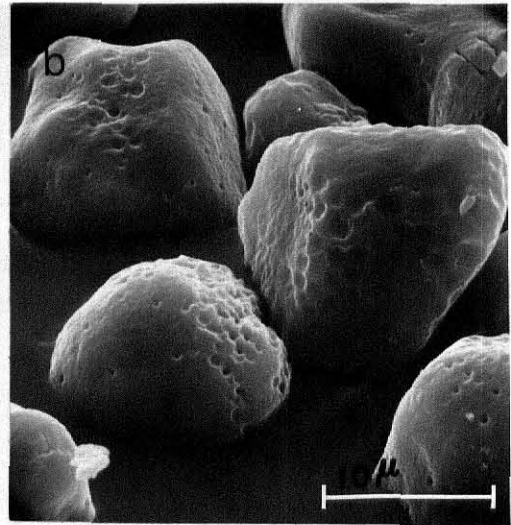
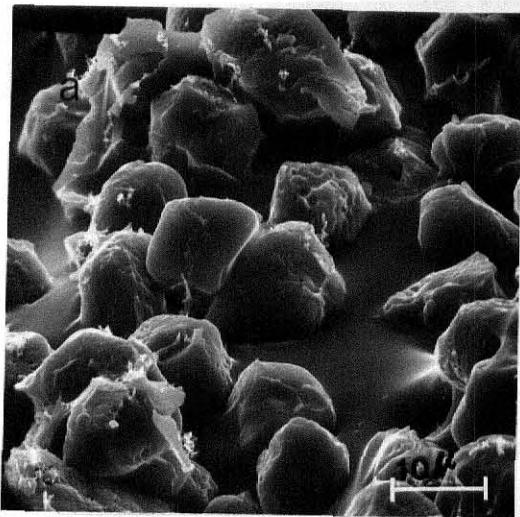
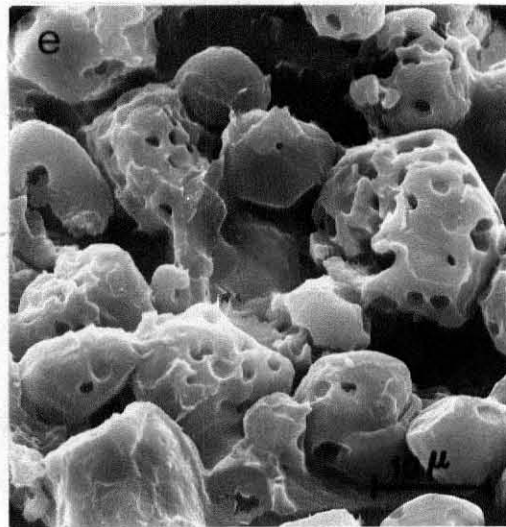
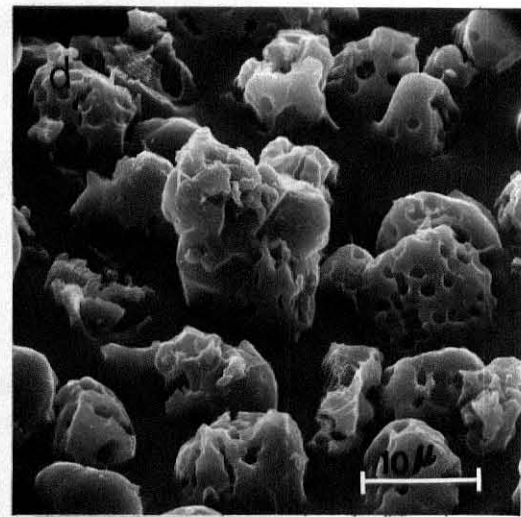
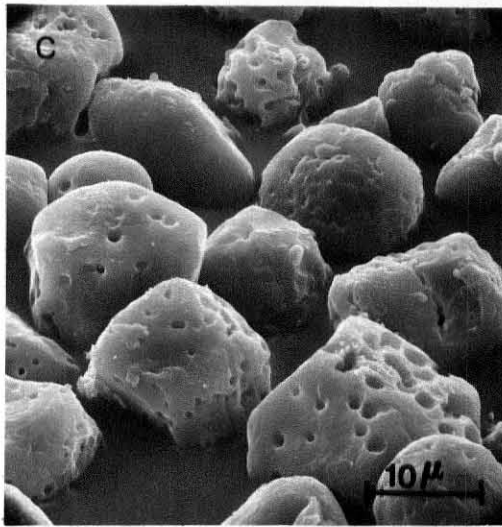
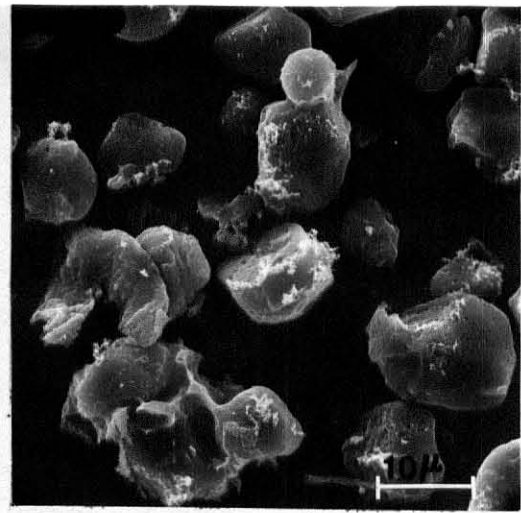
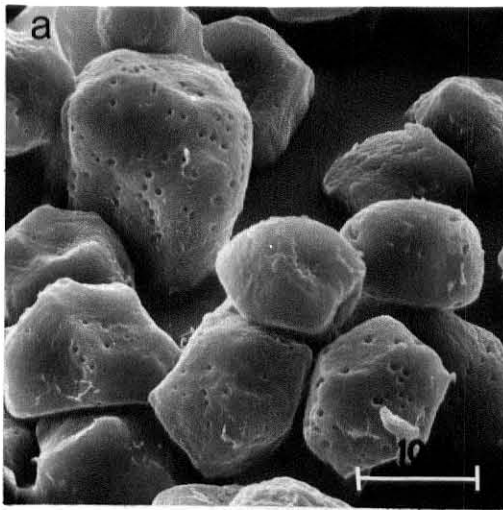


FIGURE 12. Action of glucoamylase from R. niveus on corn starch (see Table IV). Incubation periods: a. 2 hours; b. 4 hours; c. 8 hours; d. 16 hours; e. 16 hours.



Evers (71-73) were not apparent in our scanning electron micrographs of wheat starch. Equatorial grooves were seen in a small number of granules in the control sample (Figure 1c), but it is possible that these were produced by α -amylase in the endosperm of the wheat kernel.

The glucoamylases used in our study did not produce extensive attack along the equatorial region. The equatorial groove could be due to a structural weakness, a difference in the arrangement of amylose and amylopectin, or a combination of the two. Evers (50) has attributed the susceptibility of the equatorial groove to attack by α -amylase to an inturning of the concentric shells at this point presenting a weakness. If this were true, we would expect to see glucoamylase II readily attack the equatorial groove since it apparently cannot hydrolyze every portion of the granule and should attack areas of weakness. However this was not the case. We think a more plausible explanation is simply the availability and accessibility of internal α -1,4-links at the granule surface for enzymatic attack.

The difference in patterns of attack and degree of hydrolysis between glucoamylase I and II (A. niger) on the two starches can possibly be explained by a debranching effect as described by Ueda et al. (37). However, less suppositions would be required if molecular arrangements were discussed rather than simply bond-splitting abilities. If glucoamylase has weak native starch-hydrolyzing activity then attack should be centered on the weakest part of the granule or most favorable molecular arrangement. If the fibrillar concept of starch granule structure is accepted, then the weakest points should be lines radiating from the center of the granule (12). In support of this, we observed considerable attack by glucoamylase II along radial lines in both starches. Radial weakness has also been observed in gelatinized potato

starch (22). We feel this probably explains the pattern observed in degradation of starch granules by glucoamylase II and provides additional evidence in support of the fibrillar concept (72) of granule structure.

Radial weakness apparently isn't observed in attack of granules by glucoamylase I because the enzyme is not as limited in the type of substrate it requires. Thus the enzyme can attack about any portion of the granule and the radial grooves are not seen.

Another possible explanation for observed differences in attack of starch granules by glucoamylase I and II is the affinity of the enzymes for the starch substrate. Ueda et al. (37) found that glucoamylase II had almost no affinity for native corn starch granules. Smiley et al. (36) reported that glycogen was such a poor substrate for glucoamylase II (A. awamori and A. niger) that kinetic parameters of K_m and V_{max} could not be determined. Using soluble starch as substrate they found the K_m of glucoamylase II to be from 2 to 6 times greater than the K_m of glucoamylase I.

No readily apparent explanation can be offered as to why combining glucoamylases I and II increase hydrolysis above the average of the two individual rates. Perhaps the two enzymes are individually inhibited by some substrate or intermediate product, but when combined that inhibition is removed.

One general observation for all the degraded starches was the great differences in resistance to "burning" when exposed to the electron beam of the scanning electron microscope. This effect (i.e. "burning") has been studied by Kiribuchi and Nakamura (67) on nonenzymatically attacked granules from about ten different starch sources. "Burning" is actually an artifact produced by too high an accelerating voltage, magnification, or by excess

exposure of the granules to "reduced area."¹² The result is a swelling, at this point, giving the granule a "bump" and also cracking the coating. In nondegraded starches we also found this to be a problem and had to limit the magnification to around 2000 diameters. However, when viewing an extensively degraded granule the magnification could be increased considerably without "burning." We interpreted this to result (in a general sense) from the reduction of amorphous regions in the degraded starch granules.

¹²"Reduced area" is usually used for focusing the sample when taking a photograph. The scan speed is increased considerably in a "reduced area" which means the sample area is exposed to much larger amounts of beam electrons.

REFERENCES

1. Fleming, I.D., (1968), in *Starch and Its Derivatives*, Radley, J.A., Ed., London, Chapman and Hall, p 498.
2. Sandstedt, R.M. (1955), *Brewers Digest* 30, 69.
3. Buttrose, M.S. (1960), *J. Ultrastructure Res.* 4, 231.
4. Evers, A.D., Gough, B.M., and Pybus, J.N. (1971), *Staerke* 23, 16.
5. Manners, D.J. (1974), *Essays in Biochem.* 10, 37.
6. Williams, J.M. (1968), in *Starch and Its Derivatives*, Radley, J.A., Ed., London, Chapman and Hall, p 91.
7. Manners, D.J. (1962), *Adv. Carbohydrate Chem.* 17, 371.
8. Greenwood, C.T. (1970), in *The Carbohydrates*, Pigman, W., and Horton, D., Eds., Vol. II B, New York, Academic Press, 471.
9. Manners, D.J. (1973), in *Phytochemistry*, Miller, L.P., Ed., Vol. I, New York, Van Nostrand Reinhold, 177.
10. Bathgate, G.N., and Palmer, G.H. (1972) *Staerke* 24, 336.
11. Badenhuiizen, N.P. (1965), in *Starch: Chemistry and Technology*, Whistler, R.L. and Paschall, E.F., Ed., Vol. I, New York, Academic Press, 65.
12. Sterling, C. (1968), in *Starch and Its Derivatives*, Radley, J.A., Ed., London, Chapman and Hall, 139.
13. Banks, W., Geddes, R., Greenwood, C.T., and Jones, I.G. (1972), *Staerke* 24, 245.
14. Buttrose, M.S. (1963), *Staerke* 15, 213.
15. Badenhuiizen, N.P. (1969), *The Biogenesis of Starch in Higher Plants*, New York, Appleton-Century-Crofts.
16. Badenhuiizen, N.P. (1963), *Nature* 197, 464.
17. Robin, J.P., Mercier, C., Charbonniere, R., and Guilbot, A. (1974), *Cereal Chem.* 51, 389.
18. Muhlethaler, K. (1965), *Staerke* 17, 245.

19. Gruber, E., John, K., Alloush, S., and Schurz, J. (1972), *Microsc. Acta* 72, 189.
20. Meyer, K.H. (1952), *Experientia* 8, 405.
21. Banks, W., Greenwood, C.T., and Muir, D.D. (1973), in *Molecular Structure and Function of Food Carbohydrates*, Birch, G.G., and Green, L.F., Ed., Essex, Applied Science Publishers, 177.
22. Sterling, C. (1974), *Staerke* 26, 105.
23. Mussulman, W.C., and Wagner, J.A. (1968), *Cereal Chem.* 45, 162.
24. Nordin, P., Moser, J., Rao, G., Giri, N., and Liang, T. (1970), *Staerke* 22, 356.
25. Leach, H.W., and Schoch, T.J. (1961), *Cereal Chem.* 38, 34.
26. Whistler, R.L., Goatley, J.L., and Spencer, W. W. (1959), *Cereal Chem.* 36, 84.
27. Pazur, J.H., and Kleppe, K. (1962), *J. Biol. Chem.* 237, 1002.
28. Pazur, J.H., and Ando, T. (1960), *J. Biol. Chem.* 235, 297.
29. Pazur, J.H., and Ando, T. (1959), *J. Biol. Chem.* 234, 1966.
30. Pazur, J.H., and Okada, S. (1967), *Carbohydrate Res.* 4, 371.
31. Tsujisaka, Y., Fukumoto, J., and Yamamoto, T. (1958), *Nature* 181, 770.
32. Anonymous, *Technical Bull.*, Gluco-amylase, 2X Crystallized (from Rhizopus Niveus), Miles Laboratories Inc., Elkhart, Ind.
33. Lineback, D.R., Russell, I.J. and Rasmussen, C. (1969), *Arch. Biochem. Biophys.* 134, 539.
34. Andrzejczuk-Hybel, J., and Kaezkowski, J. (1971), *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* 19, 313.
35. Andrzejczuk-Hybel, J., and Kaezkowski, J. (1971), *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* 19, 451.
36. Smiley, K.L., Hensley, D.E., Smiley, M.J., and Gasdorf, H.J. (1971), *Arch. Biochem. Biophys.* 144, 694.
37. Ueda, S., Ohba, R., and Kano, S. (1974), *Staerke* 26, 374.
38. Marshall, J.J. and Whelan, W.J. (1970), *F.E.B.S. Lett.* 9, 85.
39. Marshall, J.J. (1975), *Adv. Carbohydrate Chem. Biochem.* 30, 257.

40. Kimoto, S. (1972), JEOL News 10e, 2.
41. Wivinis, G.P., and Maywald, E.C. (1967), in Starch: Chemistry and Technology, Whistler, R.L., and Paschall, E.F., Ed., Vol. II, New York, Academic Press, 649.
42. Everhart, T.E., and Hayes, T.L. (1972), Sci. Amer. 226, 55.
43. Anonymous, (1972), JEOL News 10e, 42.
44. Hall, D.M., and Sayre, J.G. (1970), Textile Res. J. 40, 147.
45. Hall, D.M., Patten, E.V., Brown, J.L., Harmon, G.R., and Nix, G.H. (1971), Ind. Eng. Chem. Prod. Res. Develop. 10, 171.
46. Hill, R.D., and Dronzek, B.L. (1973), Staerke 25, 368.
47. Aranyi, C., and Hawrylewicz, E.J. (1968), Cereal Chem. 45, 500.
48. Hall, D.M., and Sayre, J.G. (1970), Textile Res. J. 40, 256.
49. Hall, D.M., and Sayre, J.G. (1971), Textile Res. J. 41, 880.
50. Evers, A.D., and McDermott, E.E. (1970), Staerke 22, 23.
51. Dronzek, B.L., Hwang, P., and Bushuk, W. (1972), Cereal Chem. 49, 232.
52. Palmer, G.H. (1972), J. Inst. Brew. 78, 326.
53. Bathgate, G.N., Clapperton, J.F., and Palmer, G.H. (1973), Proc. European Brewery Convention 183.
54. Kiribuchi, S., and Nakamura, M. (1973), J. Japan Soc. Starch Sci. 20, 201.
55. Kiribuchi, S., and Nakamura, M. (1973), J. Japan Soc. Starch Sci. 20, 193.
56. Manners, D.J. (1971), Biochem. J. 123, 1P.
57. Rasper, V., Perry, G., and Duitschaeffer, C.L. (1974), Can. Inst. Food Sci. Technol. J. 7, 166.
58. Shetty, R.M., Lineback, D.R., and Seib, P.A. (1974), Cereal Chem. 51, 364.
59. Wolf, M.J. (1963), in Methods in Carbohydrate Chemistry, Whistler, R.L., Ed., Vol. 4, New York, Academic Press, 6.
60. Watson, S. A. (1963), in Methods in Carbohydrate Chemistry, Whistler, R.L., Ed., Vol. 4, New York, Academic Press, 3.
61. American Association of Cereal Chemists, Approved Methods (1969), St. Paul, Minnesota.

62. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. (1956), *Anal. Chem.* 28, 350.
63. Pirt, S.J., and Whelan, W.J. (1951), *J. Sci. Food Agr.* 2, 224.
64. Anonymous (June 1974), Glucostat reagent set for quantitative enzymatic determination of glucose, Technical Brochure, Worthington Biochemical Corp. Freehold, New Jersey.
65. Banks, W., and Greenwood, C.T. (1971), *Staerke* 23, 222.
66. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951), *J. Biol. Chem.* 193, 265.
67. Miller, G.L. (1959), *Anal. Chem.* 31, 964.
68. Bonitati, J., Elliott, W.B., and Miles, P.G. (1969), *Anal. Biochem.* 31, 399.
69. Anonymous, Laboratory Manual: Advanced ion-exchange celluloses, Whatman Biochem. Ltd., Clifton, New Jersey.
70. Simmonds, D.H., Barlow, K.K., and Wrigley, C.W. (1973), *Cereal Chem.* 50, 553.
71. Evers, A.D. (1969), *Chem. Ind. (London)*, 200.
72. Evers, A.D. (1969), *Staerke* 21, 96.
73. Evers, A.D. (1971), *Staerke* 23, 157.
74. Kiribuchi, S., and Nakamura, M. (1973), *J. Japan Soc. Starch Sci.* 20, 206.

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HYDROLYSIS OF NATIVE WHEAT AND CORN STARCH GRANULES BY
GLUCOAMYLASES FROM ASPERGILLUS NIGER AND RHIZOPUS NIVEUS

by

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The action of glucoamylase I and II from Aspergillus niger and the glucoamylase from Rhizopus niveus on native wheat and corn starch granules was followed by scanning electron microscopy (SEM) and by measurement of the glucose released by enzymatic attack. Two distinct patterns of attack were observed. Glucoamylase I and the glucoamylase from R. niveus gave relatively uniform attack of the granule surface resulting in large disc-like depressions. Glucoamylase II, while showing some disc-like depressions, exhibited extensive "pin-holing" with small grooves perpendicular to the edges of the granule. Similar patterns were observed for both corn and wheat starch granules, except that attack by glucoamylase I and the glucoamylase from R. niveus on wheat starch granules also developed along the area of the equatorial groove. Measurement of glucose released indicated that hydrolysis with glucoamylase I and the glucoamylase from R. niveus was nearly equal and was about twice that observed with glucoamylase II (in both starch samples). The equatorial groove previously reported for wheat starch granules was not predominant until the granules were exposed to enzyme solutions.