# EFFECTS OF GERMINATION OF WHEAT, OATS AND PEARL MILLET ON α-AMYLASE ACTIVITY AND STARCH DEGRADATION

by

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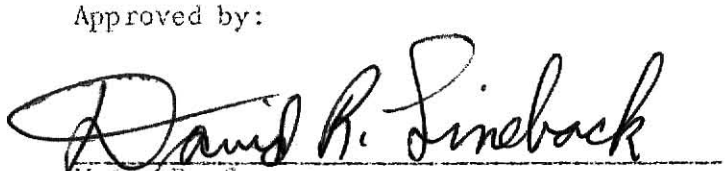

Major Professor

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# INTRODUCTION

For centuries, cereal malts have been widely used as one of the most important raw materials for production of beer and liquors and in supplementation of flours for bread baking. This is due to the production during germination of a large amount of a starch-degrading enzyme, $\alpha$-amylase, which is practically absent in ungerminated grain (1). This enzyme functions as a hydrolase catalyzing the breakdown of reserve starch in the endosperm (2,3); hence, it is active both *in vivo* and *in vitro* (4). $\alpha$-Amylase is unique in being the only plant enzyme capable of degrading native starch granules; other starch-degrading enzymes, such as $\beta$-amylase, $\alpha$-glucosidase, and limit dextrinase (5), have no action on intact starch granules (5,6).

In cereal fermentation processes, particularly with respect to the brewing and bread industries, the changes which occur to cereal starches during malting are an essential preliminary to conversion of starch into fermentable sugars for production of carbon dioxide and alcohol by yeast. *In vivo* studies on morphological changes of starch granules in germinating barley and wheat (7,8) revealed that $\alpha$-amylolytic attack on small starch granules differed from that on the larger ones; non-cereal starch granules from various sources were found to be considerably less susceptible to *in vitro* $\alpha$-amylolytic degradation (9). Preferential attack of small barley starch granules, lower susceptibility of small wheat starch granules, and higher resistance of non-cereal starch granules to $\alpha$-amylolysis can influence the rate of mash conversion, the rate of wort separation, and may considerably affect the dough system when wheat flour is replaced by 15 to 20% of non-cereal starches (9). These observations have recently stimulated much

research on physico-chemical properties, physiological behavior, and α-amylolytic degradation of starch granules from higher plants and in germinating grains. With the introduction of scanning electron microscopy to examination of the morphology of starch granules (10), studies of the shape, size and structure of starch granules and their relation to enzymic attack have been successfully accomplished.

Badi et al. (11) observed that when a portion of wheat flour in a bread formula was replaced by flour from ungerminated millet, malt or sugar was not required to produce an acceptable loaf of bread. This observation infers the presence of a very active α-amylase system in pearl millet.

Our investigation was initiated to study α-amylase activity in germinating wheat, oats and pearl millet and the effects on native starch. Oats were included since it is known that oat α-amylase is not suited as a supplement for baking purposes (12). Starch damage, the presence of starch degradation products (soluble sugars), and α-amylase activity were determined in flours prepared from germinated wheat, oats and millet. Endosperm and starch granules, isolated from germinated grains, were investigated by scanning electron microscopy (SEM) to determine the nature and extent of attack by α-amylase on the starches.

It was hoped that information from this study would better explain previous observations with oats and millet in relation to replacement of more expensive malted wheat or barley flour.

# LITERATURE REVIEW

Starch is a reserve carbohydrate found in plastids of higher plants, in chloroplasts of leaves, and in leucoplasts of storage tissues such as cereal endosperm and potato tuber. It is a spherocrystal (13) consisting of a mixture of two polysaccharides, amylose and amylopectin, which form a para-crystalline network within the granule. Amylose is a linear polymer of $\alpha$-1, 4-linked glucose residues. Amylopectin is a branched molecule in which linear chains are interlinked by $\alpha$-D-1,6-glucosidic bonds. In the granule, amylose and amylopectin are deposited in an unknown manner such that the starch appears to be arranged in a radial direction in concentric shells (14).

Starch occurs as water-insoluble, non-dispersible granules whose size and shape vary with the species and maturity of the plant. On heating starch in aqueous solution, gelatinization occurs as evidenced by swelling of the granule, simultaneous loss of birefringence, eventual solubilization, and increase in consistency or apparent viscosity of the starch suspension. The ratio of amylose to amylopectin content is involved in the viscosity of gelatinized starch solutions and pastes (15). The gelatinization temperature is characteristic of the plant species from which the starch is isolated and is normally in the range of 55-80°C (5).

Unless the crystallinity of starch granules is destroyed by heat or by mechanical action, intact starch granules from the endosperm of cereals are relatively resistant to amylolytic degradation. $\alpha$-Amylase has been reported (16-18) to be 165 to 7,000 times as active on gelatinized starch as on undamaged starch.

Development of Starch Granule

The development of starch granules in the endosperm of barley, oats, maize, rice and wheat has been studied by Buttrose (19,20) and Evers (21). At maturity barley endosperm contains two types of starch granules having distinct mean diameters of approximately 5 and 25 $\mu$ (7). In wheat, there exists a continuous distribution from small spherical granules of 2-10 $\mu$ diameter to large lenticular granules of 25-35 $\mu$ (22). The "A-type" or large lenticular starch granule of barley and wheat is the first to develop and each A-type granule develops within its own individual amyloplast to form a simple starch granule. Formation of "B-type" or small spherical starch granules is not initiated until the A-type have reached their maximum size. B-type granules develop under conditions of tight packing in evaginations of the membrane surrounding the A-type granule and many adopt the shape of the limited space in which they develop (23). A feature peculiar to A-type granules is the existence of an equatorial groove (24) which is more distinct in wheat than in barley. The equatorial groove was shown by Evers and McDermott (24) and Buttrose (20,21) to be a median plane of weakness for amylolytic enzyme attack and cracking. It is evident (19) that the starch in this region of granule has special properties, possibly the molecules being not as tightly packed as in other regions. The presence of an equatorial groove during growth suggested (21) that starch deposition in this region is abnormal. Possibly non-starchy material is entrapped in this region during starch crystallization, thus causing the structure to be more open and susceptible to splitting and enzyme attack (19). Evers (23) suggested that preferential enzyme attack at the equatorial groove might be due to the equatorial plane representing a discontinuity in each shell due to formation of a shallow

trough, which becomes progressively more shallow from the center outwards, around the equatorial region in each successive concentric shell.

All starchy endosperm cells arise by division of cells of the peripheral meristematic layer which later becomes the aleurone layer (25). Starch granules in the layer of endosperm immediately adjacent to the aleurone layer are the newly divided cells and are typically of a uniform size intermediate between the small and large starch granules at the center (26) which are among the oldest cells of the endosperm. Little is understood concerning the mode of biosynthesis of starch, especially regarding the way in which amylose and amylopectin are simultaneously produced within one plastid, and subsequently formed into a starch granule. However, the starch granule was shown not to arise by rapid crystallization but grew over an extended period of time (27) by apposition (28).

The development of oat and rice starch granules differ from that of barley and wheat in that, in young proplastids, separate starch granules form, rapidly grow in size, and new ones are initiated so that soon they fill the volume of the plastids to form characteristic compound starch granules (19). The whole structure continues to grow slowly, the individual granula[a] becoming tightly packed so that each granulum becomes polyhedral. Hall and Sayre (29) confirmed that most oat starch granules are composites of both round and polyhedral shape held within a compound cluster. They proposed that aggregation of oat granula was due to electrostatic attractions between the flat surfaces of a large number of small-sized starch granules. Some layering of oat starch granules was evident under the scanning electron microscope (29), but it is not clear whether this was due to radial or stacking layering.

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[a] granulum (-la) - the separate constituents of a compound granule.

Morphology of Starch Granules Modified by α-Amylase

In order to facilitate amylolytic degradation, the crystalline structure of native starch granules has to be disrupted. This was clearly demonstrated when Lelievre (30) found that amylolytic degradation increased as the amorphous content of starch was increased due to mechanical forces during milling. However, incubation of suspensions of wheat (24) and barley (19) starch with α-amylase revealed that native starch granules can be partly degraded by this enzyme (31). Evidence indicates that α-amylase acts (34) in dissolution of starch granules in two stages. Firstly, an initial dextrinization occurs during which starch macromolecules are broken down into fragments of intermediate molecular weight. These large oligosaccharides are then broken down further to maltose, maltotriose and small "limit dextrins" in a saccharification process (34).

With large barley and wheat starch granules enzyme attack occurs most readily in the equatorial plane and at certain points on the major surfaces of the granule (25) corroding a channel into the center of the granule where the starch is apparently much more susceptible to enzymic attack. Susceptibility to attack is evidently a property of each individual granule. Large starch granules become rough in appearance from dissolution of part of the outer surface. Small starch granules were found to be more resistant to α-amylase (7,23,33), particularly small wheat starch granules. Small barley starch granules, though less susceptible to amylolytic attack than large granules, are more easily hydrolysed than small wheat granules. It was reported that small barley starch granules have higher amylose content (22) but it is not yet certain that this is responsible for the difference in amylolytic susceptibility. The inner portion of small granules are more

readily attacked than peripheral portions (19,34,35). This observation was explained by the observation that, as the granule grows by apposition, the central (oldest) portion is less dense and more loosely packed than the outer regions (15,27). The enzyme appeared to enter the center of the small granule at one or two sites and to completely digest the interior core of the granule, leaving the outer shell unattacked (16,19).

The in vivo degradation of barley and wheat starch granules during germination has been investigated by many research workers (3,7,14,33,34,36). Evidence indicates that $\alpha$-amylase produced during germination is the predominant enzyme involved in starch degradation (37) and the extent to which starch is degraded parallels the development of $\alpha$-amylase activity (34,36). Modifications of both small and large starch granules due to $\alpha$-amylolysis during sprouting were similar to that observed in vitro (36). Kiribushi and Nakamura (34) reported that in germinating barley circular pits appeared randomly distributed over the surface of large starch granules; pits appeared preferentially in the equatorial region. Small starch granules of barley disappeared during the early stages of germination. With progress of germination, erosion progressed through the layers of the large starch granules towards the interior where the starch appeared to be more susceptible to enzymic attack than the outer layers. This is of major importance to the brewing industries in that the modified large starch granules in malt are hydrolysed at a faster rate during mashing than those from ungerminated barley, and small starch granules in malt can be degraded to a greater extent than those from ungerminated barley due to partial removal during malting of protein in which they are embedded (23).

Malting of oats caused changes to the starch granule indicated by a

limited decrease in the molecular size of amylose, and a small, but significant, reduction in the exterior chain length of amylopectin (37); changes which are similar to those observed when oat starch granules are subjected to limited $\alpha$-amylolysis *in vitro* (37). Such changes could be due to limited $\beta$-amylolysis of both starch components. Sutcliffe and Baset (38) have shown that the rate of disappearance of starch from endosperm of oat seedlings was closely correlated with the rate of increase in amylase activity. Hence it was concluded that $\beta$-amylase was the principal enzyme involved in starch degradation in oats during germination.

The $\alpha$-amylolytic corrosion of rice and oat starch has been reported by Buttrose (19) who indicated that individual granules had a very resistant outer portion while the center of the granules was rapidly degraded. While the individual granula in compound rice or oat granules are polyhedral, enzyme attack often clears more of a spherical area; possibly the inner wall exposed by enzyme attack follows the outline of a shell which, away from the periphery, may be spherical.

Until recently, only limited information was available concerning the structure of millet starch granules and its relation to amylolytic attack. An attempt has been made by Rasper *et al.* (9) to reveal the mode of *in vitro* enzymic attack on granules of non-cereal starches, as well as millet and sorghum. They reported, using scanning electron microscopy, that enzymic erosion of millet and sorghum granules appeared as discrete, more or less circular holes penetrating through several layers of the granule into the interior with corrosion of the surface of the starch granules to give a sponge-like appearance. $\alpha$-Amylase was found to degrade millet starch selectively, severely attacking some granules while others remained unchanged. Neither

the existence of an equatorial groove nor preferential enzyme attack in this region has been reported for millet starch.

SEM of the Endosperm of Cereals during Germination

During the germination of cereals, a number of significant biochemical changes occur. These include the synthesis of several hydrolytic enzymes, including both carbohydrases and proteases (39,40), changes in the solubity of the endosperm cell walls, and limited degradation of reserve starch and protein.

Unless the endosperm cell walls are degraded it might be difficult for the $\alpha$-amylase in aleurone cells (7,36) or pericarp (41) to pass into the endosperm and, hence, for initial starch degradation to occur. $\alpha$-Amylase is secreted into the endosperm, together with proteases and dextrinases, during malting (3). Thus, the first starch-containing cell layers adjacent to the aleurone cells are particularly rich in $\alpha$-amylase and are found to show a higher degree of amylolytic attack than those in the inner endosperm. It has been suggested that reserve starch in ungerminated grain might be subjected to enzymic degradation during the ripening of barley seed (42).

During the early stages of barley germination, the cementing protein, in which the starch granules are embedded, disappeared; starch granules freed from protein and small granules of barley starch then disappeared (42). As germination progressed starch granules appeared to be covered by amorphous appearing material, suggested (42) to be dextrins; equatorial grooves were apparent in large granules in the endosperm near the scutellum and were found mainly in the proximal (embryo) half of the kernel rather than the distal end (7). A small portion of severely degraded granules and dextrins remained during the later stages of germination giving an amorphous mass (42). In

germinating wheat, Dronzek et al. (36) showed that α-amylolytic attack was confined to large lenticular type granules during the early stages of growth; both large and small starch granules were severely eroded when the seed was germinated for 8 days at 20°C. Enzymic degradation was accompanied by production of free sugars and contributed to the starch-damaged values for flours milled from sprouted wheat. The mode of enzymic attack in wheat starch granules was similar to that observed in malted barley.

No information was found regarding morphological changes of starch granules and endosperm during germination of oats and millet.

# MATERIALS AND METHODS

## MATERIALS

Three different cereal grains were used for this study: hard red winter wheat (Scout 1973), oats and pearl millet. The genetic backgrounds and conditions under which wheat and oats were grown were not obtainable. Pearl millet were a randomly mated population of $F_3$ x $F_4$ generation obtained from a cross of Serere 17 and Tift 239 grown at the Agricultural Experiment Station of Kansas State University in 1974. Chemicals used for analytical purposes were reagent grade unless otherwise specified. Scanning electron microscopic observations were made using an ETEC Autoscan scanning electron microscope at an accelerating voltage of 20kv.

## METHODS

Malting and Milling. Germination of 30-g. samples of wheat, oats and millet was accomplished in a test tube according to Whitmore and Sparrow (43). Samples (120-150 g.) of wheat and oats were cleaned and sieved to obtain kernels of uniform size. Broken kernels were manually removed and the sound grains were steeped with three changes of tap water at 15°C for 48 hr. After filtering, eight 30-g. samples were weighed and placed in 6 in. x 1 in. Pyrex test tubes. The tubes were stoppered with corks containing a 3/16 in. diameter hole. The grains were germinated at 15 $\pm$ 1°C. in an upright position for differing periods of time. After 0,2,4,6,8,10,12 and 14 days, samples (30 g.) were removed, frozen, and freeze-dried for 48 hr. Rootlets and coleoptiles were removed from the samples by rubbing the dried malts in a plastic bag and sifting the material on a wire sieve (8-14 mesh). Moisture content

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was measured in all wheat samples (control, soaked, and seven germinated) by drying a 0.5 to 1.0-g. sample at 130°C for 1 hr. (44). After tempering wheat and millet samples to 15.5% moisture, the germinated grains were milled into a straight-grade flour on a Quadrumat Junior experimental mill; oat samples were milled directly.

Germination of millet was slightly modified by steeping the grains in water containing 0.3% calcium hypochlorite for 6 hr. Samples were rinsed with this solution every other day during the germination period. This modification was used to surface sterilize the millet to prevent mold infestation which occurred to a large extent in the absence of this procedure.

Identical germination procedures were also accomplished at 20°C. to compare the effect of germination temperature on α-amylase production and starch degradation during germination.

Determination of α-Amylase Activity. A method recently developed by Barnee and Blakeney (45) was used for the determination of α-amylase activity. This method enabled the determination of α-amylase in the presence of β-amylase by employing a synthetic, partially-hydrolysed potato starch, cross-linked by 1,4-butandioldiglycidether to form a granular substrate which is susceptible to α-amylase attack but resistant to degradation by β-amylase. The substrate is commercially available as Phadebas tablets (Pharmacia Laboratories, Inc., Piscataway, N. J.). Maleate buffer (0.05 M), pH 6.0, was used to lower the pH of a solution of the commercial Phadebas substrate from 7.0 to 6.25. A partially-purified wheat α-amylase was prepared from commercially malted wheat (Ross Industries, Inc., Wichita, Ks.) according to Greenwood and Milne (46) and was assayed for α-amylase activity by the procedure of Robyt and Whelan (47). The latter procedure for α-amylase activity involves adding

increasing aliquots of an α-amylase solution (up to 1 ml) to 1 ml of starch solution, prepared according to Strumeyer (48). The solution was incubated in (0.1 M) acetate buffer, pH 5.5, containing 0.001 M calcium chloride at 25°C. for 10 min. Reducing substances were measured by the Nelson copper reducing method (47). The activity was determined in International Enzyme Units (EU) per ml of enzyme solution.

The Phadebas α-amylase method was accomplished as follows. Aliquots (0.1-1.0 ml) of the suitably diluted standardized enzyme preparation were diluted to 5-ml. with maleate buffer (0.05 M), pH 6.0, containing calcium chloride (0.2 g./l.). The solutions were placed in a water bath for 15 min. at 50°C. to equilibrate. A Phadebas tablet was added to each solution and was completely dispersed by gentle shaking. The digest was then incubated for 15 min. with manual shaking each 5 min. On the completion of incubation, 1 ml. of 0.05 M sodium hydroxide was added. The volume was diluted to 10 ml. with distilled water, filtered, and the absorbance measured at 620 nm.

When the absorbance exceeded the scale of the spectrophotometer, the filtrate was diluted accordingly. A maximum absorbance of 5 may be read; beyond this, the substrate is limiting. A standard curve was obtained by plotting absorbance versus milli-Enzyme Units per 10 ml of colored solution.

For the determination of α-amylase activity in germinated samples, suitable amounts of malted flours (0.1-0.5 g.), depending on the α-amylase content, were added to 20 mls of 0.05 M maleate buffer, pH 6.0, containing calcium chloride (0.2 g./l.). The suspension was gently shaken on a mechanical shaker for 5 min. and centrifuged. An aliquot (0.1-2.0 ml) of the clear, suitably diluted supernatant solution was added to a test tube, and the volume was diluted to 5 ml with the same buffer. The solutions were placed in a

water bath at 50°C. and allowed to equilibrate for 15 min. A Phadebas tablet was added to each tube, manually shaken and the method was completed as described above. $\alpha$-Amylase activities were obtained by converting absorbance values into milli-Enzyme Units per 10 ml. of colored solution using the standard curve. $\alpha$-Amylase activity was then calculated and expressed as Enzyme Units per g. of malted flour.

Determination of Total Free Sugars in Flours. Free sugars in the flours were extracted as recommended by Shannon (49). The sugar content of the extract was then determined by the phenol-sulfuric acid method (50). Free sugars from 0.1 g. of flour were extracted with three 25-ml. portions of a mixture of methanol:chloroform:water (13:4:3, v/v/v) by suspending the flour in the solvent at 0-4°C. and shaking for 5 min. using a mechanical shaker. The suspension was centrifuged 10 min. at 1000xg and the resulting supernatant solution decanted. The extracts were combined and examined for total free sugars using the phenol-sulfuric acid procedure (50). Phenol (1 ml. of 5%) was added to 1 or 2 ml. of sugar solution containing 10 to 70 ug of sugar. Concentrated sulfuric acid (5 ml) was then added rapidly with thorough mixing. After standing for 10 to 20 min. in a water bath at 25°-30°C. the absorbance was measured at 490 nm. The amount of total free sugars was expressed as mg. glucose per g. flour using a standard curve constructed with glucose (20-120 ug.).

Determination of Damaged Starch. The percentage damaged starch in the flours was determined by the iodine-staining method of Williams and Fegol (51) using the following extracting solution and iodine-staining reagent:

1. Formamide-sodium sulfate stock solution. A 1.41 M solution of sodium sulfate was prepared by dissolving 400 g. anhydrous sodium sulfate

in 1500 ml of distilled water, and diluting this to 2 l. Formamide (300 ml) was diluted to 2 l. with this sodium sulfate solution. This stock solution is stable for about 1 month.

Extracting solution. Sulfosalicylic acid (2.0 g.) was dissolved in 1 l. of formamide-sodium sulfate stock solution. This must be prepared fresh daily.

2. Iodine stock solution. Iodine crystals (5.5 g., AR grade) and 11.0 g. of potassium iodide crystals (AR grade) were dissolved in the minimum amount of water (about 25 ml) and diluted to volume in a tinted 250-ml. volumetric flask. This solution must be stored in the dark.

Iodine reagent. Stock iodine solution (10 ml.) was diluted to 100 ml. with distilled water. This reagent must be prepared fresh daily.

3. Gelatin solution. Powdered gelatin (0.5 g., reagent-grade) was dissolved in 100 ml. of recently boiled, distilled water and filtered through glass wool.

Diluting solution. Gelatin solution (50 ml.) and 2.5 ml. of 30% hydrogen peroxide (AR grade) solution were diluted to 500 ml. with recently boiled, distilled water.

Procedure. Flour samples (1 g.) were extracted with 25 ml. of extracting solution for 15 min. at 50°C. with thorough shaking at 5-min. intervals. Optimum reproducibility was achieved by timing the addition of the extracting solution to the samples so that each sample was in the water bath for exactly 15 min. About 0.25 g. Celite filter aid (Johns Manville, Fisher Scientific Co., N. J.) was added to the suspensions, shaken briefly, allowed to stand for 1 or 2 min., and filtered through Whatman No. 4 filter paper. Aliquots (10 ml.) were pipetted into test tubes containing 10 ml. of

diluting solution. To each tube, 0.5 ml. of iodine reagent was added, mixed thoroughly, and allowed to stand in a water bath at 30°C. for 15 min. Absorbance was determined at 555 nm. against a reagent blank and was converted into percentage of damaged starch, expressed in Farrand units, by the following regression equation (51):

$$X = 0.286 + 50.3(Y)$$

where X = damaged starch (% Farrand)

Y = absorbance

Scanning Electron Microscopy (SEM). Two types of specimens were prepared for viewing by SEM: isolated starch and endosperm. Starches were isolated, after suspending flour in 0.01 M mercuric chloride to inactivate $\alpha$-amylase activity (8), by centrifuging the suspension and washing the starch from the precipitate through a bolting cloth (16XX, 72-74 u openings) with distilled water. After centrifuging, the isolated starch was thoroughly washed with 0.02 M sodium hydroxide to remove associated protein (23), and rewashed with distilled water. The particles were dehydrated in absolute ethanol to prevent agglomeration of the granules (9) and dried in a vacuum oven for 1-2 hr. at temperatures not exceeding 40°C. Samples were prepared for SEM by sprinkling starch onto double-coated Scotch tape attached to specimen stubs; excess starch was removed with compressed air carefully blown over the sample holder. Kernels were transversely sectioned with a razor blade, producing a fracture rather than a clean cut. The half-kernels were mounted on aluminum stubs with Delco No. 93 colloidal silver. All samples were coated with 60 Å of carbon and then covered with 100 Å of gold-palladium evaporated onto their surfaces in high vacuum. Coated samples were viewed and photographed in an ETEC Autoscan scanning electron microscope at an accelerating voltage of 20 kv.

# RESULTS AND DISCUSSION

Initially, an attempt was made to investigate the influence of temperature on the development of α-amylase in germinating wheat, oats, and millet. It was found that germination at 20°C. did not increase the amount of α-amylase but slightly increased the rate at which the enzyme was produced as compared to germination at 15°C. These results are in close agreement with those obtained by Kneen et al. (52). Table I shows the effect of temperature on the development of α-amylase in germinating wheat.

It was also observed that mold infestation occurred much more readily at 20°C. than at 15°C. Since molds are known to be excellent sources of -amylases, it is apparent that determinations of cereal α-amylase activity on mold-infested germinated grain may be in error. Thus, analytical data presented in this thesis are based on germination at 15°C; SEM micrographs are from samples germinated at both 15° and 20°C.

## Analytical Data

α-Amylase activity of each germinated sample was obtained using the calibration curve shown in Figure 1. Relative and total enzyme activities for germinated wheat, oats and millet are shown in Table II. Data from Table II indicate that α-amylase activity of wheat increases over 100-fold during the germination time investigated. Since the total α-amylase activity was nearly constant after 8 days, enzyme production had probably reached a maximum. Oats and millet produced lower amounts of α-amylase. After soaking the grain, wheat and oat α-amylase activity decreased to about one-half of that originally present in the dormant grains. Our results do not agree with

TABLE I. INFLUENCE OF TEMPERATURE ON DEVELOPMENT OF α-AMYLASE IN GERMINATING WHEAT

| Length of Germination (Days) | α-Amylase Activity (EU/g. flour) at 15° C. | 20° C. |
|---|---|---|
| Control | 1.3 | 1.5 |
| Soaked | 0.5 | 0.4 |
| 2 | 12.8 | 12.0 |
| 4 | 22.4 | 62.5 |
| 6 | 112.0 | 120.0 |
| 8 | 132.0 | 129.0 |
| 10 | 136.0 | 142.0 |
| 12 | 135.0 | 152.0 |
| 14 | 140.0 | 154.0 |

Figure 1. Calibration of Phadebas tablets against partially purified wheat $\alpha$-amylase.

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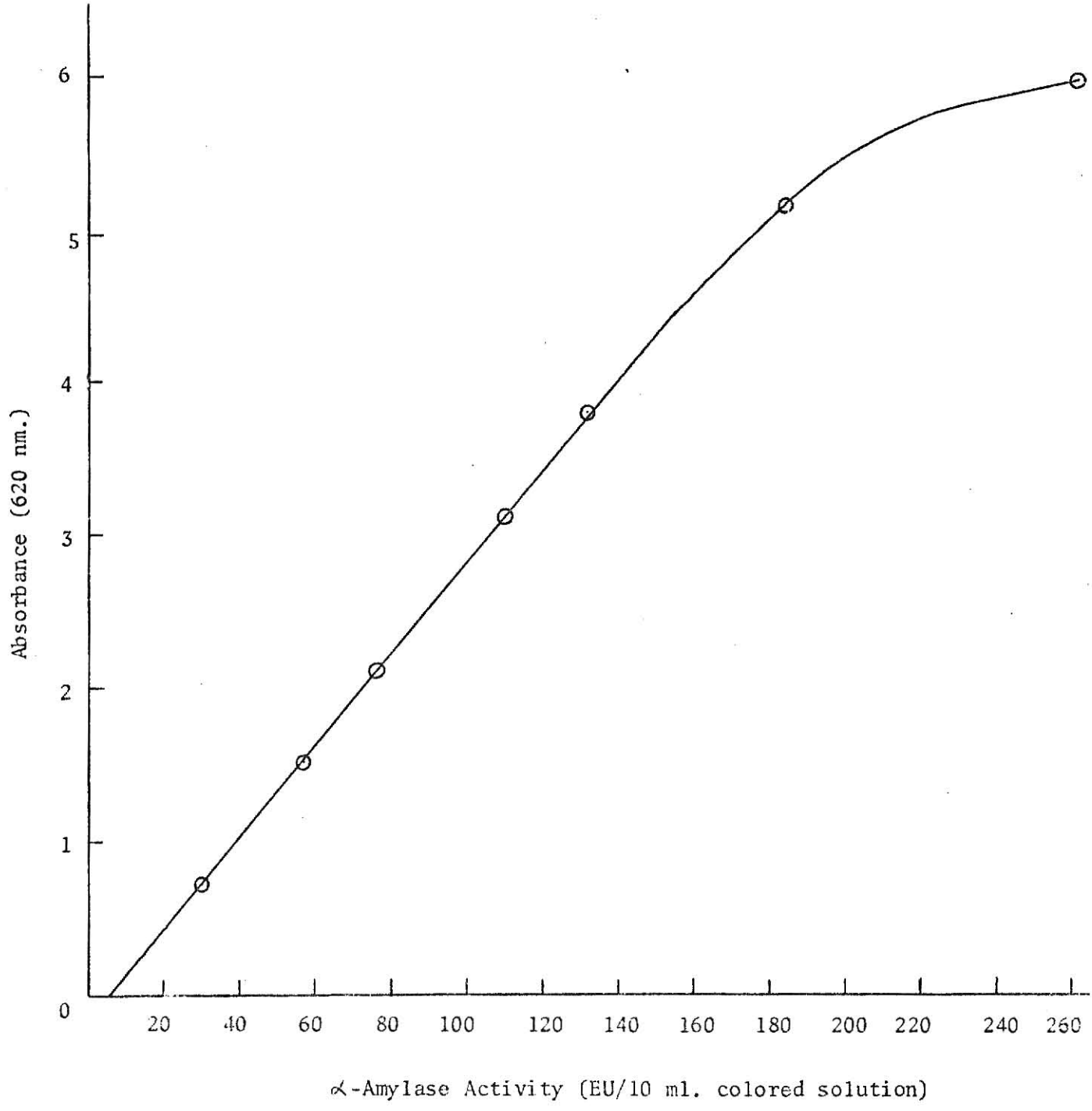

Absorbance (620 nm.)
0
1
2
3
4
5
6
20
40
60
80
100
120
140
160
180
200
220
240
260
∝-Amylase Activity (EU/10 ml. colored solution)

TABLE II. $\alpha$-AMYLASE ACTIVITY OF CEREALS DURING GERMINATION AT 15° C.

| Length of Germination (Days) | $\alpha$-Amylase Activity (Relative) | | | $\alpha$-Amylase Activity (Total)[a] | | |
|---|---|---|---|---|---|---|
| | Wheat | Oats | Millet | Wheat | Oats | Millet |
| Control | 1.0 | 1.0 | 1.0 | 1.3 | 0.7 | 0.6 |
| Soaked | 0.4 | 0.6 | 1.7 | 0.5 | 0.4 | 1.0 |
| 2 | 9.7 | 0.8 | 2.4 | 12.8 | 0.5 | 1.4 |
| 4 | 16.9 | 3.8 | 6.8 | 22.4 | 2.5 | 4.0 |
| 6 | 84.8 | 9.2 | 11.5 | 112.0 | 6.1 | 6.8 |
| 8 | 100.0 | 22.9 | 15.2 | 132.0 | 15.1 | 9.0 |
| 10 | 103.0 | 43.3 | - | 136.0 | 28.6 | - |
| 12 | 102.4 | 50.6 | - | 135.0 | 33.4 | - |
| 14 | 106.1 | 61.2 | - | 140.0 | 40.4 | - |

[a]EU/g. Flour

those of Dronzek et al. (36) who found that α-amylase activity increased 750 fold after Canadian hard red spring wheat (Manitou) was soaked and continued to increase exponentially with germination time. This apparent disagreement may be due to many factors including, in our study: the lower temperature at which the grains were soaked (15°C.) which decreased the rate of cereal germination, leaching out enzyme during soaking which would be lost in the soaking water, and the variety of wheat (hard red winter) used in this study which is relatively low in α-amylase content (53). Since the variety of millet available was susceptible to mold growth during germination, samples were collected and α-amylase activity was determined not later than 8 days of germination. After soaking, α-amylase activity increased in millet in contrast to wheat and oats. This suggests that a certain degree of sprouting might have occurred during harvesting of millet.

The percentages of damaged starch in flours prepared from grain germinated at 15°C. were determined by the Williams and Fegol procedure (51) and are shown in Table III. The conventional Blish-Sondstedt method (54), based on the enzyme susceptibility of damaged starch, is not applicable to this study since the flours were milled from malted cereal high in α-amylase activity which contributes to considerably higher amounts of maltose production as well as the amount of damaged starch. The procedures described by Farrand (55), Sandstedt and Mattern (56), involving addition of excess α-amylase to digest damaged granules initially, after which sound starch is digested at a constant rate, require from 2 to 5 hr. We chose to measure damaged starch in the flours by the rapid and reproducible method of Williams and Fegol (51) based on the observation that amylose is more rapidly extracted from damaged granules than from sound granules by saturated sodium sulfate;

TABLE III. DAMAGED STARCH IN WHEAT, OAT AND PEARL MILLET FLOURS FROM GRAINS GERMINATED AT 15° C.

| Length of Germination (Days) | Damaged Starch (% Farrand) | | |
|---|---|---|---|
| | Wheat Flour | Oat Flour | Millet Flour |
| Control | 24.1 | 6.1 | 10.1 |
| Soaked | 14.4 | 6.0 | 7.3 |
| 2 | 22.5 | 6.1 | 7.7 |
| 4 | 27.5 | 5.8 | 11.4 |
| 6 | 33.8 | 6.1 | 14.5 |
| 8 | 30.1 | 6.5 | 16.1 |
| 10 | 31.1 | 8.6 | - |
| 12 | 33.8 | 9.8 | - |
| 14 | 36.1 | 9.6 | - |

the intensity of the color developed in the extract by addition of iodine can be measured quantitatively. Absorbances obtained from this colorimetric test were converted into percentages of damaged starch, expressed in Farrand units, by the regression equation shown in Materials and Methods.

Alcohol-soluble sugars were extracted from flours at 0-4°C. with a methanol:chloroform:water (13:4:3, v/v/v) solution and were determined by the phenol-sulfuric acid method (50). The results (Table IV) were expressed as mg. glucose per unit weight of flour using a standard curve (Figure 2) prepared with 20 to 120 ug. glucose.

Figures 3,4, and 5 graphically summarize the analytical data obtained from flours milled from grains germinated at 15°C. With all three grains $\beta$-amylase activity increased slowly during the initial stages of germination and then increased at an accelerated rate as the seedling developed. The activity of wheat $\alpha$-amylase (Figure 3) tended to level off after 8 days of germination while oat $\alpha$-amylase continued increasing during the later stages of sprouting (Figure 4). As germination progressed the amount of free sugars generally followed the same trend as enzymic activity; both $\alpha$-amylase activity and the amount of free sugar increased with increasing germination time for wheat, oats, and millet. With wheat and millet, soaking the grains produced a decrease in the amount of damaged starch in the milled flours from 24.1 and 10.0% to 14.4 and 7.3%, respectively (Table III). The damaged starch content of oat flour remained constant up to 8 days of germination. The decrease in damaged starch content in flour from wheat and millet might result from partial dissolution of the protein or amorphous matrix embedding the starch granules in the endosperm, thus resulting in softened kernels with less resistance to the grinding action of the mill rolls. Hardness of wheat has been reported

TABLE IV. TOTAL FREE SUGARS (mg./g. flour) IN WHEAT, OAT AND PEARL MILLET FLOURS FROM GRAINS GERMINATED 15° C.

| Length of Germination (Days) | Wheat Flour | Oat Flour | Millet Flour |
|---|---|---|---|
| Control | 5.2 | 7.5 | 5.9 |
| Soaked | 5.8 | 6.4 | 4.3 |
| 2 | 7.7 | 8.7 | 4.6 |
| 4 | 22.5 | 16.9 | 4.3 |
| 6 | 35.9 | 20.2 | 6.6 |
| 8 | 49.5 | 25.5 | 10.9 |
| 10 | 59.1 | 38.5 | - |
| 12 | 62.2 | 41.3 | - |
| 14 | 68.9 | 45.9 | - |

Figure 2. Standard curve for determination of total free sugars by phenol-sulfuric acid procedure (50)

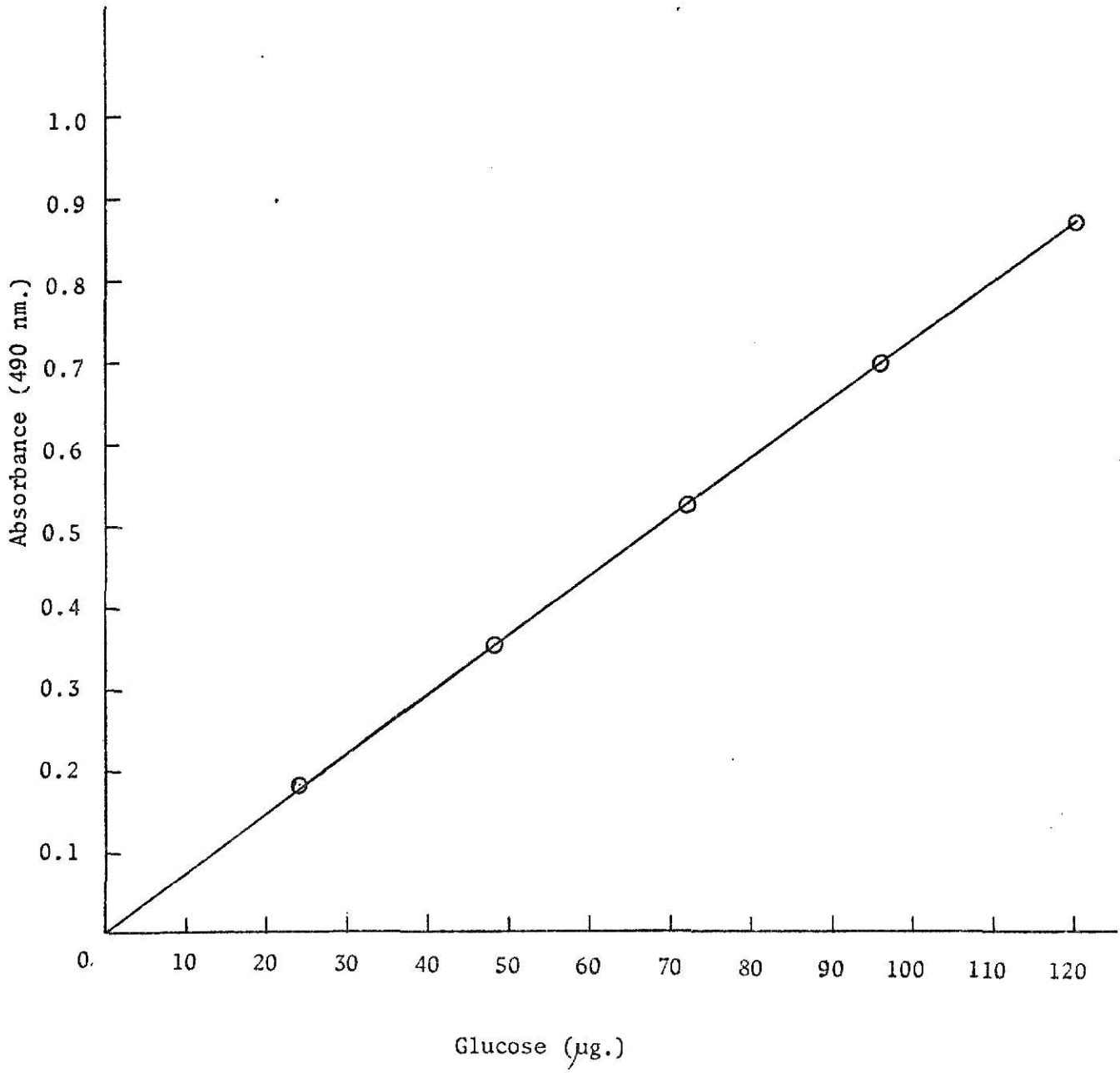

Absorbance (490 nm.)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
90
100
110
120
Glucose (µg.)

Figure 3. $\alpha$-Amylase activity, free sugar content and damaged starch content in flour milled from wheat germinated at 15° C. for varying lengths of time.

⊙ - % Damaged starch

⊡ - $\alpha$-Amylase activity

△ - Total free sugars

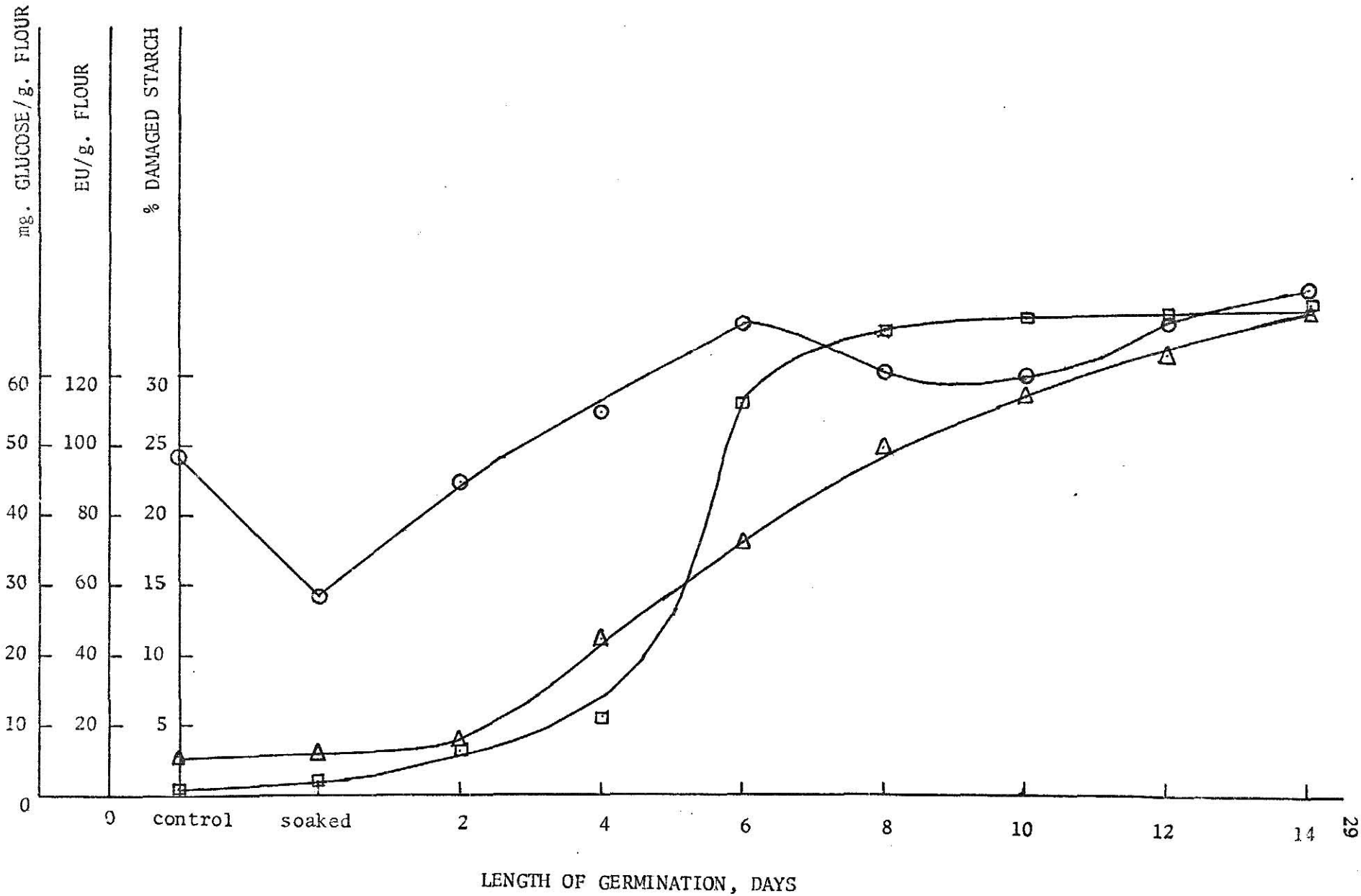

mg. GLUCOSE/g. FLOUR
EU/g. FLOUR
% DAMAGED STARCH
60
50
40
30
20
10
0
120
100
80
60
40
20
30
25
20
15
10
5
0
control
soaked
2
4
6
8
10
12
14
29
LENGTH OF GERMINATION, DAYS

Figure 4. $\alpha$-Amylase activity, free sugar content and damaged starch content in flour milled from oats germinated at 15° C. for varying lengths of time.

⊙ - % Damaged starch

⊡ - $\alpha$-Amylase activity

△ - Total free sugars

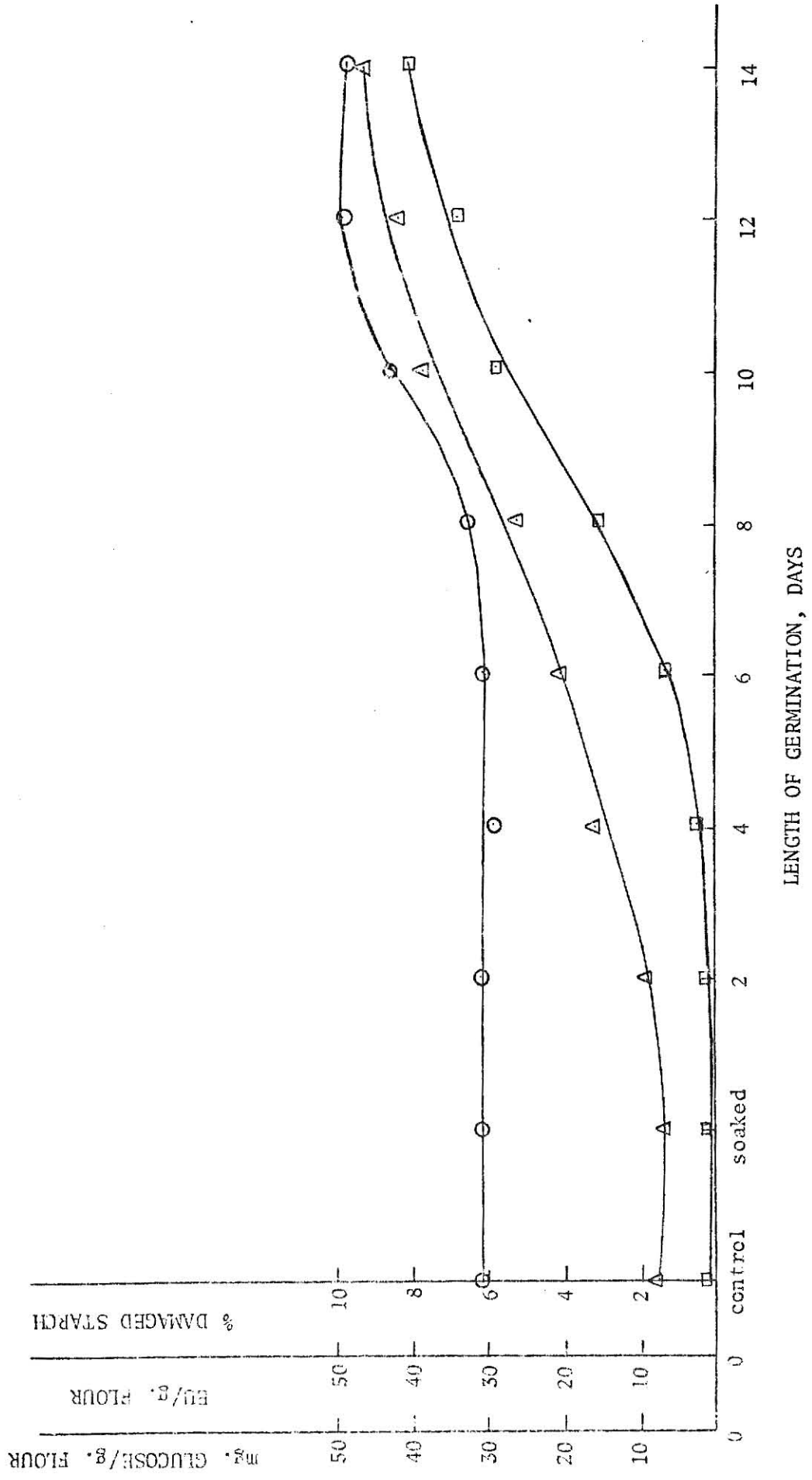
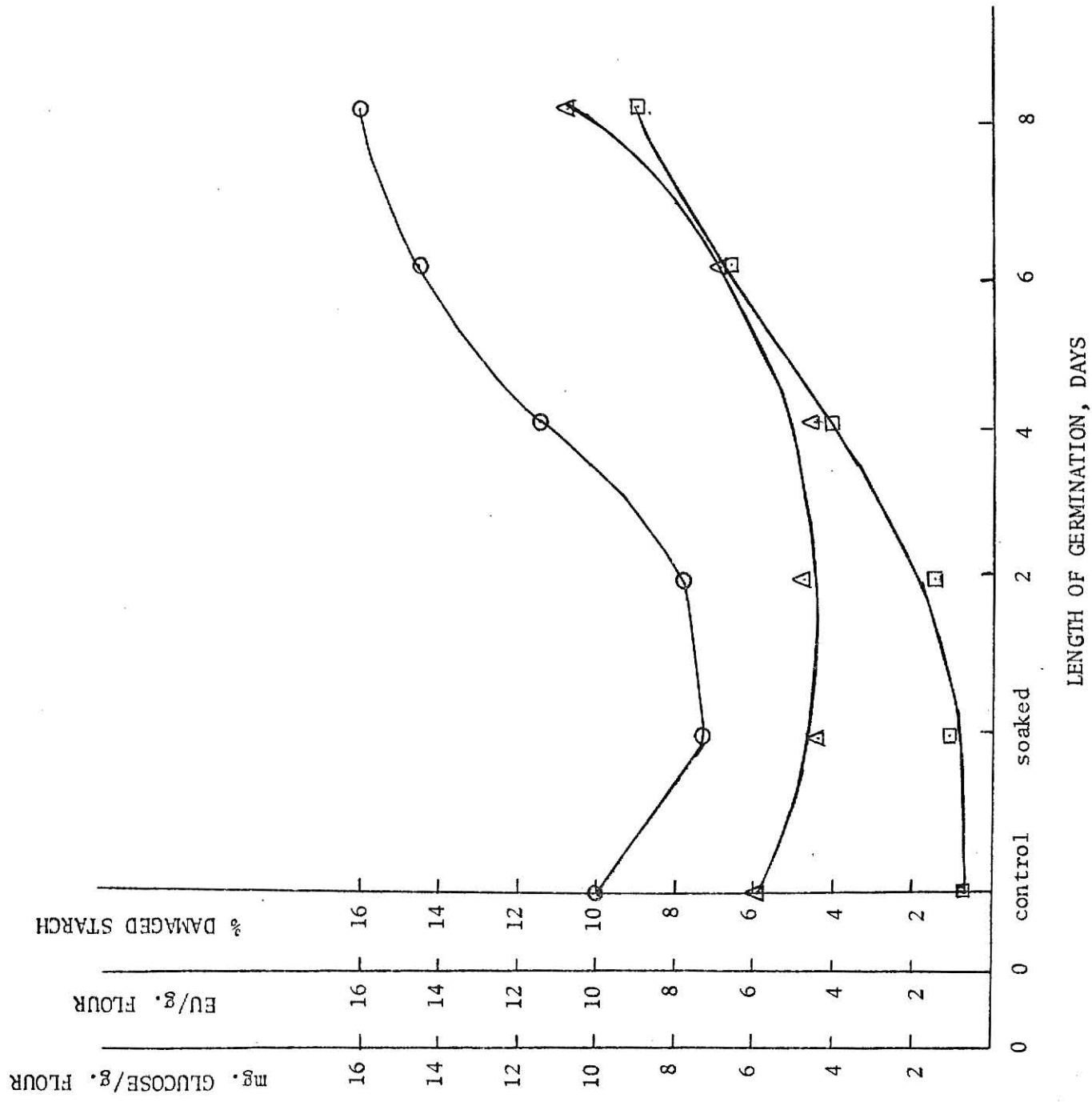

mg. GLUCOSE/g. FLOUR
EU/g. FLOUR
% DAMAGED STARCH
10
8
6
4
2
50
40
30
20
10
0
control
soaked
2
4
6
8
10
12
14
LENGTH OF GERMINATION, DAYS

Figure 5. $\alpha$-Amylase activity, free sugar content and damaged starch content in flour milled from millet germinated at 15° C. for varying lengths of time.

⊙ - % Damaged starch

□ - -Amylase activity

△ - Total free sugars


mg. GLUCOSE/g. FLOUR
EU/g. FLOUR
% DAMAGED STARCH
2
4
6
8
10
12
14
16
0
control
soaked
2
4
6
8
LENGTH OF GERMINATION, DAYS

to be related to the adhesion of starch and protein (57) and damaged starch content increases with increasing hardness of wheat (58). However, with further germination the amount of damaged starch in flour from all three cereals increased. These data indicate that starch in the endosperms of wheat and millet were probably gradually degraded by α-amylase as sprouting progressed, resulting in damaged starch granules. Oat starch probably is less susceptible to amylolytic degradation as indicated by the relatively small amount of damaged starch present in the flour and also by the much smaller amount of α-amylase formed. Microscopic evidence supporting these interpretations is presented in the following section.

SEM Microscopy

The operating principle of the scanning electron microscope differs from that of the transmission electron microscope in that, instead of examining the transmitted electron beam which has been changed by absorption, contrast and scattering effects of the sample placed in the path of the electron beam, the image created by secondary electrons emitted from the surface of the sample being scanned with the primary electron beam, is observed (10). An image is produced on the screen of a cathode-ray tube connected to the scintillator-photomultiplier system that detects the secondary electrons. Photomicrographs of the images can be made with a Polaroid camera.

The scanning electron microscope has many advantages for studying the structure of starch granules and enzymic attack of granules, as compared to ordinary light or transmission electron microscopes. These include the possibility of obtaining a large depth of focus, of obtaining a three-dimensional view of the starch granule, and of determining which details seen in the light microscope are due to surface structure and which are due to

internal structure. Furthermore, much larger samples can be viewed with the scanning electron microscope and no sectioning methods are necessary for sample preparation. However, some limitations to application of SEM to the study of starch granules have been observed (10). First, since starch is a non-conductive material which must be lightly coated with a conductive material such as gold-palladium alloy, only surface detail is obtained. In order to obtain details of the inner structure of the starch granule, the granule has to be ruptured or crushed. Second, some starch granules cannot be scanned beyond a certain degree of magnification without severely cracking the particles as a result of heating in the electron beam. The micrographs presented in our work, however, are essentially free of this artifact. Third, small hairline artifacts in some micrographs are produced as a result of an accumulation of negative charge on the starch surface by the primary electrons. This can be prevented by using an antistatic agent (59) which results in lower resolution as compared to the metal-shadowing technique, but the danger of morphological changes is minimized.

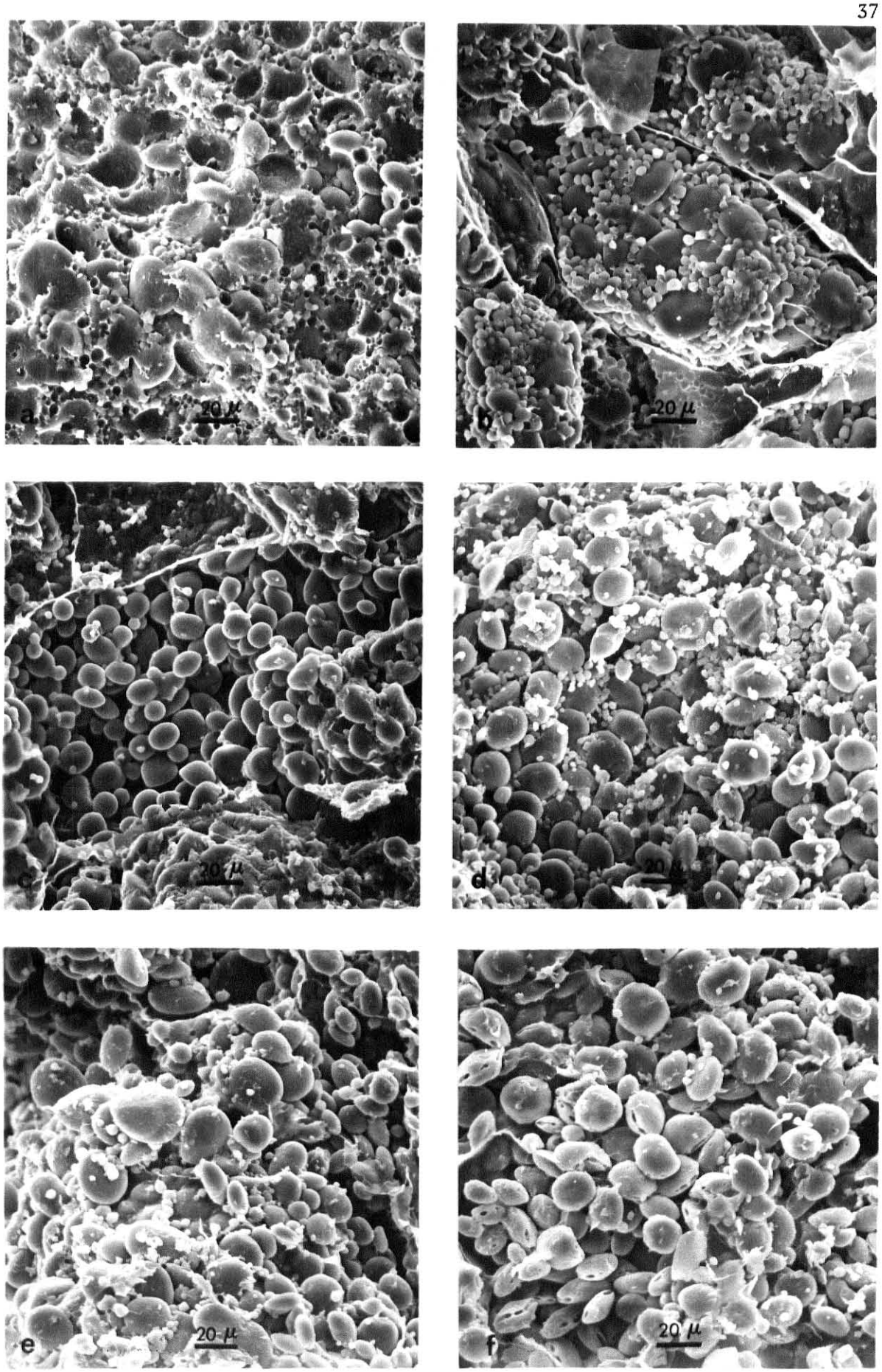
In order to obtain a truly representative picture of all various sizes and shapes of starch granules in any one sample, one micrograph at low magnification is included to show typical shapes and variations in size of a large number of granules. The remaining micrographs are at higher magnifications to show surface detail and to show the pattern of amylolytic degradation of the starch granule in greater detail. To reduce the number of illustrations, the micrographs included were selected from a large number of micrographs to be as representative as possible.

SEM Observations of the Endosperm of Wheat, Oats and Millet during Germination

Figure 6 contains scanning electron micrographs of endosperm from

Figure 6. Endosperm from ungerminated and germinated (20° C.) wheat

a = ungerminated
b = soaked
c = germinated 2 days
d = germinated 4 days
e = germinated 8 days
f = germinated 14 days


a
20 μ
b
20 μ
c
20 μ
d
20 μ
e
20 μ
f
20 μ

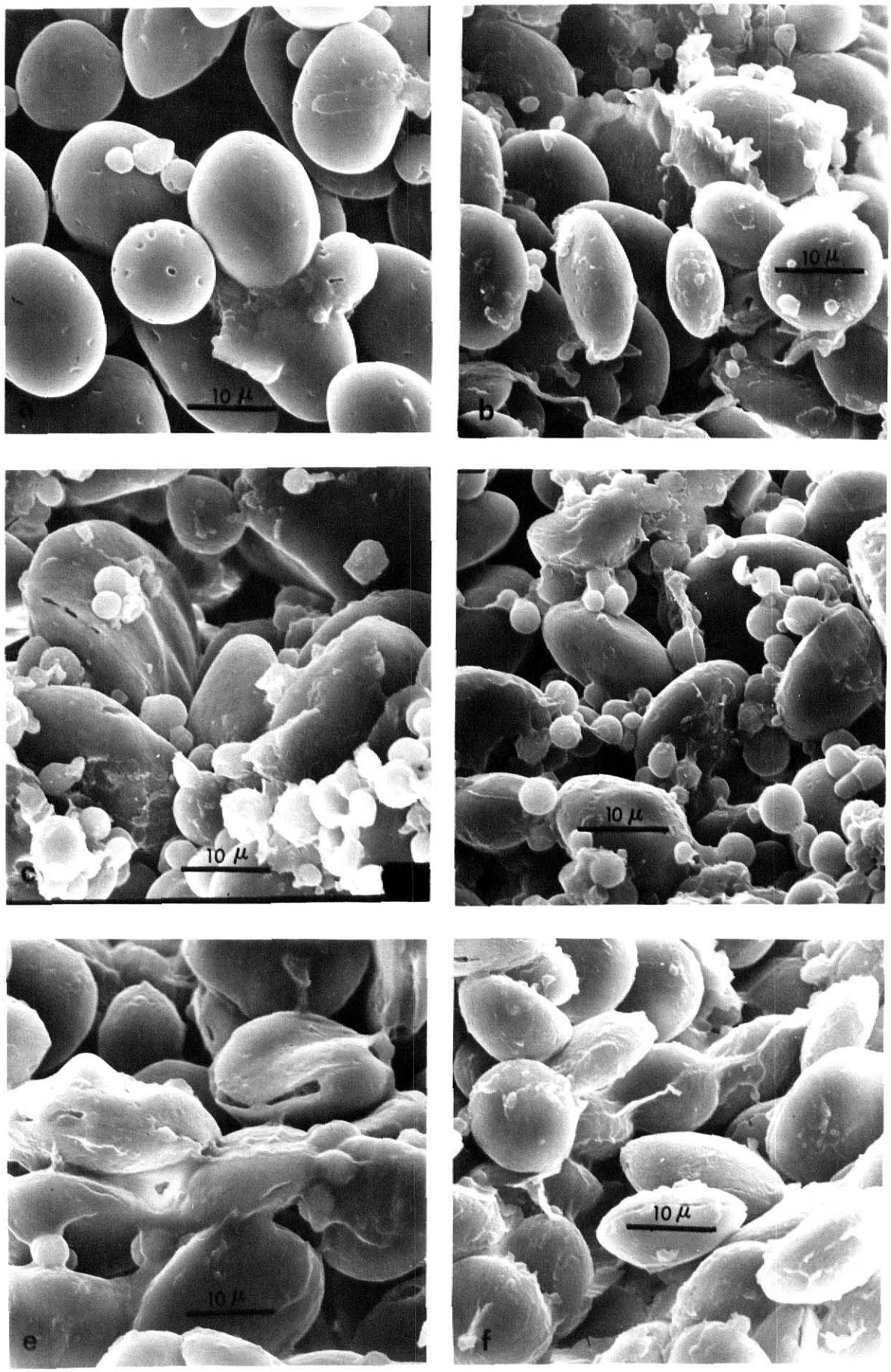
ungerminated wheat (Figure 6a), soaked wheat (Figure 6b) and wheat germinated at 20° C. for various periods of time (Figures 6c-6f) at low magnification. The endosperm was exposed by fracturing the kernel transversely with a razor blade. It is evident from the control sample (Figure 6a) that there is an apparent bimodal distribution of granule size in wheat consisting of large lenticular granules and small spherical granules, ranging in size from 2 u to 30 u, embedded in amorphous cementing materials. Small spherical indentations seen in the endosperm (Figure 6a) suggested that small spherical starch granules have been removed from these areas during the fracturing procedure. The amorphous material appears similar to the "wedge protein," which occupies the interstitial spaces between starch granules, described by Hess (60). This material appeared to decrease after soaking (Figure 6b) and totally disappeared during the early stages of germination (Figure 6c). The lamellar membranes surrounding the endosperm cell wall are readily apparent in Figure 6b. There was no evidence of enzymic attack on starch granules until samples were germinated for 2 days. An endosperm cell from wheat germinated for 2 days containing mostly intermediate size starch granules, is shown in Figure 6c. The photomicrographs appear to support the analytical data presented previously concerning the development of $\alpha$-amylase activity. As germination time increases, both small and large starch granules become separated from the materials in which they are embedded in the endosperm. Enzymic erosion of starch granules was obviously visible in wheat germinated for 14 days (Figure 6f), particularly in the region of the equatorial groove. Enzymic degradation of starch granules was first observed in cells adjacent to the aleurone layer which might be expected since $\alpha$-amylase is formed there.

Figure 7 shows, at higher magnification than Figure 6, the endosperm of wheat germinated at 15° C. for 2 to 14 days. Enzymic erosion observed in isolated starch definitely occurred *in vivo* instead of *in vitro* during the isolation process. Comparing Figures 6 and 7 indicates that α-amylase in wheat germinated at 15° or 20°C. showed comparable activity in degrading starch granules. Figures 7a to 7d illustrate that enzymic degradation of large starch granules occurs at germination times from 2 to 14 days as evidenced by surface pitting and erosion at the equatorial groove. The surface of small starch granules showed much less erosion suggesting that they are less susceptible to amylolytic degradation. The latter observation agrees with results previously reported by other investigators (36).

A small portion of endosperm cell wall is apparently seen in the lower left corner of Figure 7b suggesting that solubilization of the cell wall by hydrolytic enzymes such as β-glucanases, pentosanases and proteolytic enzymes was not yet completed after 6 days of germination. In later stages of germination (Figure 7e and 7f) erosion of starch granules progressed after severe hydrolysis of the endosperm cell wall had occurred. This indicates that, unless the endosperm cell wall is degraded, it is difficult for α-amylase which is produced in aleurone cells to pass into the endosperm and initiate starch degradation. The absence of small starch granules in Figures 7e and 7f was not due to preferential amylolytic attack since small granules are seen in the corresponding micrograph at lower magnification and in a micrograph of isolated starch from the same sample. The surface of starch granules in endosperm of wheat germinated for 12-14 days appeared to be covered by an amorphous mass. This material was suggested by Kiribushi and Nakamura (42) to be dextrins resulting from starch degradation by amylase.

Figure 7. Endosperm from germinated (15° C.) wheat

a = germinated 2 days
b = germinated 6 days
c = germinated 8 days
d = germinated 10 days
e = germinated 12 days
f = germinated 14 days


10 μ
a
10 μ
b
10 μ
c
10 μ
10 μ
e
10 μ
f

Micrographs, at different magnification, of oat endosperm samples germinated at 20° and 15° C. are displayed in Figure 8. Oat endosperm appears to contain two types of starch granules varying in size from 3 to 25 u. It will be seen later, from the micrographs of isolated oat starch (Figure 12), that the large granules are compound starch granules. Protein is visible in the endosperm from the control sample (Figure 8a) and disappeared as samples were germinated for 4 days at 20°C. (Figure 8b). Such behavior is similar to that observed for wheat. No evidence of enzymic attack can be seen on the surface of oat starch granules in any of the micrographs in Figure 8. However, amorphous material appeared to cover starch granules in the endosperm of oats germinated at 15° C. for 8 days (Figure 8c). These observations suggest that oat starch either is less susceptible to attack by $\alpha$-amylase than wheat starch or that there is not sufficient $\alpha$-amylase formed during germination to degrade the starch. These micrographs indicate that increasing germination temperature from 15° to 20° C. did not appear to increase $\alpha$-amylolysis of the starch (Figure 8d-8f). In spite of the absence of $\alpha$-amylolytic attack on oat starch granules, oat -amylase activity increased with germination time and was accompanied by an increase of free sugars. This suggested that oat $\beta$-amylase might be responsible for the disintegration of compound oat starch granules.

Figure 9 clearly illustrates the manner in which starch granules are packed in endosperm cells of germinated millet. Most starch granules are spherical with a diameter of approximately 10 $\mu$, except for starch granules near the center of the endosperm which are relatively smaller in size (about 5 $\mu$). The starch near the aleurone layer are more tightly packed so that the opposing faces flattened and granules become polyhedral in shape (Figure 9b).

Figure 8. Endosperm from ungerminated and germinated (20° and 15° C.) oats.

a = ungerminated
b = germinated 4 days, 20° C.
c = germinated 14 days, 20° C.
d = germinated 2 days, 15° C.
e = germinated 8 days, 15° C.
f = germinated 14 days, 15° C.

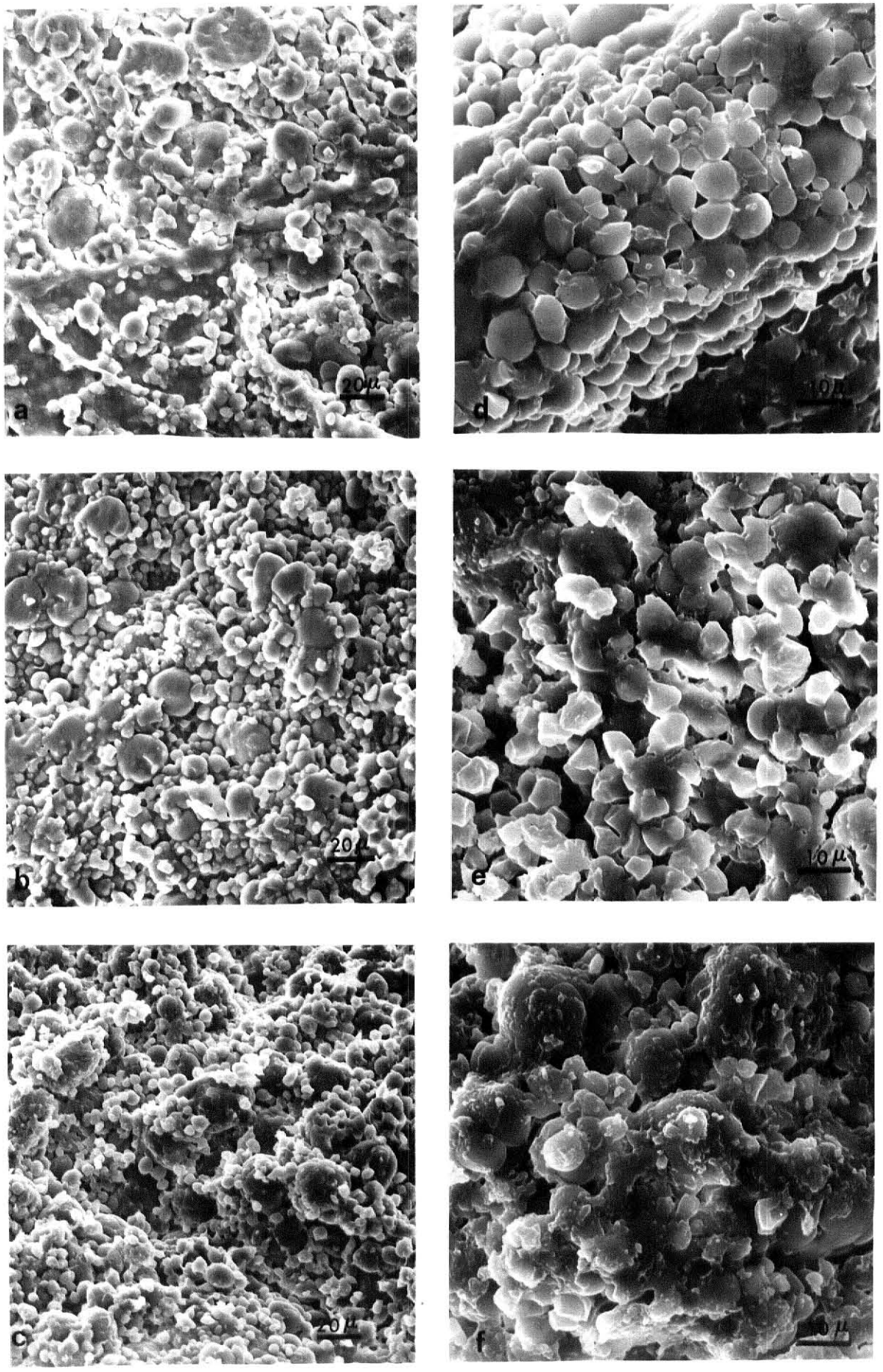
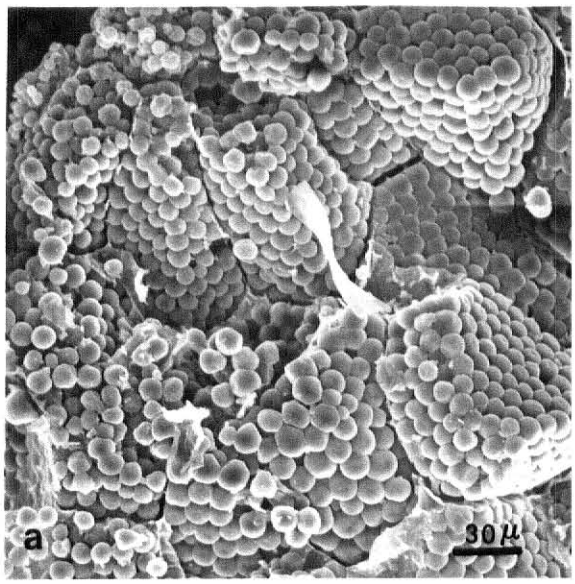
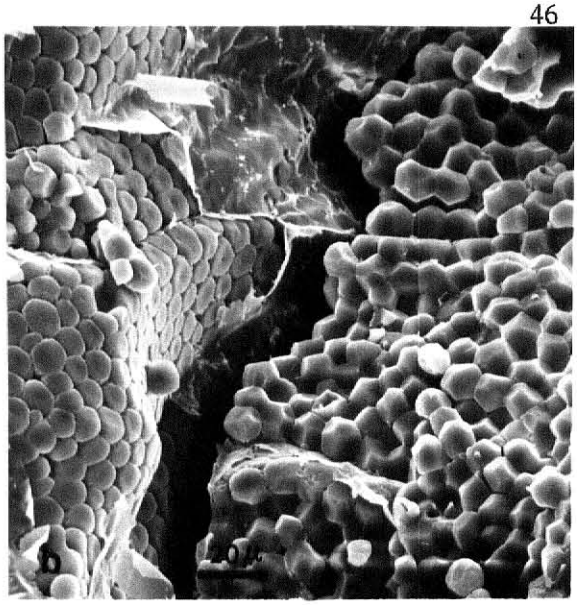
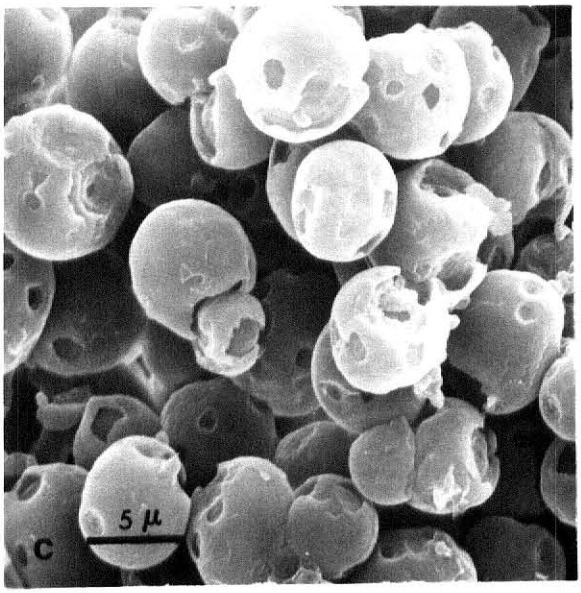

a
20μ
d
10μ
b
20μ
e
10μ
c
20μ
f
10μ

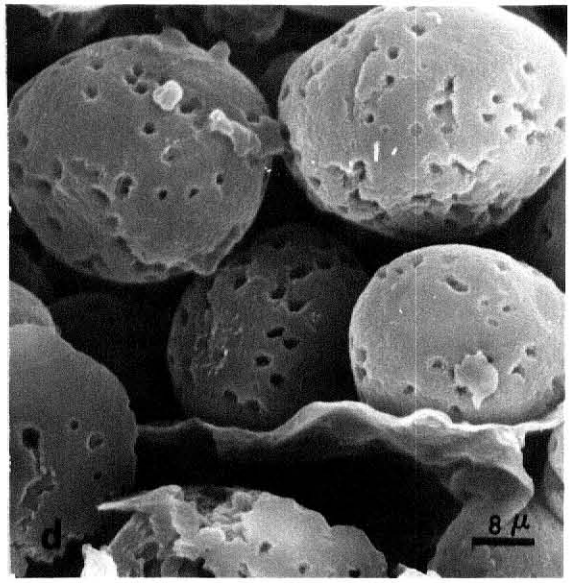
Figure 9. Endosperm from a single kernel of ungerminated millet.

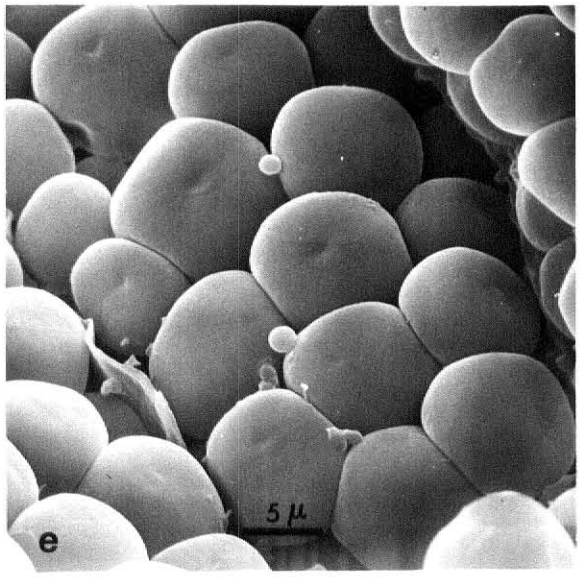
a = spherical granules of soft endosperm
b = interface region of soft (left) and hard (right) endosperm containing spherical and polyhedral starch granules
c,d = amylolytic degradations of spherical granules near scutellum of the embryo
e = a portion of soft endosperm containing intact spherical granules


a
30 μ


b


c
5 μ


d
8 μ


e
5 μ

Considerable erosion and pitting, believed to be due to the enzymic attack, was visible on the surface of small starch granules adjacent to the center of the endosperm of ungerminated millet (Figure 9c). Degradation of the starch in ungerminated millet diminished as distance from the aleurone layer increased (Figure 9d) and completely disappeared at the periphery of the endosperm (Figure 9e). This might be explained by the postulation of Kiribushi and Nakamura (42) that reserve starch in the endosperm is subjected to degradation during the process of ripening. Changes occurring in the endosperm of millet during germination at 20° C. up to 8 days are illustrated in Figure 10. After soaking and in grains germinated for 2 days (Figure 10a and 10b), enzymic erosion of granules was observed in the same regions of the endosperm as in ungerminated millet, except erosion was more severe and more uniformly distributed. As germination progressed beyond 4 days (Figure 10c,d and 10e,f), amylolytic degradation proceeded toward the polyhedral starch granules at the periphery of the endosperm (Figure 10d and 10f). In contrast to wheat and oats, cell walls of millet endosperm remained unhydrolyzed after 8 days of germination, at a time when enzymic erosion of starch granules has spread throughout the endosperm. The extent of enzymic digestion of starch appears to parallel the amount of $\alpha$-amylase activity during germination of millet.

Modification of Starch during Malting

Micrographs of starch granules isolated from flour milled from wheat germinated up to 14 days at 15° C. are shown in Figure 11. Since $\alpha$-amylase activity in wheat, oats, and millet increased markedly upon germination (Table II), the high correlation between an extent of enzymic erosion of the starch granules and $\alpha$-amylase content, reported by Dronzek et al. (36) for

Figure 10. Endosperm from germinated (20° C.) millet.

a = soaked
b = germinated 2 days
c,d = germinated 4 days
e,f = germinated 8 days

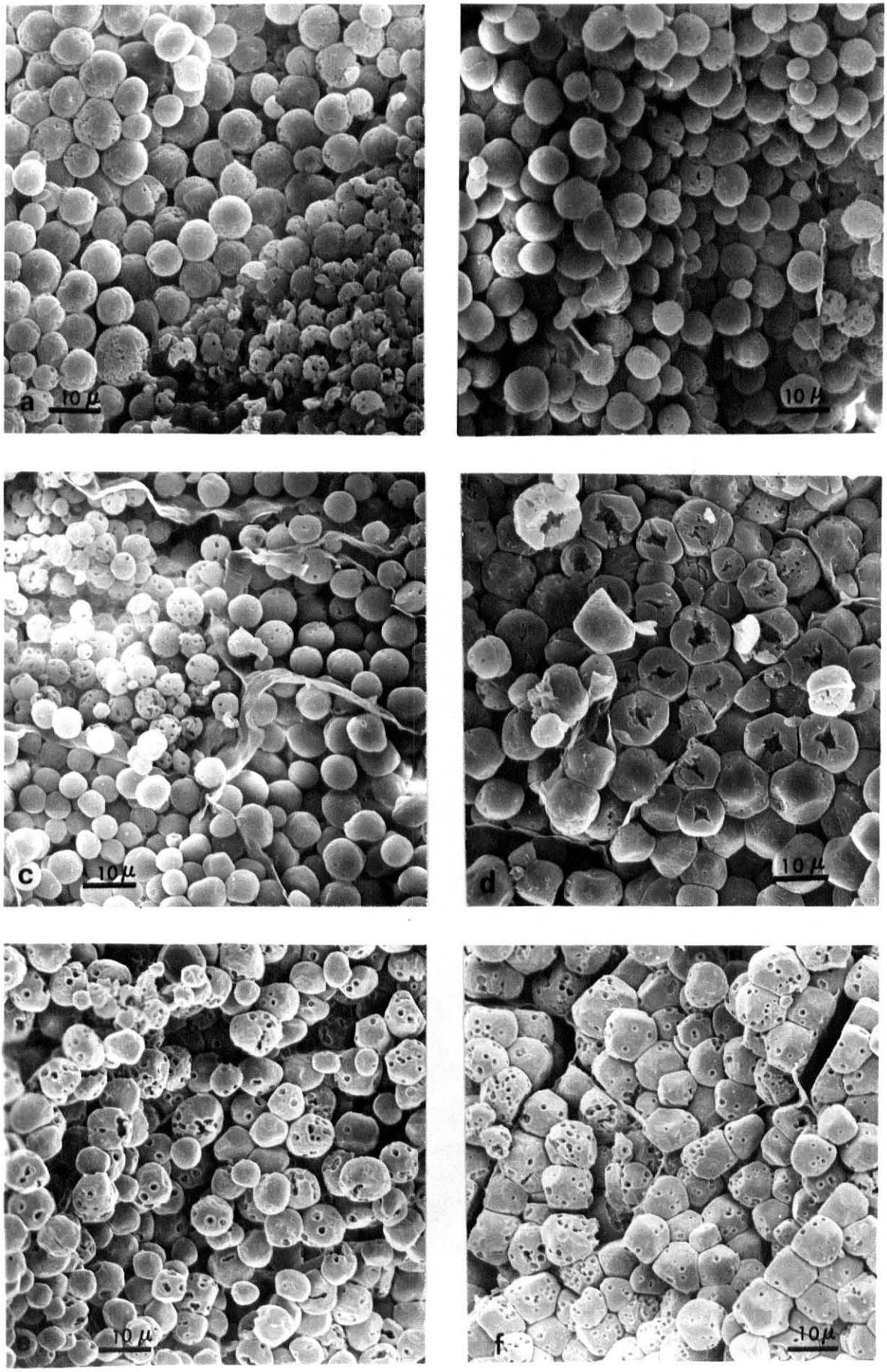
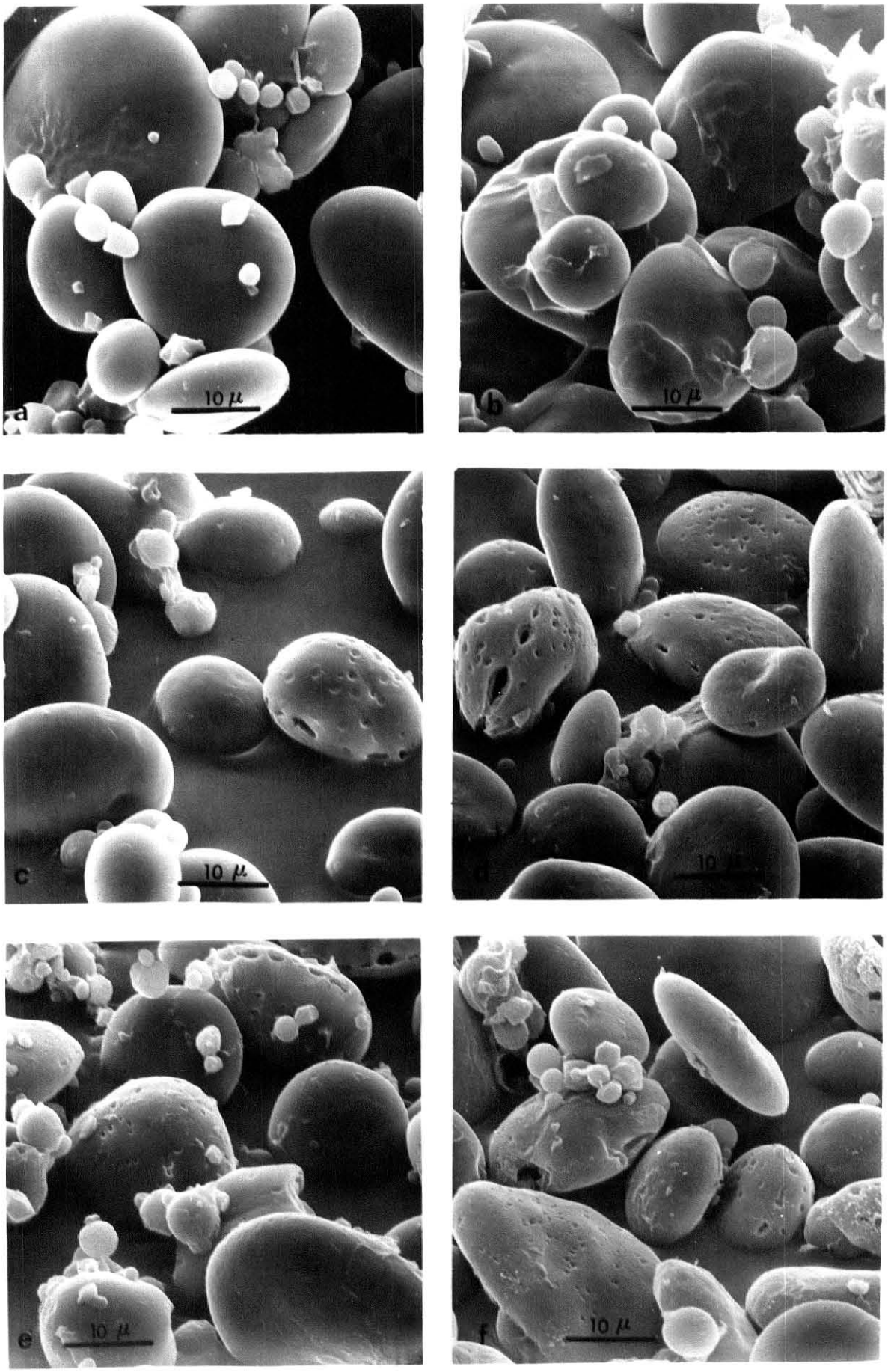

a
10 μ
10 μ
c
10 μ
d
10 μ
10 μ
f
10 μ

Figure 11. Starch granules isolated from ungerminated and germinated (15° C.) wheat.

a = ungerminated
b = soaked
c = germinated 2 days
d = germinated 4 days
e = germinated 8 days
f = germinated 14 days


a
10 μ
b
10 μ
c
10 μ
d
10 μ
e
10 μ
f
10 μ

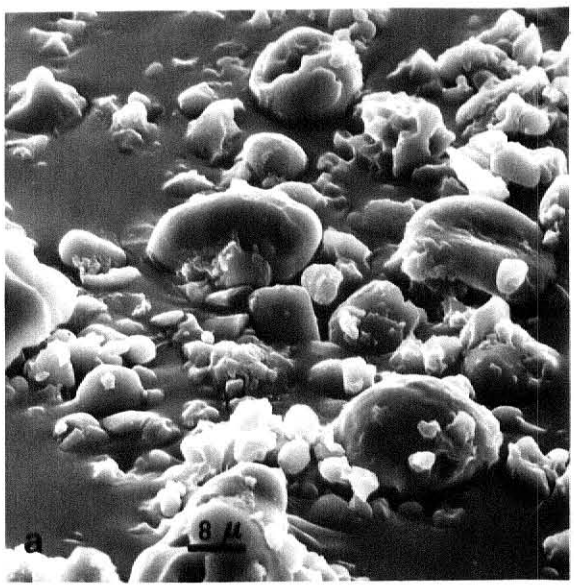
wheat, was expected. However, it is evident in Figure 11 that wheat starch granules were not severely degraded during germination even after 14 days. Dronzek et al. (36) showed extensive degradation of hard red spring wheat granules after 8 days of germination accompanies by a 27,000-fold increase in $\alpha$-amylase activity during that time. Differences between the types of wheat used in these two studies might be one of the factors responsible for these apparent discrepancies. However, while the majority of starch granules showed no surface erosion. $\alpha$-Amylolytic attack on large starch granules became apparent after two days of germination (Figure 11c) as evidenced by pitting on the granule surface and in the region on the equatorial groove.

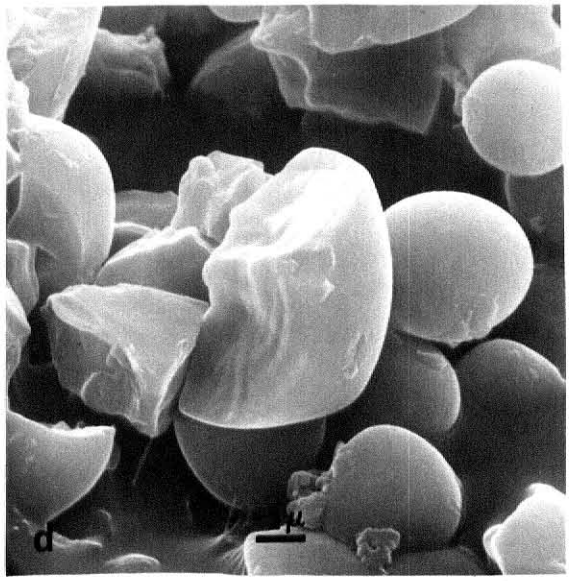
Enzymic attack increased with longer germination times as indicated by surface erosion of a larger number of starch granules (Figure 11d) while corrosion at the equatorial groove became intensive in some granules leading towards the center of the granule (Figures 11d, 11e and 11f), revealing the inner shell structure (Figure 11e). Erosion of the granule surface did not occur uniformly; in some granules enzyme penetration occurred on the major surface and on some granules penetration took place at both the equatorial groove and the major surface. Small wheat starch granules appeared to be more resistant to the action of $\alpha$-amylase (Figures 11c, 11e and 11f) and did not disappear after germination for two weeks; some are still visible in Figures 11e and 11f. The amorphous material covering starch granules in the endosperm at latter stages of germination are not evident in the isolated starch (Figures 11e and 11f), suggesting that this material is readily solubilized and is lost during the isolation procedure.

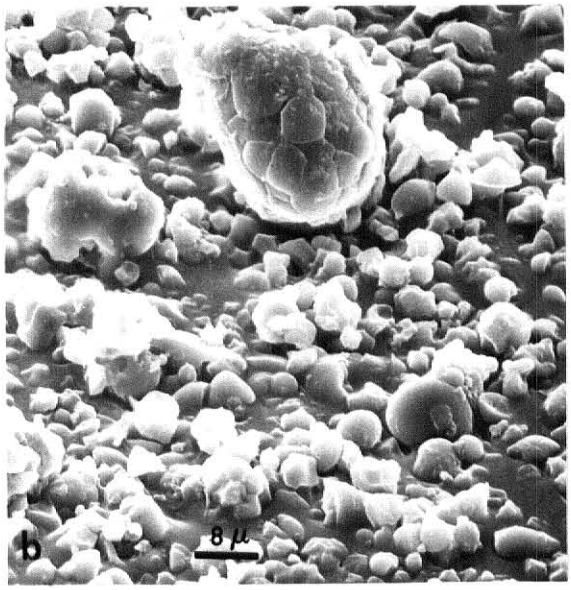
Micrographs of starch granules isolated from ungerminated and germinated (15° and 20° C.) oats are shown in Figure 12 at both low and high

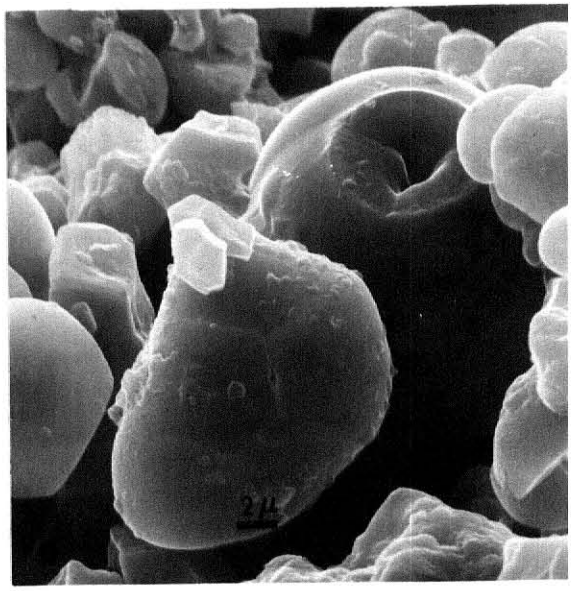
Figure 12. Starch granules isolated from ungerminated and germinated (20° and 15° C.) oats.

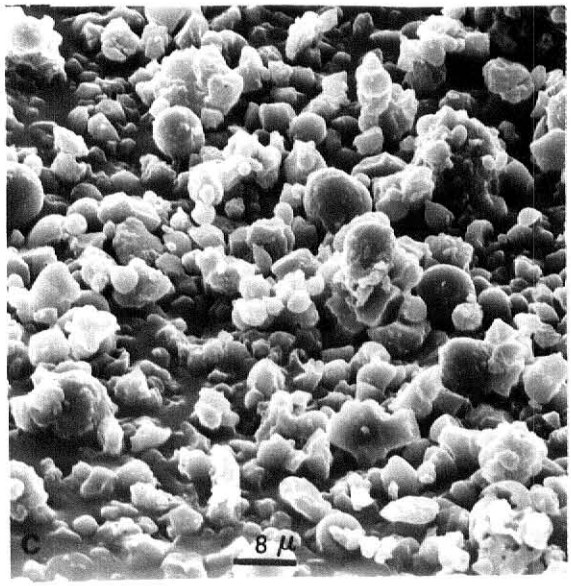
a = ungerminated
b = germinated 6 days, 20° C.
c = germimated 14 days, 20° C.
d = ungerminated
e = germinated 8 days, 15° C.
f = germinated 14 days, 15° C.

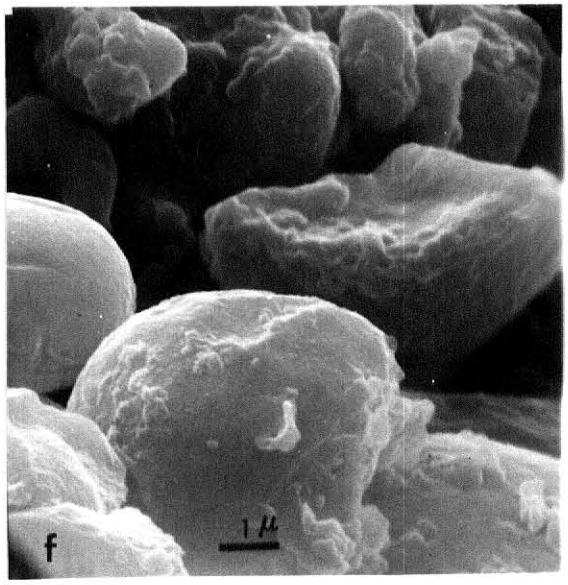

a
8 μ


d
1 μ


b
8 μ


2 μ


c
8 μ


f
1 μ

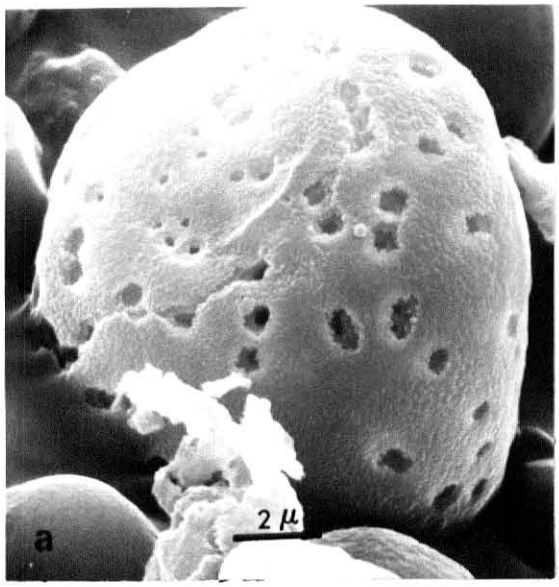
magnification. Prior to germination (Figure 12), most oat starch granules are compound granules composed of a large number of small granula. This observation agrees with studies of Hall and Sayre (29). As oats were germinated the compound granules were gradually disintegrated revealing the manner in which small granula are held together in a compound cluster (Figure 12b). Ultimately, the smaller oat starch granula were freed in later stages of germination (Figure 12c). No evidence of enzymic attack on the surface of oat starch granules was observed even when micrographs of starch isolated from oats germinated at 15° C. were taken at higher magnification (Figures 12d-12f).

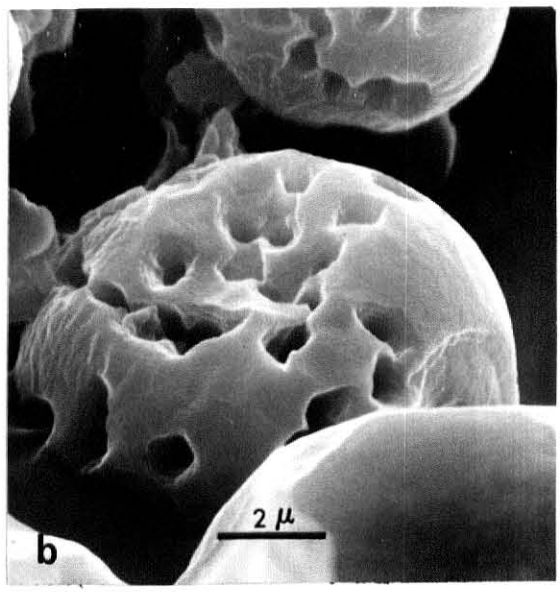
It was possible to examine oat starch with the scanning electron microscope at magnifications as high as 13,000x without severely cracking the granules as a result of heating in the electron beam. This suggested that oat starch granules may be bound by very cohesive forces which might be responsible for the apparent resistance to α-amylolysis of oat starch. However, starch isolated from oats germinated for 14 days exhibited a mildly corroded, rough surface (Figure 12f). It is not clear that the center portion of the broken oat starch granule seen in Figure 12f, was subjected to more α-amylolysis than the outer portion of the granule as reported by Buttrose (19) when oat starch granules were degraded in vitro by α-amylase.

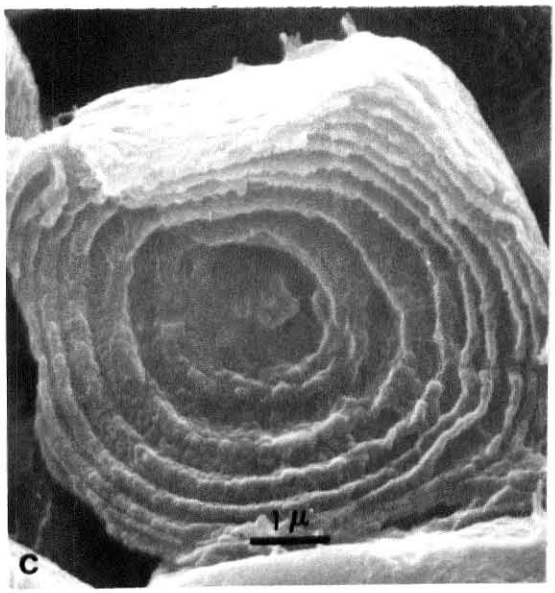
Millet starch granules modified by α-amylolysis are shown in Figure 13. The nature of the attack by α-amylase as well as some details of interior architecture are clearly revealed. As mentioned earlier millet starch granules are rather uniform in size with spherical granules observed in the center of the soft endosperm and polyhedral granules in the periphery of more tightly packed hard endosperm. The concentric shells (rings) in the interior

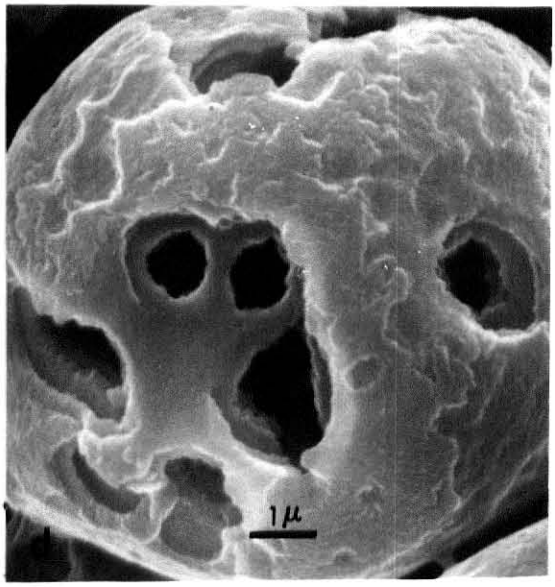
Figure 13. Endosperm and isolated starch from germinated (15° and 20° C.) millet.

a = starch, germinated 6 days, 15° C.
b = starch, germinated 4 days, 15° C.
c = endosperm, germinated 6 days, 15° C.
d = endosperm, germinated 8 days, 20° C.


2 μ
a


2 μ
b

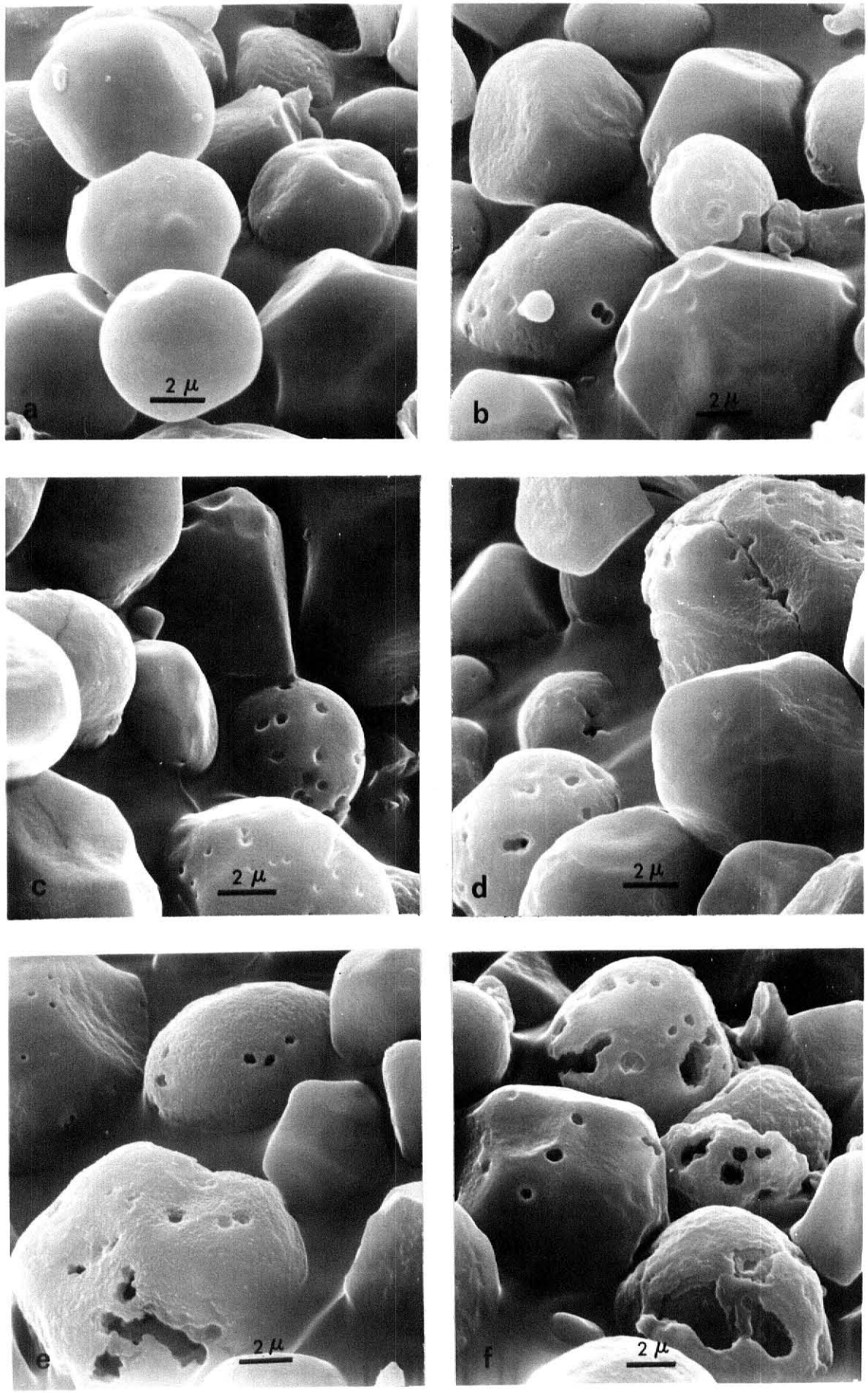

1 μ
c


1 μ

of a millet starch granule are clearly visible in Figure 13c. This starch granule was isolated from millet germinated for 6 days at 15° C. The thickness of the concentric rings appears to be relatively uniform. The distance between adjacent shells was greater near the center of the granule than that at the periphery. If the area between the shells contains starch of a more amorphous nature than that in the shells, this might be responsible for preferential enzymic attack on the interior portions of the granule. In contrast to wheat starch granules, there was no evidence of a "furrow" or a shallow trough in the equatorial region of millet starch granules. The mode of $\alpha$-amylolytic degradation of millet starch granules during germination was similar to the in vitro degradation reported by Rasper et al. (9). Although malted millet flours contained relatively low levels of $\alpha$-amylase (Table II), the enzyme appeared to be the most active based on pronounced modification of starch granules. This observation implies that the commercially available granula substrate (Phadebas) employed for the determination of $\alpha$-amylase activity might not be suitable for this purpose for malted millet flour. It is evident in Figure 13a, 13b and 13d which revealed that erosion of millet starch granules appeared as discrete holes penetrating deeply through several layers of the granules into the interior. The holes on the surface enlarged in size and tended to junction with each other as the extent of $\alpha$-amylolysis increased. Comparison of micrographs of starch from millet germinated for various periods of time at 15° C. confirmed that the development of circular pits on the surface of the granules (Figure 14) generally paralleled the development of $\alpha$-amylase activity in the endosperm (Table II). Enzymic erosion is visible in starch from ungerminated millet (Figure 14a) as previously observed in the endosperm (Figure 10).

Figure 14. Starch granules isolated from ungerminated and germinated (15° C.) millet.

a = ungerminated
b = soaked
c = germinated 2 days
d = germinated 4 days
e = germinated 6 days
f = germinated 8 days


2 μ
a
2 μ
b
2 μ
c
2 μ
d
2 μ
e
2 μ
f

On one of the polyhedral starch granules isolated from millet germinated for 4 days (upper right of Figure 14d) a crack aligned along the circular pits is clearly visible. This might be a region of structural weakness in the granule which was susceptible to enzymic erosion and mechanical splitting. However, it was not observed in other granules and may represent an artifact. During latter stages of germination ( 6 to 8 days), the enzyme appeared to penetrate through several layers of the concentric shells resulting in severe digestion of the central core of the granule (Figure 14f).

## SUMMARY

Hard red winter wheat, oats and pearl millet were germinated at 15° C. and 20° C. for as long as 14 days. Endosperm areas in kernels fractured with a razor blade and starches isolated from the malted flours were examined with a scanning electron microscope to determine the morphology of starch granules and the nature of α-amylolytic attack of the granules. Free sugars, damaged starch content and α-amylase activity of the flours were determined to establish relationships between physical changes in the cereal grains and elaboration of α-amylase during germination.

Germination at 20° C. did not significantly increase the amount of α-amylase activity but did slightly increase the rate of its appearance. Starch granules in wheat and millet were gradually degraded by the action of α-amylase in vivo accompanied by production of free sugars. This enzymic degradation contributed to the damaged starch values of flours milled from the germinated grains. Oat starch granules appeared to be most resistant to α-amylase attack during germination as confirmed by the small damaged starch content of flour milled from malted oats. The increase of free sugars in malted oat flour paralleling the increase of α-amylase activity during germination suggested that α-amylase might be responsible for disintegration of oat compound starch granule to smaller simple granules.

Starch granules in the endosperm near the aleurone layer were attacked at earlier stages of germination than were those in the inner endosperm. After soaking and during early stages of germination, the amorphous protein matrix in which wheat starch granules are embedded disappeared, facilitating access to starch granules by degradative enzymes. Amorphous-appearing

materials were observed to cover the surface of wheat starch granules during later stages of germination. Oat endosperm also showed a decrease in the cementing material holding the starch granules together and the appearance of amorphous material after germination for 14 days. There was no evidence of enzymic degradation in scanning electron micrographs of the endosperm. In millet, starch granules of distinct sizes and shape were found at different location in the endosperm. In contrast to wheat and oats, millet contained very little or none of the amorphous protein matrix in which starch is embedded and the cell walls of the amyloplast still remained unhydrolyzed after 8 days of germination. Enzymic attack was visible on millet starch granules in ungerminated kernels and progressed during germination from the spherical granules adjacent to the embryo to the polyhedral starch granules at the periphery of the endosperm. The extent of attack of millet starch granules appeared to parallel the increase in $\alpha$-amylase activity during germination.

Wheat starch granules were not severely attacked even though wheat had the greatest increase in $\alpha$-amylase activity during germination. Some degradation of wheat starch granules was observed in a manner similar to that reported by Dronzek et al. (36). No channelling or pitting of the granule surface was visible as a result of $\alpha$-amylase action on oat starch granules. The compound granules did disintegrate during germination releasing the individual small oat starch granules. $\alpha$-Amylase of germinated millet was the most active enzyme on the basis of starch granule degradation during germination.

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# EFFECTS OF GERMINATION OF WHEAT, OATS AND PEARL MILLET ON α-AMYLASE ACTIVITY AND STARCH DEGRADATION

by

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Endosperm area in kernels fractured with a razor blade and starches isolated from germinated wheat, oats and millet were examined with a scanning electron microscope for changes and modifications of the starch granules by $\alpha$-amylolytic attack during germination. Starch granules in these three cereals were degraded during germination by the action of $\alpha$-amylase accompanied by production of free sugars and increase in the damaged starch values of flours milled from the germinated grains. The cementing material embedding the starch granules in the endosperms of wheat and oats decreased and disappeared during germination while millet contained no such material. Amorphous-appearing material was found to cover starch granules in the endosperm of wheat and oats during later stages of germination. In spite of having the highest $\alpha$-amylase activity among the three cereal grains, a lower extent of enzymic degradation than expected was observed on the surfaces of wheat starch granules. Compound oat starch granules were relatively resistant to enzymic attack and no evidence of starch erosion was seen; the small granula were released from the compound granules during germination. $\alpha$-Amylase from millet was the most active enzyme in terms of extent of degrading starch granules. It corroded the surface of millet starch granules to form discrete holes leading to the interior of the granule. Inner concentric shell structures were observed in starch granules of wheat and millet but no such structures were observed in oat starch granules.