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SMALL ANGLE X-RAY SCATTERING BY HYDRATED WHEAT STARCH GRANULES

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Abstract

Wheat starch:water (1:2) dispersions were studied by small angle x-ray scatter diffractometry (SAXS), and by light, polarizing microscopy, and scanning electron microscopy (SEM). X-ray scatter data were collected in the 64-661 A range. Radii of gyration (Rg) of spherically shaped regions and d-spacings were calculated for samples that were treated as follows: 25° C and run at 25° C; heated to 88° C and run at 82° C; heated to 88° C, stored at 25° C for 2 h or 3 days and run at 25° C; heated to 88° C, stored at 2° C for 3 days and run at 6° C. Samples comparable to those used for SAXS experiments were stored without x-ray irradiation to evaluate the effects of x-ray exposure on starch structure. All samples were evaluated for differences in granule morphology and loss of birefringence using light, SEM and polarizing microscopy.

SAXS results indicated that a well-defined d-spacing that was seen between 260-296 A in unheated granules is related to birefringence of the starch granule and was not found again after heating, cooling, and storage. These observations indicate that a more random order was present after heating. The 260-296 A spacing fits the 3 cluster, ordered model for amylopectin in which an extended amylopectin molecule has a diameter of 500 A. The Rg data further support this model because an Rg value for unheated starch was found at 249 A. A maximal Rg change occurred for starch heated to 88° C and stored for 3 days at 2° C (Rg 175 A). These data suggest that heating, cooling and storage result in a molecular reorganization extending over a relatively large distance and involving both a loss of order (shown by loss of the 260-296 A spacing) and a tightening of the structure (shown by the decrease in the Rg values).

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Introduction

Changes in starch granule structure take place when starch granules are hydrated, heated and subsequently cooled and stored. These changes result from molecular rearrangements of starch granule molecules involving distances of a few A between neighboring atoms or up to 40 μm involving the entire starch granule. Wide angle x-ray scattering often is used to study repeating distances up to 15 A. Small angle x-ray scattering (SAXS) techniques are useful in the study of macromolecules for which repeating distances lie between 30 and 800 A. Small angle x-ray scattering techniques are also useful since they do not require chemical pretreatment as do other analytical methods that have been used to elucidate structure of starch (Robin et al. 1974, Kassenbeck 1978, Yamaguchi et al. 1979). However, there have been very few studies using SAXS for starch-based food systems (Sterling 1962, Traub et al. 1957, Grosskreutz 1960).

If one assumes that some macromolecules or regions within macromolecules are globular (or spherical), the size of these regions can be estimated by calculating the radii of gyration (Rg) of these regions. Regularly repeating distances within and between the molecules of starch granules likewise can be estimated from small angle x-ray scatter data.

Information from SAXS is useful when coupled with data obtained from light, polarizing and scanning electron microscopy (SEM) studies in which changes in shape, swelling and birefringent properties of starch are evaluated. The purpose of this study was to evaluate the interrelationship of the changes in the 30 to 800 A region of the starch granule, as calculated from SAXS data, to the changes in the whole granule as seen by light, polarizing and scanning electron microscopy.

Materials and Methods

Sample Preparation and Treatment

Wheat starch (Aytex, General Mills) - water samples were prepared using 35% wheat starch and 65% glass distilled water. The starch-water samples were weighed in 5.00 g lots, and injected into glass capillaries (1 mm diameter, Glas Berlin-West Co.). The capillaries were

sealed by heating and coating with quick-setting epoxy.

The heating and storage conditions are summarized in Table 1. Unheated samples were held and examined at 25° C. Heated samples were brought to 88° C and held at 88° C for 2 h prior to x-ray or microscopy. Samples were examined at 82° C or at 25° C immediately after the 88° C holding period. Other heated samples were cooled to 25° C, held either 2 hours or 3 days before examination by SAXS at 25° C. A third group of 88° C heated samples were cooled to 2° C and examined at 6° C after storage for 3 days at 2° C. All treatments were observed by microscopy at 25° C.

For each capillary used for SAXS experiments, another capillary was stored at the same temperature outside of the camera in order to evaluate the effects of x-ray exposure on starch structure. The data presented are based on three or four replications of each treatment combination except those for the sample heated to 88° C and run at 82° C. Capillaries containing the latter treatment samples frequently ruptured during the SAXS data collection run.

Small Angle X-ray Scattering

Small angle x-ray scattering data were collected between 64 and 661 A using an Ubar Kratky Camera modified according to Kaler (1982) and a TEC model 210 position sensitive detector. The camera was completely interfaced with a PDP 11/60 minicomputer via a Computer Automated Measurement and Control (CAMAC) data highway. The camera modifications included: 1. a lengthened sample-to-detector distance (129 cm); 2. an adapted sample holder which controlled and monitored sample temperature and held liquid or solid samples; and 3. an adapted sample chamber (without Mylar windows) for decreased background x-ray scatter. A movable beamstop allowed control of the amount of x-ray beam reaching the detector. CuKα radiation (λ=1.514 A) was used.

After the samples were heated and stored according to the conditions shown in Table 1, each capillary was loaded into a temperature controlled sample holder. The holder was placed in the camera and secured. A vacuum was slowly applied to the system and maintained at 40 to 60 mTorr throughout data collection. The capillary position was adjusted to optimize its position relative to the x-ray beam. The beamstop was adjusted for most efficient collection of x-ray scatter. The voltage was adjusted to 40 kV and amperage was adjusted to 3 mA. Data were collected for the first 2 h close to the detector in order to optimize the zero angle scatter for channel numbers 725 to 800. The x-ray beam and vacuum system were then turned off and the capillary was checked for breakage. The x-ray beam and vacuum system were turned back on and the beamstop was moved further from the detector. Thus, a greater number of counts could be obtained to optimize peak intensity between channels 0 to 725. The amperage was once again adjusted to optimize data collection. Data were collected for 6 h and used to plot scatter curves of intensity (I) of x-rays scatter at some scattering angle versus Channel Number, which can be translated into d-spacings and Rg values.

Scattering data were also plotted as I vs. h^2, where h is $4 \pi \sin \Theta/2\lambda$, and Θ is the scattering angle, and λ the incident x-ray wavelength. These plots can be used to find Rg values from the slope of the line of the best least-squares fit to values measured at the lowest values of h as h approaches zero, according to the Guinier equation (Guinier 1963, Glatter and Kratky 1982):

$$\ln I(h) = \ln I(0) - 1/3\, h^2\, Rg^2 \qquad (1)$$

I (0) is defined as the intensity of the scattered x-rays as the scatter angle approaches zero. Scattering data plotted as relative intensity (I/I_0) versus channel number represent the ratio of intensity at a specific scatter angle to the intensity as the scatter angle approaches zero. A predominant scattering angle for a particular sample then can be estimated. These scattering angles are related to the d-spacing by the Bragg equation:

$$n\lambda = 2\, d \sin \Theta \qquad (2)$$

where n is 1, and d the repeating distances. A main assumption for the Rg calculations is that the effective mean radius results from a spherical or globular area.

Microscopy

When SAXS data collection was completed, the irradiated and nonirradiated capillaries were immediately broken onto glass microscopy coverslips. Pieces of coverslip and sample were viewed using a Unitron microscope in the light or polarized light fields.

Other portions of the cover slips were mounted on silver painted aluminum stubs, placed in a desiccator over calcium sulfate and held for at least 24 h, coated with Au/Pd and viewed using a Philips Model 500 scanning electron microscope at 6 kV.

Results and Discussion

From the SAXS experiments, typical curves plotting I versus h^2 can be found in Figure 1a for starch samples at 25° C, and in Figure 1b for starch samples heated to 88° C prior to data acquisition. Other plots for different heat treatments are similar to those seen in Figure 1b.

Radii of Gyration (Rg)

The Rg values were calculated from the types of curves found in Figure 1. The averaged Rg values (Table 1) represent spherical regions with overall radii ranging from 249 $\pm$ 13 A for unheated starch samples that had x-ray data obtained at 25° C to 175 $\pm$ 24 A for starch samples that were heated to 88° C and stored for 3 days at 2° C prior to collection of x-ray data.

From Table 1, it can be seen that, initially, effective mean Rg values show large radii that change very little (18 A) upon heating to 88° C. After cooling and longer storage, however, there is a progressive decrease in the effective mean radii of the spherical regions, the least effect being in samples cooled for 2h at 25° C with an average

Table 1. Sample heating, cooling, storage conditions and effective mean radius of gyration (R_g).

Temperature of Sample Treatment(1)	Temperature and Duration of Storage Treatment	Temperature of SAXS (2)	R_g (A)
25° C	25° C for 2 h.	25° C	249 ± 13
88° C	88° C for 2 h.	82° C(3)(4)	231
88° C	25° C for 2 h.	25° C	215 ± 20
88° C	25° C for 3 days	25° C	190 ± 15
88° C	2° C for 3 days	6° C(3)	175 ± 24

(1) All samples held for 2 h prior to storage treatment.
(2) All samples were run on SAXS for 8 h. Similar capillaries were held for 8 h at the same temperature as the SAXS experiments to evaluate the effects of prolonged x-ray irradiation on starch microstructure when compared to normal holding.
(3) SAXS temperature could not be adjusted higher than 82° C or lower than 6° C.
(4) Replications were not possible due to repeated capillary breakage.

change of 34 A, followed by samples held for 3 days at 25° C with an average change of 59 A, then followed by samples which were cooled for 3 days at 2° C with an average change of 74 A.

Some variations in the effective mean radii are to be expected due to the complex nature and size differences of the starch granules as well as orientation of individual starch granules to the x-ray beam. Starch granules can also swell, disintegrate or agglomerate differently resulting in a wide range of Rg values. This variation was attributed to milling and processing damage, differing amylose to amylopectin ratios, and differing growth conditions.

Repeated Spacings

From the SAXS scattering data, typical plots of I versus channel number, and d-spacings, are seen in Figure 2a and 2b for unheated starch run at 25° C and starch heated to 88° C for 2 h prior to collecting data. From these plots, repeating spacings in the 30-800 A range can be studied. Except for an unresolved peak at about 260-296 A in unheated samples, distinct peaks were not found. All samples that had undergone heat treatment lost this peak and never regained it. This result could be supportive of the Rg data showing that the spherical or globular regions are spaced at regularly repeating distances.

Since distinct d-spacings were not found, general intensity differences for spacings between 64-199 A (Figure 3) and 260-661 A (Figure 4) were compared. (The slope of the scatter curves seemed to change within these 2 ranges). The ranges of relative intensity for d-spacings are shown in Figures 3 and 4 for each sample treatment.

The relative intensity data for the 64-199 A spacings (Figure 3) indicate that the starch samples that were unheated and run at 25° C; heated to 88° C and run at 82° C; and heated to 88° C and cooled at 25° C for 2 h and run at 25° C had a greater number of smaller repeating

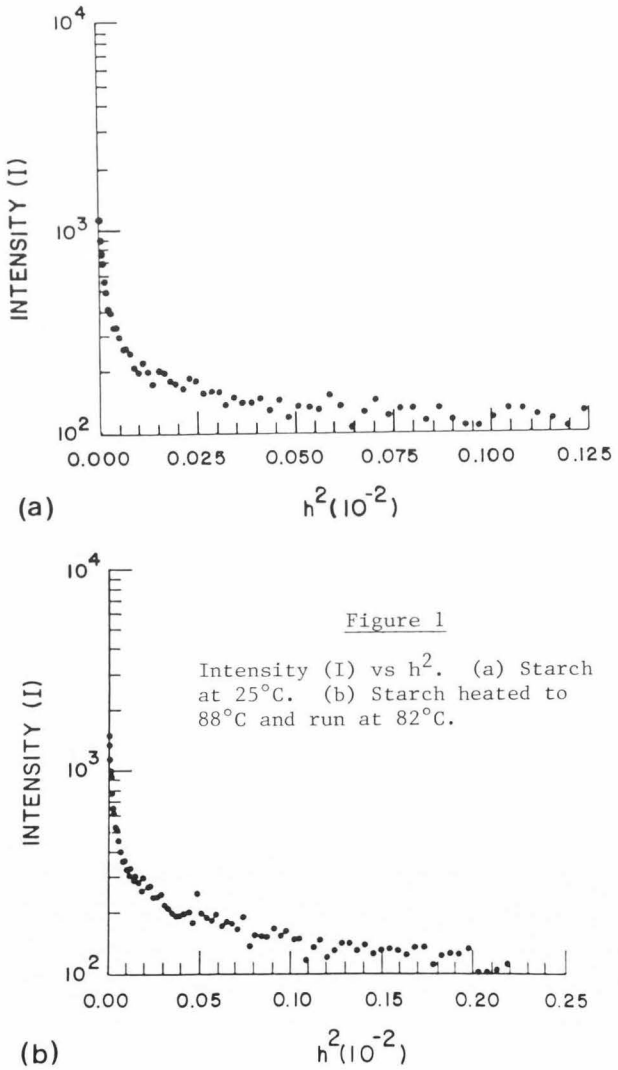

Figure 1

Intensity (I) vs h^2. (a) Starch at 25°C. (b) Starch heated to 88°C and run at 82°C.

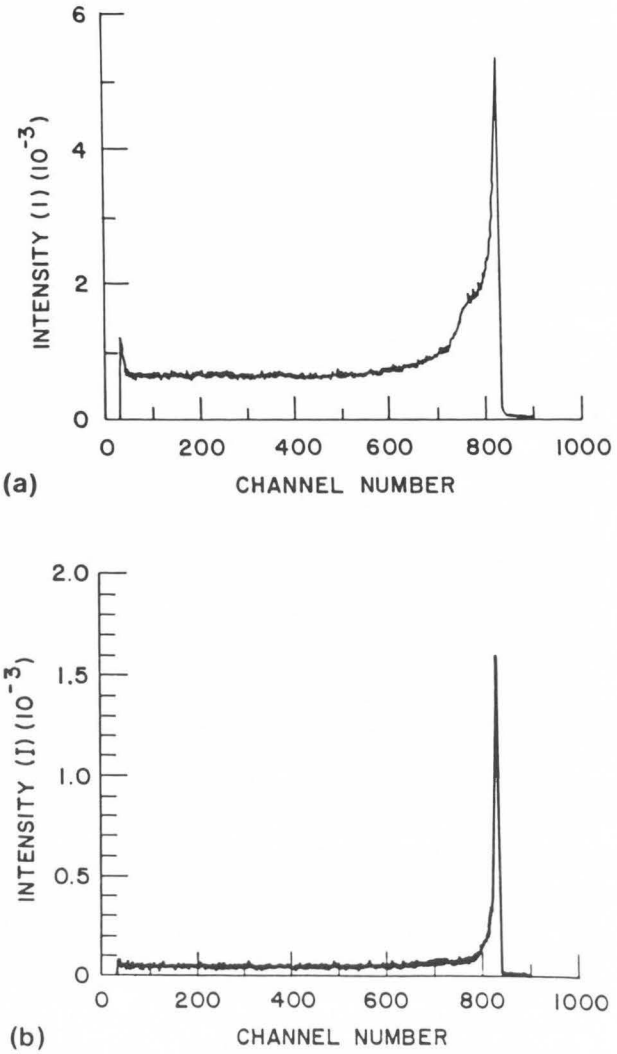

Figure 2. Intensity (I) vs channel number.
(a) Starch at 25° C.
(b) Starch heated to 88° C and run at 82° C.

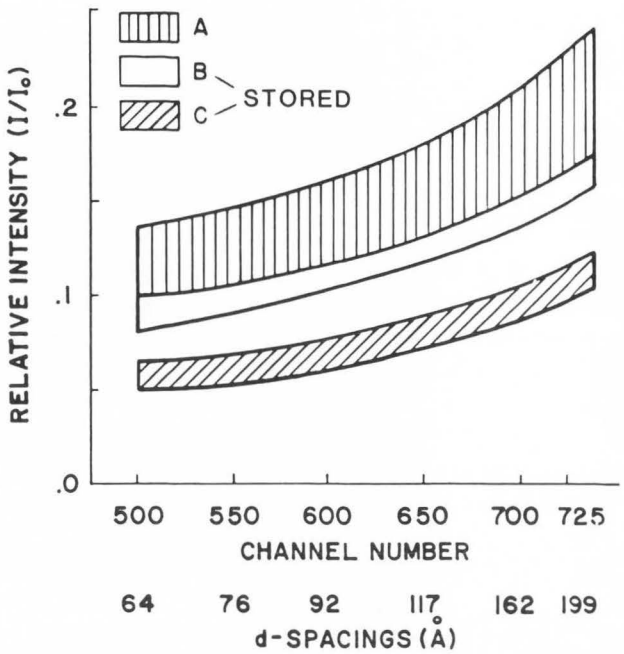

Figure 3. Relative intensity (I/I_o) vs. channel number, equivalent to spacings from 64 to 199 A. Cross-hatched regions show blocks where data fall for various treatments.
(a) 25° C, 88° C, and 88° C for 2 h at 25° C treatments.
(b) 88° C for 3 days at 25° C treatment.
(c) 88° C for 3 days at 2° C treatment.

distances than samples which were heated to 88° C and cooled for 3 days at 2° C and then run at 6° C.

The relative intensities in the 260-661 A range (Figure 4) showed that samples heated to 88° C, then stored for 3 days at 25° C and run at 25° C, or stored at 2° C and run at 6° C, had more of the larger d-spacings within this space range and a fewer number of smaller d-spacings when compared to samples which were unheated or heated to 88° C, held for 2 h and run at 25° C. Thus, in samples which were stored for 3 days, there was a general trend to a greater number of longer d-spacings as compared to those present in samples which were unheated, or were heated to 88° C and stored for minimal time (2 h).

It appears that storage of the previously heated samples results in decreases in the size of the spherical regions, as measured by the Rg values, and increases in the d-spacings, as measured by the relative intensities at the various d-spacings. This observation implies that some critical bonds are broken during heating that are crucial to maintaining the larger spherical areas. Once these bonds are broken, a molecular reorganization or compacting can result in smaller spherical radii and larger d-spacings, when the granule is stored for longer periods of time.

This interpretation of Rg values as being indicative of polymeric conformational changes is similar to that for proteins by Lewis and Sligar (1983) and Stivala and Khorramian (1982) for complex polysaccharides. Thus, we used Rg values to estimate which structural units may contribute to the calculated Rg values based on scattering data.

The Rg values found are supportive of the Kassenbeck and French models for amylopectin. The Kassenbeck model for amylopectin (Kassenbeck, 1978) is based on three cluster groups, each 50-70 A in length, which gives a total unit length between 150-210 A. French (1972, 1984) suggested that in the extended amylopectin model, the diameter of an amylopectin molecule would be about 500 A. This value is close to two times the Rg value found in our experiment. The radial length of 1000 A or more per cluster layer would be out of the range of our SAXS method. Once these clusters are broken, the population of well-defined d-spacings become smaller and the structures are more randomly oriented. The swollen granule would bring

the well-defined d-spacings out of the range of SAXS. This probably parallels the events seen when the granule loses birefringence and has an enthalpic change as seen by calorimetric studies in the 55-75° C range (Donovan and Mapes, 1980; Burt and Russell, 1983).

In the case of the present study, the x-ray data on scatter spacings indicate that a structure at about 296 A disappears upon heating and does not reappear after cooling. If the granule swells, it would be out of SAXS range. However, some spherical structure exists before heating, as measured by the Rg, but those areas tighten up on themselves and become smaller. If the Rg value is attributed to amylose, one would expect it to be larger after heating as the helix uncoils and is disrupted. For this reason, it is believed that the Rg calculation may reflect the amylopectin extended cluster model of French (1972), where the site could become smaller after bonds break and yet would be indicative of less order or crystallinity, which is the original contributory factor to birefringence.

Sterling (1962) and Traub et al. (1957) reported a spacing in the 90-100 A range for dry endosperm of wheat, rye, maize, groats, rice, and barley, and in 95-115 A range for wetted specimens. They also found similar spacings in dry or wetted wheat starch, flour, and dough, but not in gluten. Because the 90-100 A spacings were found in lipids extracted from flour with petroleum ether, but not in the starch from lipid-extracted flour, Traub et al. (1957) interpreted these spacings as being due to lipids closely associated with starch. The absence of these spacings in our starch samples may be due to the starch preparation methods.

Changes in structure, as measured in the 4 to 12 A range by wide angle x-ray scattering, are not necessarily at variance with the results reported here for small angle x-ray scattering. Wide angle x-ray scattering data for wheat starch show that the original intensity peaks seen between 4 and 6 A are replaced by different scatter patterns after storage, going from an A to a V pattern and/or a B pattern. These wide angle patterns are attributed to starch conformational changes and to complexing of amylose with intrinsic lipids which are present in the starch granule in the case of V pattern generation (Zobel, 1964; Hellman et al. 1954). Starch granules considered to have zero amylose, amylose, and amylose-lipid complexes all give wide angle x-ray diffraction patterns in the 4-12 A range (Banks and Greenwood, 1975). Thus it would be expected that changes in structures involving small distances are accompanied by perturbations of the larger structural units whose dimensions and spacings are measured SAXS. This viewpoint is consistent with observations of structure by other methods notably SEM and selective enzymic degradation of the granule.

Wide angle scatter differences for stored bread crumb have also been noted (Pisesookbunterng et al. 1983; Dragsdorf and Varriano-Marston 1980). The concomitant changes in the larger structural units have not been studied by SAXS, although our studies of stored gels show that changes at this level of organization occur in the starch-water model systems.

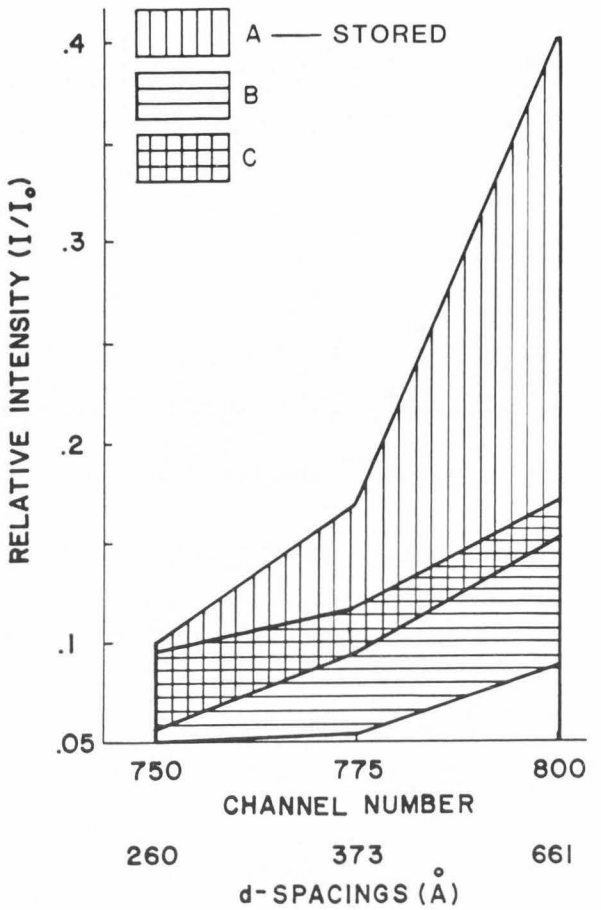

Figure 4. Relative intensity (I/I_o) vs channel number, equivalent to spacings 260-661 A. Cross-hatched regions show blocks where data fall for various treatments.
(a) 88° C for 3 days at 25° C or 2° C.
(b) 25° C, 88° C, 88° C for 2 h at 25° C.
(c) Blocks where data appeared for all treatments.

Microscopy

By light and polarizing micoscopy, unheated starch samples appeared sphere to disk shaped and birefringent. By SEM (Figure 5), unheated starch samples (Fig 5a) appeared sphere to disk shape and unswollen. After 8 h of x-ray data collection, the appearance of the irradiated was the same as the non-irradiated sample.

Granules from samples taken after heating to 88° C appeared swollen, irregularly shaped and not birefringent as seen by light and polarizing microscopy. By SEM all heated samples, except for the samples held for 3 days at 25° C or at 2° C, had structures similar to those seen in Figure 5b in which the granules appeared swollen and irregularly shaped. Granules which are more defined and puckered (arrow) as seen in Figure 5c for samples stored at 25° C for 3 days, and more ridged (arrow) as seen in Figure 5d for samples stored for 3 days at 2° C. All samples which were used for x-ray data acquisition exhibited the same features as before x-ray exposure and will not be presented here. Thus, samples

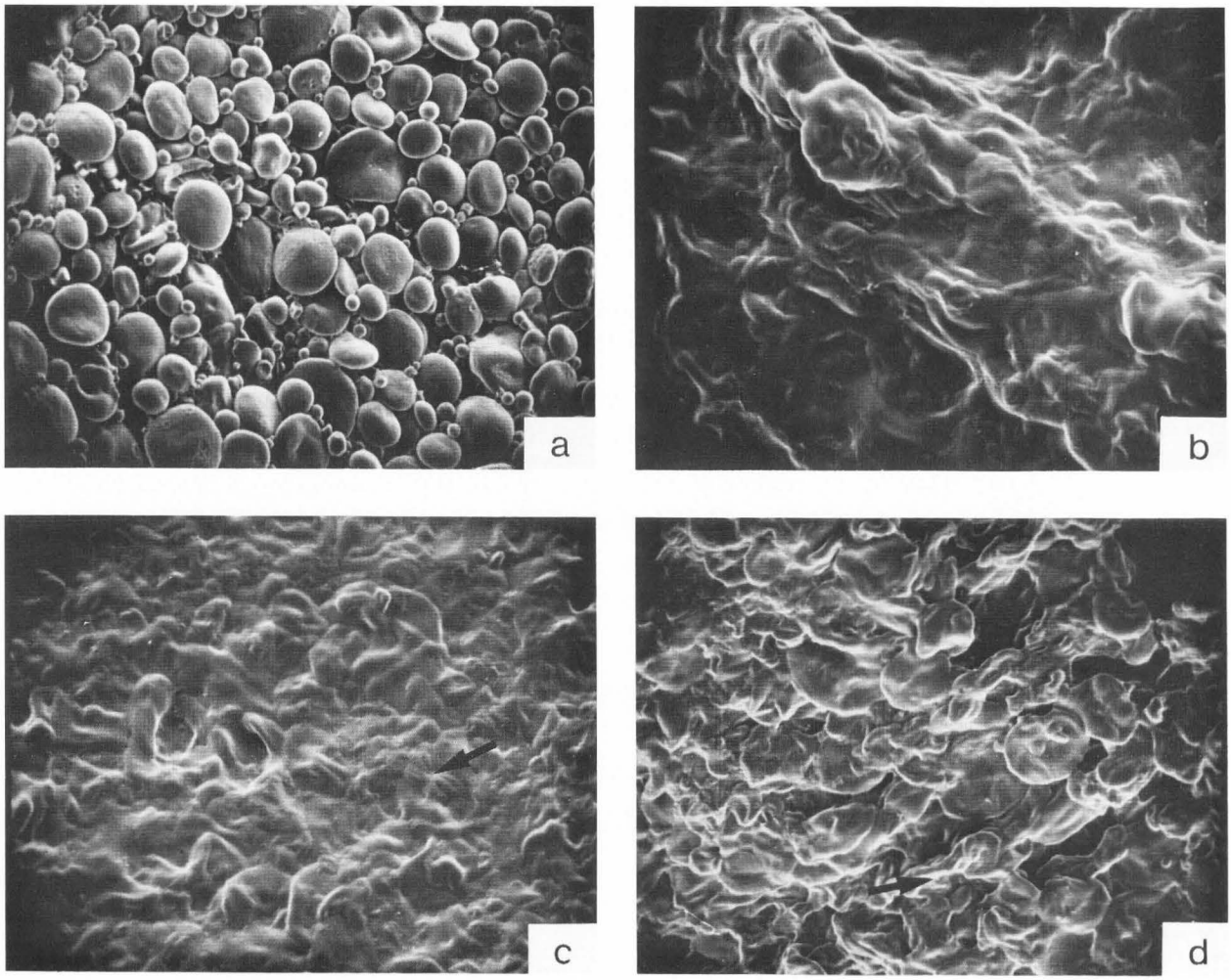

Figure 5. Scanning electron microscopy of starch granules. 15 μm
(a) Unheated at 25° C.
(b) Heated to 88° C and held at 88° C an equivalent time as x-ray runs at 82° C.
(c) Heated to 88° C and stored at 25° C an equivalent time as x-ray runs (arrow shows puckering).
(d) Heated to 88° C and stored for 3 days at 2° C for an equivalent time as x-ray runs. (Arrow shows ridges).

viewed by SEM appeared to develop some granule and/or matrix changes after storage that were not seen by light and polarizing microscopy. These differences were not due to dehydration because the capillaries were sealed during storage, although the variously treated samples may have responded differently to dehydration during preparation for SEM (Davis and Gordon, 1984).

Thus, microscopy confirmed the overall changes expected from the sample treatments, and, compared to the resolution of SAXS, permitted evaluation of changes over a still larger organizational domain.

Conclusions

SAXS results indicate that the well-defined d-spacing between 260-296 A may be related to birefringence of the starch granule since this spacing, as well as birefringence, disappeared upon heating. Once the molecules reorganize upon cooling, the granule is more randomly ordered and birefringence does not recur. The 260-296 A spacing closely fits the 3 cluster, ordered model for amylopectin in which an extended amylopectin molecule has a diameter of about 500 A. Rg data indicate that there are radii that range between 249 A for unheated starch and 175 A for heated and 3 day stored at 2° C samples. This supports the hypothesis that molecules, after heating, cooling and storage, have undergone a molecular reorganization that results in a more random structure with smaller repeating distances than are found in the native granule structure.

The implications of these findings are that

consideration should be given in product formulation to control of reorganization at the amylopectin cluster level in order to lessen staling associated with retrogradation in starch-containing cereal-based products.

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Discussion with Reviewers

H. F. Zobel: What assurance do you have that the SEM photos, 5d in particular, do not reflect artifacts of dehydration?

Authors: We agree that dehydration is always a possibility. In other studies, we have used other methods of sample preparation, but have felt that these other methods also had potential for dehydration. Some of these comparative studies are described by Davis and Gordon (1984).

H. F. Zobel: Did you take wide-angle pictures of aged gels to show recrystallization to forms different from the native A structure?

Authors: No. We agree that this is an essential next step since much more data are available for the wide-angle x-ray diffraction. We also, as you suggest, need to correlate any changes that occur over the very short distances with changes that involve organization extending over the longer ranges.

H. F. Zobel: What is your picture of a randomly ordered, 3 dimensional, higher order molecular reorganization?

Authors: We are referring to a level or organization often referred to as tertiary in the protein literature; i.e. "higher order". But we also want to stress that the individual elements within this structure also show a certain amount of random

order. Finally, the Rg calculation gives an idea of the size of regions either occupied by the macromolecules or by regions within the macromolecules.

H. F. Zobel: How do you relate the 100 A diffraction spacings reported by others and/or explain their absence in your results?

Authors: Differences in starch source or treatment may be one reason for our not observing this spacing. However, in the preliminary studies we used a longer flight tube than in the studies reported here. With the longer flight tube, we had some indications of the presence of smaller spacings, but we were not able to detect them with the shorter flight tube. The shorter flight tube was used to minimize damage to the detector since long time x-ray exposure was necessary. Also, alignment with the longer flight tube was a problem and we were not sure whether the data were real or an artifact of parasitic scatter.