

# Location of Sites of Reaction Within Starch Granules<sup>1</sup>

Kerry C. Huber<sup>2,3</sup> and James N. BeMiller<sup>2,4</sup>

## ABSTRACT

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To observe granular reaction patterns within modified starch granules, starch derivatives were converted to thallium(I) salts and viewed by scanning electron microscopy compositional backscattered electron imaging. Observation of phosphorylated potato and sorghum starches and a hydroxypropyl analog of waxy maize starch revealed that granular patterns of reaction were influenced by both starch and reagent types. In waxy maize and sorghum starches, flow of reagent into the granule matrix occurred

from channels (laterally) and cavities (from the inside outward). In potato starch granules, which do not possess channels, reagent diffused inward through exterior granule surfaces. Phosphoryl chloride (highly reactive) reacted to a large extent at granule surfaces, while the propylene oxide analog (less reactive) appeared to diffuse into the granule matrix prior to reacting.

Although chemical modification of granular starch has been investigated extensively, complete characterization of starch reactions has proven to be both elusive and challenging. A major barrier to understanding starch chemical modification is the complexity of the granule (Gallant and Bouchet 1986; BeMiller 1997; Gallant et al 1997) and the fact that the complex granule structure varies, not only between genotypes but also within a genotype (i.e., there is structural heterogeneity of starch granules). Neither the granule microstructure nor the ultrastructure, which ultimately determine the accessibility of individual starch molecules within starch granules to chemical reagent molecules, is completely understood. This lack of understanding is compounded by the response of the granule structure to various reaction conditions (i.e., the components of the chemical reaction system have the potential to alter granule structure and, thereby, to influence patterns of reaction). Additionally, chemical modification itself can alter granule microstructure over the course of reaction, so aside from strictly structural considerations, appreciable complexity regarding reaction locale is likely introduced by the various modifying reagents themselves, which vary in their properties and levels of addition. Also, the low levels of reagent typically required to impart desired functionality to starch contribute to the difficulty of directly detecting and locating granular modification sites.

## Intrinsic Factors in Starch Granule Reactions

Although starches from various sources exhibit fundamental structural similarities, they differ in the specific details of their micro- and ultrastructures. Differences between starch types with respect to granule morphology (size; shape; presence or lack of pores, channels, and cavities), molecular structure (amylose and amylopectin fine structures), and composition (amylose-to-amylopectin ratio, content of nonstarch components) lead to variations in starch properties (X-ray diffraction pattern, gelatinization temperature range, gel properties, retrogradation tendency, granule swelling power and pattern, etc.), which are indications of structural divergence.

Structural differences also have the potential to affect starch chemical modification processes. Singh et al (1993) found that root and tuber starches were more susceptible to structural modification than were either cereal or legume starches. While preparing carboxymethyl derivatives of corn and amaranth starches, Bhattacharyya et al (1995) observed that the optimum reaction conditions differed

for the two starches. Azemi and Wootton (1995) reported that hydroxypropylated common corn, waxy maize, and high-amylose corn starches differed with regard to their distribution of substituent groups on starch polymer molecules. These same three corn genotypes also varied in response to acid-catalyzed hydrolysis (Mussulman and Wagoner 1968).

## Extrinsic Factors in Starch Granule Reactions

Because starch granule microstructure ultimately directs patterns of chemical modification, the fact that various components of the reaction system have the potential to influence granule microstructure over the course of starch derivatization, and, as a result, affect the granular reaction locale, is also significant. In particular, the reaction medium, presence of catalytic base, addition of salts, reaction temperature, and both the amount and type of added reagent (since derivatization itself alters granule structure and behavior, even though the derivatized granules remain birefringent) are likely to influence starch granule reactions.

While investigating the substitution of granular corn starch with fatty acyl chlorides under anhydrous reaction conditions, Hauber et al (1992) determined that some swelling of granules was necessary to achieve chemical derivatization and that in the unswollen state not even surface hydroxyl groups were available for reaction. The necessity for granule swelling with respect to starch derivatization was corroborated by Kweon et al (1996), who in the course of examining the preparation of cationic starch, found a low reaction efficiency for starch reacted in anhydrous ethanol compared with that reacted in aqueous ethanol (all reactions NaOH-catalyzed). The fact that any derivatization occurred in the anhydrous reaction system might be explained by the presence of NaOH, which could have produced a small degree of granule swelling, perhaps surface-layer swelling. In contrast, Whistler et al (1998) reported reaction of granule surfaces in carbon tetrachloride using triethylamine as a catalyst; the degree of swelling, surface or otherwise, is unknown. However, based on available evidence, it can be concluded that the extent of granule reaction is a function of the properties of the reaction medium and probably, therefore, the magnitude of granule swelling.

Reaction efficiency with ethylene oxide improved with increasing alkali levels (below levels of granule gelatinization), which also resulted in elevated granule swelling (van Wamers et al 1994). Salts, which were added to the reaction system to protect starch granules against gelatinization during derivatization, were reported to decrease efficiency slightly. For hydroxypropylation of corn starch in an aqueous medium, Villwock (1996) determined that both salt concentration and type influenced reaction efficiency, presumably by controlling the degree of granule swelling and, thus, the access of reagent to starch molecules. Shi and BeMiller (2000) reported that, in the presence of citrate, hydroxypropylation of common corn starch required 1.8 times the amount of reagent that was required

<sup>1</sup> Contribution No. 16304 of Agricultural Research Programs, Purdue University.  
<sup>2</sup> Whistler Center for Carbohydrate Research, 1160 Food Science Building, Purdue University, West Lafayette, IN 47907.  
<sup>3</sup> Current address: Department of Food Science and Toxicology, University of Idaho, Moscow 83844.  
<sup>4</sup> Corresponding author. E-mail: bemiller@purdue.edu

when sulfate, a less lyotropic salt, was present. In contrast, the addition of Na<sub>2</sub>SO<sub>4</sub> was reported to improve the efficiency of the cross-linking reaction of waxy barley starch with POCl<sub>3</sub> (Wu and Seib 1990), but whether the improvement was due to an effect on the reagent, starch granule, or water is unknown.

Increased reactivity of potato starch with acetic anhydride after autoclaving of granules was attributed to granule swelling (Vasanthan et al 1995), and Hauber et al (1992) and Kweon et al (1996) reported that changing the temperature of the reaction system during derivatization modified granule reactivity, presumably via changes in the extent of granule swelling.

### Investigation of Granular Reaction Locale

Yeh and Yeh (1993) reported that cross-linking rice starch with POCl<sub>3</sub> decreased the subsequent extent of hydroxypropylation, while in contrast incipient hydroxypropylation increased the subsequent degree of cross-linking with POCl<sub>3</sub>. In addition, hydroxypropylation altered the gelatinization behavior of rice starch by increasing internal plasticization within granule amorphous regions and lowering the onset temperature for glass transition, while the melting of starch crystallites was largely unaffected (Seow and Thevamalar 1993; Yeh and Yeh 1993). This evidence suggests that the reaction took place almost exclusively within amorphous regions.

At the molecular level, substituent groups are unevenly distributed both between starch molecules and over the molecules themselves, both of which indicate that starch granules react heterogeneously (Hood and Mercier 1978; Steeneken and Smith 1991; Steeneken and Woortman 1994; Kavitha and BeMiller 1998; Shi and BeMiller 2000).

The distribution of cationic substituents within potato starch granules, which is in dispute, may vary according to the preparation method. In evaluating potato starch commercially derivatized dry or suspended in water or organic solvent, Vihervaara et al (1990) detected differences in the patterns of reaction. Both the organic solvent- and water-cationization processes yielded starch derivatives with uniform distributions of substituents, while the dry preparation process favored greater derivatization at granule surfaces. In contrast, Hamunen (1995) detected no differences between the nitrogen distributions of dry- and water-cationized potato starch. For laboratory-prepared starches, the inner region of the granule was most heavily derivatized, while for commercial starches derivatization appeared to be uniform throughout the granule. Whistler et al (1998) subjected corn starch granules to carboxymethylation with chloroacetic acid under anhydrous conditions. Under their conditions, reagent did not appear to penetrate the granule matrix, and the reaction was apparently limited to exterior and cavity surfaces. Indirect evidence for exclusive reaction of Remazolbrilliant blue R dye (RBB) at granular surfaces of wheat starch granules in aqueous slurry was obtained by Seguchi (1995) and Seguchi and Kanenaga (1997).

The distribution of phosphate in POCl<sub>3</sub>-modified granular corn starch was investigated by converting sites of derivatization to mercury(II) phosphate salts and visualizing their granule locale using transmission electron microscopy (Whistler and Spencer 1960). Although granules appeared to be most highly derivatized at exterior granule and cavity surfaces, reaction throughout the granule matrix was hypothesized, based on the appreciable loss of iodine-binding capacity for even slightly modified starches. Derivatization sites for POCl<sub>3</sub>-modified legume starches have been hypothesized to occur in the amorphous regions of the granule, because no difference is apparent between the x-ray diffraction patterns of modified and unmodified starches (Hoover and Sosulski 1986). Berghofer and Klaushofer (1977) investigated the granular distribution of phosphate cross-links in potato starch modified with sodium trimetaphosphate (STMP) in the semidry state. Modification with STMP produced a uniform pattern of reaction throughout the granules. Variation between the reaction patterns could be explained by differences in reaction conditions (aqueous starch slurry vs. semidry starch), reagent type (POCl<sub>3</sub> vs. STMP), and starch type (corn

vs. potato). The most likely explanation is differences in reagent type (i.e., the highly reactive POCl<sub>3</sub> reacts with the first starch molecules it encounters, those near surfaces, while in the other reaction granules are first impregnated throughout with STMP, then dried and heated).

For corn starch sulfated with triethylamine-sulfur trioxide complex in either aqueous sodium hydroxide saturated with sodium sulfate (Whistler et al 1959) or benzene-pyridine (Whistler and Spencer 1960), exterior granule and cavity surfaces were determined to be the primary regions of chemical modification. Whistler et al (1959) also reported that granule reactivity was inversely related to the extent of previous drying, even when modification was done under aqueous conditions, although the extent of cavitation was directly proportional to the extent of drying. Potato starch esterified with citrate was reported to have a homogeneous distribution of citrate throughout the granule based on detection of bound calcium (Berghofer and Klaushofer 1977).

In conclusion, few, if any, methods have successfully and conclusively located derivatization sites within modified starch granules. Yet, complete understanding of starch chemical modification requires a reliable method of elucidating the locale of starch granule reaction sites. Development of such a method would allow granular patterns of reaction to be observed and, perhaps, better products to be produced through control and modification of starch derivatization processes. The objective of this research was to develop a method for labeling starch granule reaction sites for two specific types of reactions and visualize patterns of reaction in granules.

## MATERIALS AND METHODS

### Starch Sources

Commercial waxy maize and potato starches were donated, respectively, by the A. E. Staley Manufacturing Company (Decatur, IL) and Penford Food Ingredients (Englewood, CO). Sorghum starch isolated from *hl* sorghum grain (genotype P-851171) was used because of its numerous, large, and clearly seen channels (Huber and BeMiller 1997).

### Starch Reaction with DTAF

Waxy maize starch was derivatized with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF) (Sigma Chemical Co., St. Louis, MO) using a method similar to that described by Whistler et al (1998) for obtaining surface-reacted acylated and carboxymethylated corn starch granules. Starch (10.0 g, db) was suspended in triethylamine (18.5 mL) with constant stirring for 30 min, after which the DTAF reagent (0.0025 g) in carbon tetrachloride (15.2 mL) was added to the reaction mixture. The reaction was allowed to proceed 7.5 hr in the dark (to protect the reagent against photobleaching). Then, the mixture was transferred to a screw-capped polypropylene tube, and the modified starch was recovered by centrifugation. The colorless supernatant, which indicated that the DTAF had migrated into or onto the starch granules, was discarded and replaced with anhydrous EtOH (≈35 mL) to wash unreacted dye from the starch. The tube containing the starch-EtOH suspension was shaken briefly on a multiwrist shaker (in the dark) and centrifuged to recover the starch product. The supernatant, which was colored due to the presence of dye, was discarded and replaced with fresh EtOH. The tube was returned to the shaker, and the washing process was repeated over the course of several days until all unreacted dye had been removed. The washed starch was recovered by vacuum filtration, allowed to air-dry in the dark, and stored in the dark at 4°C. A reaction control also was generated by carrying native waxy maize starch through the same process, except no DTAF reagent was added to the initial reaction mixture.

### Starch Reaction with Phosphoryl Chloride

Both *hl* sorghum and potato starches were cross-linked to varying degrees (parameters given in Table I). Starch was suspended in

water, and the resulting slurry was adjusted to pH 11.5 with NaOH (1.0M). The required amount of phosphoryl chloride (Fisher Scientific, Pittsburgh, PA) was added to the starch slurry in a dropwise fashion to avoid generation of excessive heat. During reagent addition, reaction pH was maintained in the alkaline range (11.0–11.5) by concurrent addition of NaOH (1.0M) using a radiometer pH meter equipped with an autoburette. The reaction was allowed to proceed 2.5 hr, after which the reacted starch was recovered by vacuum filtration and washed on the filter until the washings were neutral. Recovered starch was dewatered on the filter by washing with absolute EtOH (35 mL) and allowed to air-dry. Reaction controls were prepared under the same conditions, except the phosphoryl chloride was omitted from the reaction. Phosphate levels of both control and modified starches were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) to determine approximate degrees of substitution (described later).

### Starch Reaction with Sodium 3-Chloro-2-hydroxy-1-propanesulfonate

Waxy maize starch was derivatized to different extents with sodium 3-chloro-2-hydroxy-1-propanesulfonate (Aldrich Chemical Co., Milwaukee, WI) in aqueous alcohol using a method similar to that of Kweon et al (1996). Reaction parameters used to generate the four derivatives and the reaction control are given in Table II. For each reaction, reagent was first dissolved in the EtOH plus water, with stirring, followed by addition of the appropriate amount of NaOH (7.0%, w/v); the amount of NaOH was proportional to the amount of reagent added. Starch was immediately added to the reaction mixture with rapid stirring, and the reaction was allowed to proceed for 24 hr at 50°C. After completion, the reaction mixture was neutralized with a solution of HCl (3.0M) in absolute EtOH. The modified starch was recovered by vacuum filtration, resuspended in water (150 mL) for washing purposes, again recovered by vacuum filtration, dewatered on the filter with absolute EtOH (50 mL), and allowed to air-dry. Calculated molar substitution values were based on quantitation of the sulfur content of the derivatized starch.

### Conversion of Starch Derivatives to Thallium(I) Salts

Aqueous thallium(I) acetate (0.25M, 35 mL) (Alfa Aesar, Ward Hill, MA) was transferred to screw-capped polypropylene tubes

TABLE I  
Reaction System Parameters for POCl<sub>3</sub>-Reacted Sorghum and Potato Starches<sup>a</sup>

Starch Code	Water (mL)	POCl <sub>3</sub> (mL)
Sorghum		
PS-control	9.0	0.000
PS-1	9.0	0.045
PS-2	9.0	0.400
PS-3 <sup>b</sup>	40.0	1.440
Potato		
PP-control	9.0	0.000
PP-1	9.0	0.030
PP-2	9.0	0.400
PP-3 <sup>b</sup>	40.0	1.440

<sup>a</sup> Each contained 5.0 g of starch.

<sup>b</sup> Reaction proceeded 24 hr. Reaction mixture contained sodium sulfate.

TABLE II  
Reaction System Parameters for Modification of Waxy Corn Starch with 3-Chloro-2-hydroxy-1-propanesulfonate (PO Analog)<sup>a</sup>

Starch Code	7% NaOH (mL)	Water (mL)	Reagent (g)
HP-control	12.7	7.2	0.00
HP-1	3.8	16.2	0.34
HP-2	7.7	12.3	1.70
HP-3	12.7	7.2	3.39

<sup>a</sup> Each contained 20.0 g of starch and 37 mL of EtOH.

containing a starch sample (0.6 g, as-is basis). Tubes were subsequently shaken for 24 hr on a multiwrist shaker, after which they were centrifuged to recover the starch pellet. The supernatant was decanted, and the previously described steps, beginning with the addition of thallium(I) acetate solution, were repeated twice more to ensure complete conversion of anionic groups to Tl<sup>+</sup> salts. After exchange, starch samples were carried through a series of washes to remove unbound and excess thallium(I) acetate from granules. Two wash media were independently used, either deionized distilled water or hot 85% aqueous ethanol (v/v, 65°C). Wash medium (35 mL) was added to tubes containing exchanged starch, which were shaken for 30 min on the multiwrist shaker. After a brief suspension in wash medium, the tubes were centrifuged, and the supernatant was decanted. This series of events constituted one wash cycle. The cycle was repeated multiple times until it was determined that unbound thallium(I) acetate had been adequately removed from the starch. To establish the necessary number of washes for a particular sample, Tl<sup>+</sup> concentrations in each wash medium and starch were determined by ICP-AES using a Plasma 400 ICP-AES instrument (Perkin-Elmer Cetus, Norwalk, CT) equipped with a model AS 90 auto sampler. This instrument has spectral bandwidths of 0.019 nm in the UV region and of 0.19 nm in the visible region and possesses a Czerny-Turner mount monochromator with 408-mm focal length. The two gratings used are 4,200 lines per millimeter blazed at 220 nm and 1,800 lines per millimeter blazed at 490 nm for the UV and visible regions, respectively. For the final wash cycle, starch samples were recovered by vacuum filtration immediately following the 30-min interval on the multiwrist shaker (centrifugation step omitted), washed on the filter with absolute EtOH (35 mL), and allowed to air-dry.

Wash media and thallium-exchanged starch derivatives were analyzed quantitatively for Na, K, P, Ca, Mg, and Tl using ICP-AES. Wash medium samples were analyzed directly without any predigestion, although EtOH-containing samples were evaporated and redissolved in deionized distilled water prior to analysis. Starch samples, however, required a predigestion step. For predigestion, 70% nitric acid was added to calibrated Folin-Wu

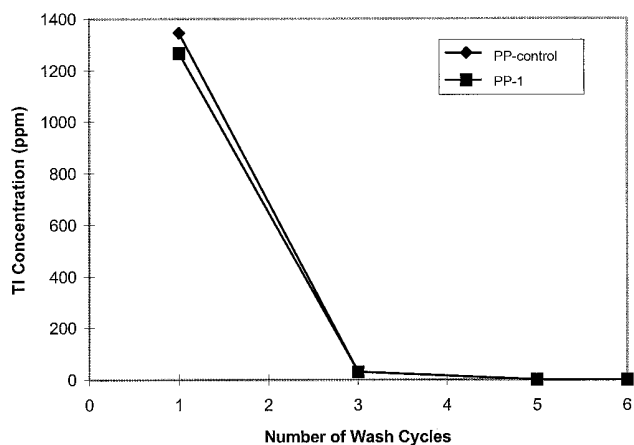


Fig. 1. Tracking of thallium wash-medium concentration for the control (PP-control) and a modified (PP-1) starch sample over the course of six wash cycles with hot 85% aqueous EtOH.

TABLE III  
Results for Thallium-Exchanged Starches Following Six Wash Cycles

Product	P (μmol/g starch)	Tl (μmol/g starch)	Tl:P Ratio	Starch DS
PP-control	27.93	54.99	1.969	0.0045
PP-1	33.51	57.09	1.704	0.0055
PP-2	162.6	210.9	1.297	0.0263
PP-3	239.5	325.9	1.360	0.0388

digestion tubes containing measured amounts of starch sample. Tubes were heated in a digestion block until nitric oxide fumes began to evolve, after which they were moved from the block to a rack and allowed to cool for at least 30 min. After the addition of hydrogen peroxide (30% solution,  $\approx 3.5$  mL), tubes were returned to the digestion block. With the advent of oxygen evolution from solution, tubes again were removed from the block until evolution had subsided. Finally, tubes were replaced in the block and their contents digested at  $180^{\circ}\text{C}$  for 1 hr or until the volume in the tube was reduced to  $\approx 1.5$  mL. After cooling, solutions were diluted to a final volume with nitric acid (5% solution) and subjected to ICP-

AES. A reagent blank was processed in the same manner, except that no starch sample was introduced.

The sulfur content of waxy maize hydroxypropyl starch analogs was determined according to Method 976.29 of AOAC International (AOAC 1995). Determinations for each derivative were conducted in duplicate.

### Microscopy

Intact DTAF starch granules were placed on a slide, and a drop of immersion oil was added. The starch suspension was overlaid with a coverslip and viewed with an Vanox photomicroscope (Olympus Corp., Lake Success, NY) equipped with fluorescence optics. The microscope head was equipped with a DM500 dichrome mirror, a 400–490 nm range exciter filter, and a 0515 barrier filter to provide blue light illumination. Photomicrographs were recorded on TX-400 film (Eastman Kodak Co., Rochester, NY).

Thallium-exchanged starch derivatives were embedded in Spurr's resin and microtome-sectioned dry (BeMiller and Huber 1997) to produce sections 1–2  $\mu\text{m}$  thick. Dry sections were mounted directly on double-sided carbon tape, which first had been adhered to aluminum stubs. Mounted specimens were carbon-coated and viewed in a SEM-840 scanning electron microscope (JEOL USA, Inc., Peabody, MA) at 10 kV using compositional backscattered electron (BSE) imaging. Images were captured with a digital acquisition system.

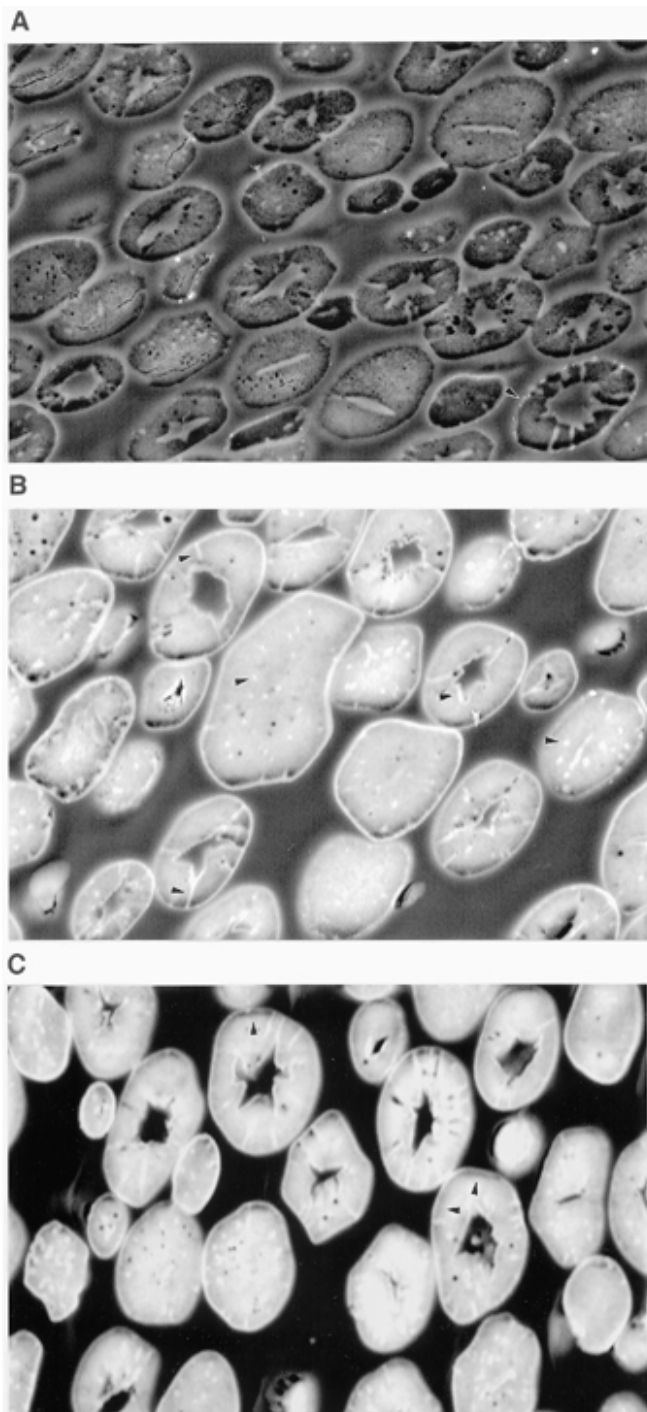
## RESULTS AND DISCUSSION

### Labeling of Granular Reaction Sites

Because starch derivatives investigated in this work were anionic in nature, the strategy for labeling such derivatives centered on conversion of substituent groups to heavy metal salts and location of heavy metal cations via electron microscopy. Preliminary efforts involved the use of mercuric chloride to prepare mercury(II) salts of  $\text{POCl}_3$ -reacted sorghum starch (Whistler and Spencer 1960). After exchange and washing (five wash cycles), starches were analyzed by ICP-AES to determine the relative amounts of mercury and phosphorus (Hg:P ratio) and provide a measure of the efficiency of mercury ion exchange onto phosphate substituents. According to Koch et al (1982), modification of starch with  $\text{POCl}_3$  introduces a mixture of both monostarch phosphate (two anionic sites per phosphate substituent) and distarch phosphate (one anionic site per phosphate substituent). As a result, a Hg:P ratio within the range of 2:1 (100% monostarch phosphate) to 1:1 (100% distarch phosphate) would be expected for a proper exchange. We found that the exchange of mercury ions onto phosphate substituents of modified sorghum starch was extremely inefficient (0.06:1.0 Hg:P ratio) and largely unsuccessful. It was speculated that this inefficiency might be related to the divalent nature of mercury(II) cations.

In an attempt to improve the efficiency of conversion of starch anionic derivatives to a heavy metal salt, thallium(I) acetate was used in place of mercuric chloride. The obtained Tl:P ratio (1.5:1.0) indicated vastly improved retention of  $\text{Tl}^+$  compared with  $\text{Hg}^{2+}$  at phosphate reaction sites and illustrated the importance of ionic compatibility in studies of this type.

Although heavy metal-labeling of reactive sites appeared to be improved by the use of thallium(I) acetate, the exact number of washes required to remove unbound  $\text{Tl}^+$  from starch granules needed to be determined. This task was complicated by the fact that exchanged starch samples did not wash to an obvious end point. Further, the true end point for  $\text{POCl}_3$ -modified sorghum starch was not readily apparent, because the monoester:diester ratio of the starch was unknown. Method development was simplified using unmodified potato starch, which contains a known level of native phosphate in the monoester form (Kasemsuwan and Jane 1996), in place of  $\text{POCl}_3$ -derivatized sorghum starch. This change accommodated a truer determination of exchange efficiency based on the known amount and state (monoester) of the potato starch



**Fig. 2.** Electron micrograph of granule sections from  $\text{POCl}_3$ -treated *hl* sorghum starch thallium salt visualized by SEM compositional BSE imaging: **A**, PS-1 (arrow points to possible outer-surface reaction site); **B**, PS-2 (arrows point to channel and cavity reaction sites); **C**, PS-3 (arrows point to least reacted sites) (Table I).

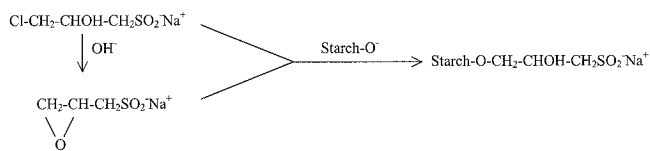
phosphate. ICP-AES analysis was used to examine both the wash medium and the starch. Native potato starch, which had been separately exchanged with sodium acetate, was subjected to eight washes with deionized distilled water. Sodium wash concentrations in the wash water decreased rapidly to baseline levels after two wash cycles (Fig. 1 and Table III). A similar curve was obtained for the washing of thallium-exchanged starch, except that four wash cycles were required. More importantly, the corresponding Na:P and Tl:P ratios within the starch samples after the stated number of wash cycles were 1.77:1 and 1.92:1, respectively, both of which approximated the theoretical 2:1 ratio expected for starch phosphate monoesters.

Another attempt to accentuate the washing end point involved the use of EtOH as a wash medium. We anticipated that the use of EtOH would 1) decrease the dielectric constant of the wash medium (relative to water) and, thereby, eliminate the effect of the phosphate  $pK_a$  value, and 2) favor removal of excess metal acetate over exchanged metal starch phosphate (based on the solubility differential of metal acetates and phosphates in EtOH). In practice, however, the use of 100% EtOH was ineffective, because granule swelling in EtOH was greatly reduced or eliminated, which greatly retarded removal of excess metal acetate. However, hot 85% aqueous EtOH (v/v, 65°C) appeared to swell granules sufficiently for removal of excess metal acetate. By tracking the thallium levels in the aqueous EtOH throughout washing, a curve similar to that previously observed for water washing was obtained, except that the washing end point for the aqueous EtOH medium was delayed (five to six washes). Again, the Tl:P ratio of the starch at the washing end point (1.97:1) approached the theoretical ratio (2:1) expected for starch phosphate monoester. Although hot aqueous EtOH appeared to be an effective wash medium, exchanged starch still did not wash to a fixed end point (based solely on starch Tl:P ratio). As a result, end-point determination remained dependent on wash water analysis, and the end point was at the break in the curve between rapidly removed cation and slowly leached cation (Fig. 1 and Table III).

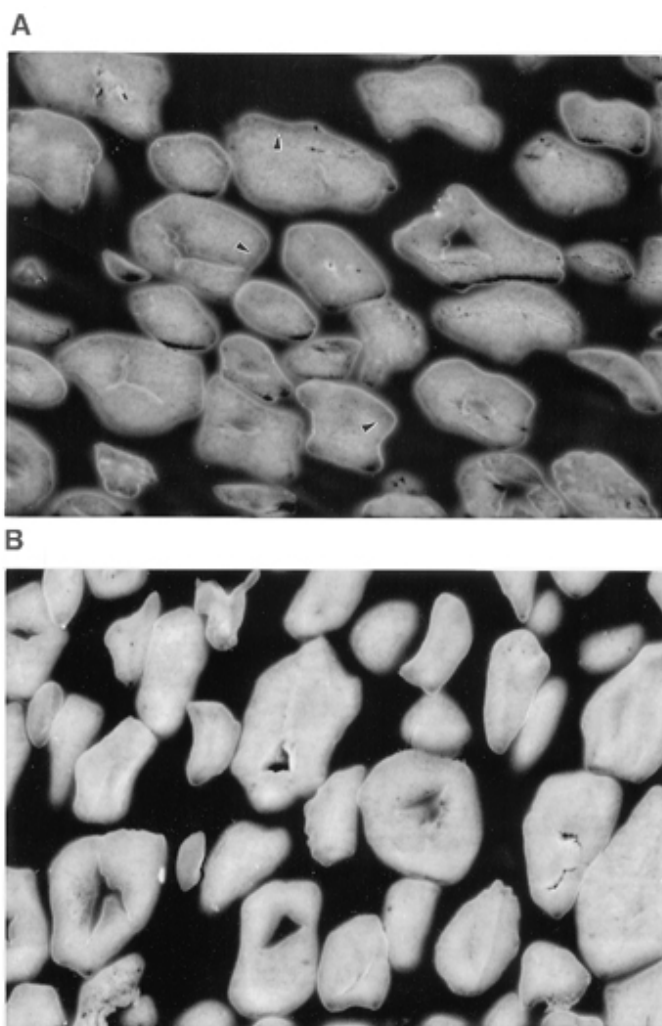
Although the observed metal ion:P ratios for exchanged starch provided some evidence for the conversion of starch phosphate substituents to thallium or sodium salts, further documentation was obtained through two additional experiments. The first experiment was designed to take advantage of the potato monostarch phosphate  $pK_a$  values, which should be approximated by those determined for glucose 6-phosphate ( $pK_{a1} \cong 0.94$ ,  $pK_{a2} \cong 6.11$  [Lim and Seib 1993]). If metal cations were truly bound to starch phosphate moieties, the final Na:P levels retained by the starch should be affected by the pH of the wash medium. Potato starch, which had been exchanged with sodium acetate, was subjected to washing with deionized distilled water at either pH 5.65 (below phosphate  $pK_{a2}$ ), 7.00 (slightly above  $pK_{a2}$ ), or 8.65 (above  $pK_{a2}$ ). Wash medium pH was adjusted using either HCl or NaOH (0.01M). After two washes, final Na:P values for exchanged starches washed at pH 5.65, 7.00, and 8.65 were 1.0, 1.3, and 1.7, respectively. Sodium cations likely were associated with starch phosphate moieties. For the second experiment, additional phosphate was incorporated into native potato starch by reaction with different amounts of  $POCl_3$ . After exchange with thallium acetate and washing (four wash cycles) with deionized distilled water, the modified starch possessed an increased thallium content (relative to the exchanged native starch), which was proportional to the incorporated phosphate. The fact that the amount of thallium retained by the starch was a function of its phosphate content provided additional evidence that the cations in exchanged starch were coupled to starch phosphate substituents.

After development of the method using native potato starch, the developed exchange and wash procedures were applied to modified sorghum, potato, and waxy corn starches (Tables I and II). (Phosphorylated starches are abbreviated PS [sorghum] and PP [potato].) Exchanged starch derivatives were collected only after

the proper number of wash cycles (sufficient to reduce Tl<sup>+</sup> concentrations in the wash medium to baseline levels) had been positively established (erred on the side of conservancy). The thallium contents of the exchanged modified starches correlated closely to the respective levels of chemical modification. All Tl:P ratios fell within the expected range (between 2:1 and 1:1) (Fig. 1 and Table III). The ratios further indicated a monoester:diester ratio of  $\cong 1:3$  for the modified starches. This ratio agrees with the report of Koch et al (1982). With regard to the sulfonated (HP analogs) of waxy corn starch, the obtained Tl:S ratios ( $\cong 0.69$ – $0.88:1$ ) were slightly lower than had been expected (1:1) but were conservatively acceptable. The lower efficiency may be due to experimental error. Starch reaction controls, particularly those of waxy corn and *hl* sorghum, did retain some residual thallium after washing. Defatting (according to the method of Morrison and Coventry [1985]) of waxy corn starch prior to thallium exchange did not improve the removal of residual thallium during washing. This baseline binding of thallium ions may be due to remnant



**Fig. 3.** Reaction that produces hydroxypropyl starch analog.



**Fig. 4.** Electron micrograph of granule sections from an analog of hydroxypropylated waxy maize starch thallium salt visualized by SEM compositional BSE imaging: **A**, HP-1 (arrows point to least reacted sites); **B**, HP-2 (Table II).

endosperm protein at starch granule surfaces. Residual thallium levels added to the background noise levels but should not have interfered with the objective, which was to observe patterns of granular chemical reactions.

### Visualization of Granular Patterns of Reaction

**DTAF Waxy Maize Starch.** The granular pattern of reaction for DTAF-reacted waxy maize starch was observed by fluorescence microscopy. As anticipated, the reaction was confined to (reacted dye was localized at) granule surfaces, including those of both channels and cavities (not shown). Whistler et al (1998) had previously reported exclusive reaction of exterior granule and cavity surfaces using the same reaction conditions, but they did not report visualization of granule channels or, apparently, directly observe a reaction at channel surfaces. Although channel surfaces were likely derivatized, channels could not be resolved by the light microscopy procedure used in their study. They did, however, credit channels for the delivery of reagent to cavities at the granule hilum.

**Phosphorylated Sorghum and Potato Starch.** Although sorghum and potato starches were both modified with  $\text{POCl}_3$  under similar reaction conditions, the two starches differed with regard to their observed granular patterns of reaction. Reaction patterns for the modified sorghum starches (PS-1, PS-2, and PS-3), observed by scanning electron microscopy (SEM) with compositional BSE imaging, are shown in Fig. 2A–C. Compositional BSE imaging, which differentiates regions of relative high and low atomic mass, allowed thallium-rich regions of the specimen (bright regions) to be distinguished. Because all three micrographs were captured using identical microscope settings, variations in brightness and contrast observed between micrographs reflect true differences in thallium contents and distributions. Perceived differences between PS-1 (lowest level of modification) and PS-control were minimal and might have been overshadowed by the residual thallium content of the control starch. (Background levels in the control starches in all cases might be due to granule-bound protein.) At best, several PS-1 starch granules (Fig. 2A) possessed small clusters of possible reaction sites at their outer surfaces, features that were not observed in the control. In contrast, the more highly modified starches (PS-2 and PS-3) exhibited obvious differences relative to the control. For PS-1 and PS-2, the primary reaction appeared to occur at granule surfaces, including those of channels and cavities (Fig. 2B and C), although the reaction likely occurred within the granule matrix as well. This finding was logical, based on the high reactivity of  $\text{POCl}_3$ . The predominant reaction of granule surfaces with  $\text{POCl}_3$  corroborates an earlier report (Whistler and Spencer 1960). Thus, the distribution of reaction sites within the granule does not appear to be homogeneous. Based on reaction patterns observed in this study, it is hypothesized that channels and cavities within sorghum starch granules play a significant role in directing the granular locale of the reaction. For derivatives PS-2 and PS-3, the least reacted areas appeared to be those located just inside granule perimeters (Fig. 2B and C). This observation would seem to suggest that penetration of reagent through the perimeter surface of granules inward was somewhat impeded, perhaps by a highly associated outer shell. In contrast, many of the most intensely reacted regions seemed to occur within the granule, particularly around channels (Fig. 2C). Thus, the primary path for reagent flow into the granule matrix over the course of the reaction likely occurs through channels (laterally) and cavities (from the inside out), as earlier theorized in a dye infiltration study (Huber and BeMiller 2000). In such a scenario, the outer regions of the granule probably would be the last to be reached by the reagent, as appeared to be the case for many granules. It is possible that the apparent resistance of an outer granule layer to reagent penetration is affected by surface impurities (lipids or proteins). Such cannot be ruled out in this work. However, because in potato starch granules (discussed below), which have

little protein and lipid content, the outermost layer was last region to react, it seems to us that this explanation is unlikely. There are other reasons for this conclusion as well. The pattern was observed in both types of granules reacted at high pH (conditions that solubilize or remove protein and lipids). In addition, it seems unlikely that there would be a uniform coating of protein or lipid on or near granule surfaces. Finally, it was concluded by Schleiden (1849) 150 years ago that the layers in potato starch granules are progressively tougher proceeding from the inside outward. Direct evidence that this is true is lacking, but it has been observed that oxidation of potato starch with sodium hypochlorite proceeds from the granule interior outward (Fischer 1977; Boruch 1985). These observations, and that of granule ghost formation, are congruent with a special highly organized structure near the granule periphery.

Because potato starch granule reaction patterns were of secondary interest in this work, obtained results are discussed without the use of electron micrographs. For potato starch, reaction with  $\text{POCl}_3$  appeared to be more uniform than had been observed for sorghum starch. In TI-exchanged native potato starch, which possesses monostarch phosphate, peripheral surfaces of the granules were labeled most intensely, although a few granules showed labeling at their granule centers. Although peripheral bands were universally observed for virtually all granules, the intensity of the bands did vary among granules. It is possible that the bright bands represent native monostarch phosphate substituents, although bands also could denote residual granule surface protein. That the native phosphate substituents of potato starch are concentrated on only a small fraction of amylopectin molecules (Abe et al 1982) and that they are concentrated at potato starch granule cores (Jane and Shen 1993) has been proposed. With even the lowest level of modification evaluated in this study (PP-1), bands at granule peripheries appeared less pronounced and somewhat overshadowed compared with those observed for native potato starch granules. The decreased contrast of the peripheral bands in the modified starch granules implied that phosphate addition during chemical modification occurred more uniformly and was less limited to granule surfaces than was the case with sorghum starch. Differences in reactivity between adjacent more dense and less dense growth rings could be perceived occasionally. Also, as observed in a few broken or cracked granules, starch material within the inner regions of potato starch granules was more susceptible to reaction than was the starch comprising the outer granule shell.

**HP Analog of Waxy Maize Starch.** Waxy maize starch was derivatized with sodium 3-chloro-2-hydroxy-1-propanesulfonate to generate a hydroxypropyl starch analog (Fig. 3), which could be converted to a thallium(I) salt. Electron micrographs depicting granular reaction patterns for the two highest substitution levels (HP-2 and HP-3) are presented in Fig. 4A and B. Micrographs of the HP-analog starch were generated using the same microscope settings; as a result, variations in the relative intensities of images depict real differences. The lowest level of substitution (HP-1) did not differ significantly from the reaction control. As was previously demonstrated for the sorghum starch phosphate derivatives, relatively little reaction occurred in the peripheral regions of granules just inside the granule exterior (HP-2, Fig. 4A). Although derivatization certainly occurred at the outermost surfaces of the granule, reagent did not appear to diffuse readily from the outer surfaces into the granule matrix. Many of the peripheral granule regions, which were not heavily derivatized at lower substitution levels (HP-2, Fig. 4A), became derivatized with the addition of more reagent (HP-3, Fig. 4B). Reagent diffusion into the granule matrix likely occurred by way of channels and cavities, as was theorized for the  $\text{POCl}_3$ -reacted sorghum starch, and peripheral regions were likely the last to react. Further, granules that exhibited little or no reaction in their interiors occasionally were encountered. This might be the result of fewer, or the complete lack of, channels within these granules or could be an artifact arising

from observation of a section that did not include the region close to the hilum. Although channels exist in waxy maize starch granules, channels highlighted by the sulfonate reagent were not frequently encountered, and were only unclearly observed in the HP-2 granules. Whereas channels within waxy maize starch granules are typically fewer in number, smaller in size, and less easily observed compared with those of sorghum starch granules (Huber and BeMiller 1997), the inability to visualize channels was probably due, at least in part, to the nature of the reagent. Because of the lesser reactivity of the sulfonate reagent (compared with POCl<sub>3</sub>), it probably diffused further into the granule matrix before reacting and reacted more evenly throughout the granule. At the highest substitution level (HP-3), the reaction, in general, appeared relatively homogeneous throughout the granule, although some granule-to-granule variation was observed. Occasional visualization of rings or shells within granules indicated that heterogeneity at finer structural levels likely existed. However, because the highest substitution level used in this work (MS  $\cong$  0.06) is low for a modified food starch (MS of hydroxypropyl groups = 0.20 allowed) and because the results of this work suggest that derivatization becomes more uniform as its extent increases, it can be assumed that hydroxypropylation, as commercially practiced, likely produces homogeneous derivatization within granules.

### CONCLUSIONS

Although channels within corn starch granules had been credited previously with delivery of reagent into the hilum (Whistler et al 1998), reaction on or near channel surfaces had not been reported. Using a reactive fluorescent dye (DTAF), reactivity of channel surfaces was demonstrated.

To observe reaction patterns within modified starch granules, a method was developed for conversion of anionic starch derivatives to thallium(I) salts. After development, the method was applied to POCl<sub>3</sub>-modified sorghum and potato starches, as well as to an analog of hydroxypropyl waxy maize starch. With the aid of SEM compositional BSE imaging, granular reaction patterns (based on the distribution of thallium salts) were determined for the various starch derivatives. Observed granular patterns of reaction were influenced by both starch and reagent type. Locations of derivatization sites are probably conservative (i.e., where increases are seen in concentrations of heavy metal ions over those in a control, there is undoubtedly an anionic group present at the highlighted locations), but other sites of derivatization may be present and not highlighted due to insufficient localized ion concentration.

Waxy maize and sorghum starch granules, which possess channels leading into their interiors, exhibited similar patterns of infiltration by reagent. Reagent entry into the granule matrix appeared to be greatest through channels (laterally) and cavities (from the inside outward), based on observed patterns of reaction. Diffusion of reagent from exterior granule surfaces into the granule matrix appeared to be hindered, perhaps by a highly associated shell at the granule periphery. Peripheral regions of the granule matrix just inside external surfaces were generally the last to be modified. In contrast, for potato starch granules, which do not possess channels, reagent must pass through the external surface to gain access to the granule matrix, and evidence for this pattern was obtained. At high levels of modification, granular patterns of reaction for potato starch seemed relatively uniform and, overall, exhibited less of a preference for granule surfaces (relative to waxy maize and sorghum starches).

Relative reactivities of reagents also appeared to impact granular reaction patterns of starch. Waxy maize starch granules, which had been derivatized with an analog of propylene oxide, displayed more homogeneous reaction patterns than did POCl<sub>3</sub>-modified sorghum starch granules. POCl<sub>3</sub>, due to its greater reactivity, was more inclined to react at granule surfaces, including those of channels and cavities. In contrast, the less reactive propylene oxide analog

likely diffused further into the granule matrix prior to reaction. In fact, few channel surfaces were highlighted by the propylene oxide analog, while the reverse was true for the POCl<sub>3</sub> reagent. Although some of the observed variability could be attributable to differences between the two starches themselves (channels in waxy corn starch granules are less frequent, smaller in size, and more difficult to observe), the reactivity of the reagent certainly appears to be a significant factor in granule reactions. Further, it is also apparent that starch granule architecture impacts the locale of granular derivatization sites.

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