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A Nonaqueous Procedure for Isolating Starch Granules with Associated Metabolites from Maize (*Zea mays* L.) Endosperm[1]

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ABSTRACT

A nonaqueous procedure using glycerol and 3-chloro-1,2-propanediol was developed for the isolation from maize of starch granules with associated metabolites. In this procedure, immature endosperm tissue was quickly frozen at −156 C, freeze-dried, homogenized in cold glycerol, filtered through Miracloth, and centrifuged through a higher density medium of 3-chloro-1,2-propanediol. The procedure was used to isolate starch granules from the endosperm of *normal* and the mutant *amylose-extender dull waxy*. Starch and water-soluble polysaccharide recovery was high with low cytoplasmic (RNA) and nuclear (DNA) contamination.

Electron microscopic examination of the isolated starch granules failed to demonstrate the presence of the amyloplast's membrane. However, based on an examination of fresh, freeze-dried, and rehydrated freeze-dried *normal* endosperm, it is suggested that the amyloplast membrane and enclosed stroma metabolites were dried onto the surface of the starch granules during the freeze-drying procedure. Chemical analysis of the glycerol-propanediol isolated granules showed the presence of alcohol-soluble sugars, inorganic phosphate, and phosphate-containing compounds. These soluble metabolites may represent amyloplast stroma metabolites which became bound to the starch granules during freeze-drying. Thus, this isolation procedure should be useful when metabolites closely associated with starch granules *in situ* are to be evaluated.

In maize endosperm, as well as in other storage tissues, starch accumulates in amyloplasts. These organelles develop from proplastids and are specialized leucoplasts (1). The plastids are surrounded by a double membrane and are thought to be the site of starch synthesis (1). However, due to the difficulties associated with the isolation of intact amyloplasts, no satisfactory determinations of metabolite and enzyme composition of amyloplasts have been made.

Attempts to isolate amyloplasts with intact membranes have been unsuccessful. Duffus and Rosie (6) and Williams and Duffus (26) used aqueous procedures to isolate a starch granule fraction from potato tubers and immature barley endosperm, respectively. Their starch granule preparations, which they called amyloplasts, contained granule-bound starch synthase. Viswanathan (24) used a buffered mannitol solution to isolate starch granules which reportedly contained all enzymes necessary to convert sucrose to starch. However, we have been unable to repeat their results (J. C. Shannon, unpublished results). Ford (7) used an aqueous procedure similar to that used by Viswanathan (24) and a nonaqueous procedure employing hexane and carbon tetrachloride to isolate amyloplasts. Neither procedure was satisfactory for the isolation of amyloplasts with associated stroma metabolites free of other cellular components. The hexane-carbon tetrachloride procedure yielded poor starch recovery and excessively high contamination with other cellular components. In the studies above (6, 7, 24, 26), there were no reports of the detection of amyloplast membranes by the electron microscopy. Thus, the presence of the bounding membrane and integrity of the isolated amyloplasts is open to question.

As part of our continuing *in vivo* study of starch biosynthesis, we wanted to determine the metabolite composition of amyloplasts in immature maize endosperm; however, there were no suitable procedures for the isolation of starch granules with associated stroma metabolites. Here, we describe a nonaqueous procedure using glycerol and propanediol which is suitable for isolating starch granules with associated metabolites. In addition the ultrastructure of fresh, freeze-dried, rehydrated freeze-dried maize endosperm tissue, and the isolated starch granule preparations were examined.

MATERIALS AND METHODS

Plant Materials. The maize (*Zea mays* L.) inbred, W64A (*normal*) and the mutant genotype *amylose-extender dull waxy* (*ae du wx*), were grown in 1976 and 1977 at The Pennsylvania State University Agricultural Research Center. The *ae du wx* had been incorporated into the IA453 inbred by backcrossing seven times. All plants were self-pollinated. At 20 days after pollination, 10 maize ears were harvested, and the kernels from the upper one-sixth and lower one-fourth of each ear were discarded. The remaining kernels were cut in half longitudinally, the pericarp and embryo were removed and discarded, and the endosperm slices were used for starch granule isolation.

Tissue Lyophilization. The endosperm slices were quickly frozen in Freon-12 (CCl_2F_2) chilled to its freezing point (−156 C) in a liquid nitrogen bath. The tissue was lyophilized in a VirTis freeze-drier (5–10 μm Hg vacuum) for 48 h. Before the vacuum was obtained, the tissue was kept below −25 C. The freeze-dried tissue was stored in a desiccator at −25 C with P_2O_5 until used (12).

Starch Granule Isolation. The three isolation procedures tested here included the aqueous and nonaqueous (hexane-carbon tetrachloride) procedures similar to those used by Ford (7) and a nonaqueous procedure using glycerol and 3-Cl-1,2-propanediol adapted from Kirsch *et al.* (12).

For the aqueous procedure, endosperm tissue (*ae du wx*) from 25 fresh kernels (approximately 4 g) was homogenized for two 10-s pulses with a Polytron (model PCU-2, Brinkmann Instrument, Westbury, NY) at a setting of 6 in 20 ml cold (0–4 C) homogenization medium [0.4 M mannitol, 50 mM sodium citrate, and 17 mM cysteine (pH 7.0)]. The homogenate was filtered through two layers of Miracloth to remove cell debris. The filtrate was subsequently centrifuged at 750*g* for 5 min (0–4 C). The pellet was

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washed twice with homogenization medium by suspension and centrifugation as described above. The pellet obtained was used as the aqueously isolated starch granule preparation.

For the hexane-carbon tetrachloride nonaqueous procedure, freeze-dried *ae du wx* endosperm tissue pooled from 10 maize ears was pulverized 30 s in a Spex mixer-mill (Spex Industries, Inc., Metuchen, NJ). A 0.5-g sample was placed in a chilled mortar and ground with a pestle using 10 ml cold (0–4 C) hexane. The mortar was rinsed three times with cold hexane. The homogenate and washes were filtered through a Nitrex monofilament nylon mesh screen with openings of 80 × 80 μm and a pad of glass wool. The filtrate was collected in a tapered centrifuge tube. The above procedures were carried out in a cold room (5–7 C). The filtrate was subsequently centrifuged at 1000*g* for 3 min (0–4 C). The supernatant was discarded and the pellet was suspended in 1 ml cold hexane. Thirty ml of a mixture of CCl_4-hexane (5:1, v/v) were placed under the suspension by using a syringe and canula, and the tube was centrifuged at 1000*g* for 3 min. The supernatant was discarded, and the pellet was washed three times by repeated suspension in hexane followed by centrifugation through the CCl_4-hexane mixture as described above. The pellet obtained was used as the hexane-CCl_4 isolated starch granule preparation.

For the glycerol-propanediol nonaqueous procedure, a 0.5-g subsample from the same pulverized, freeze-dried endosperm tissue used above was homogenized with a Polytron for four 10-s pulses in 15 ml cold (0–4 C) glycerol at a speed setting of 6. The homogenizations were carried out in a 50-ml plastic centrifuge tube held in an ethanol-ice bath. The homogenizations were interrupted to reduce heat buildup in the sample. The homogenate was centrifuged at 500*g* for 10 min at 0 to 4 C. The supernatant was filtered in the cold through two layers of Miracloth under vacuum to remove cell wall debris. The pellet was homogenized again with the Polytron for 10 s in 10 ml cold glycerol and also filtered. The homogenates were continually stirred during filtration to keep the starch granules suspended, and the Miracloth was washed with 5 ml cold glycerol. The combined filtrates (30 ml) were collected in a 50-ml plastic centrifuge tube containing 10 ml cold 3-Cl-1,2-propanediol and centrifuged at 30,000*g* for 30 min at 0 to 4 C using a Sorvall (Ivan Sorvall, Inc., Newton, CT) centrifuge equipped with a SS-34 rotor. The pellet was suspended in 10 ml glycerol. Three ml 3-Cl-1,2-propanediol were placed under the suspension by using a syringe and canula, and then the suspension was centrifuged as described above.

The glycerol-propanediol procedure was further improved using immature *normal* endosperm tissue as the experimental material. At a later stage of the study presented here, when a horizontal Sorvall HB-4 rotor was available, the centrifugation procedure was changed to 25,000*g* for 60 min. This will be specified under "Results." After each centrifugation, the supernatant was decanted and residual supernatant on the tube walls was removed with a lint-free tissue. The pellet obtained is referred to as starch granules isolated by the glycerol-propanediol procedure.

Chemical Analyses of Whole Homogenate. Samples of fresh and pulverized, freeze-dried tissue, from the same bulk samples used for the isolation of starch granules, were used for extraction and measurement of alcohol-soluble components, WSPs,[2] and starch. Subsamples of the tissue used above were taken for the extraction and measurement of RNA, DNA, and protein. All extractions were replicated three times.

The tissue samples (1 g fresh tissue or 0.1 g freeze-dried tissue) were extracted with MCF (methanol-chloroform-0.2 M formic acid, 13:4:3, v/v), 10% (v/v) ethanol, and 90% (v/v) dimethyl sulfoxide according to the procedure of Shannon (20). Before extraction, the tissue was suspended in cold MCF and held at −25 C overnight to inactivate phosphatase (2). RNA, DNA, and protein were extracted according to Smillie and Krotkov (22) and Short *et al.* (21) as modified by Liu (15).

The quantity of RNA was determined following procedures of Key and Shannon (11). DNA was assayed by the diphenylamine method (4), and protein was determined by the Lowry procedure

Table I. *Effect of Isolation Procedures on Purity and Metabolite Composition of Starch Granule Preparations from 20-day-old ae du wx Endosperm*

Chemical Components	Cellular Content		Amyloplast Content		
	WH1[a]	WH2[b]	Aqueous	Nonaqueous	
				Hexane	Glycerol
	mg/g dry wt		*% WH1*	*% WH2*	
Starch	300.1	393.5	85	66	71
WSP	222.2	117.8	42	64	64
Starch + WSP	522.3	511.2	66	65	69
Total sugars	139.7	186.6	1	48	12
Reducing sugars	37.8	39.4	5	39	14
RNA	3.9	4.1	12	37	5
DNA	0.7	0.7	10	27	3
Protein	99.0	95.2	13	25	4
Free phosphate	16.3[c]	14.9[c]	1	43	24
7-min phosphate	6.0[c]	8.4[c]	1	40	19
Total phosphate	25.0[c]	24.7[c]	1	46	25

[a] Whole cell homogenate (WH) prepared from fresh endosperm material.

[b] Whole cell homogenate (WH) prepared from freeze-dried endosperm material.

[c] Measured in μmol/g dry weight.

Table II. *Levels of Metabolites in Starch Granule Preparations following One to Four Centrifugations through 3-Cl-1,2-propanediol*

The whole cell homogenate (WH) and starch granule preparations were prepared from 20-day-old freeze-dried *normal* endosperm tissue.

Chemical Components	WH[a]	Number of Centrifugations			
		1	2	3	4
	mg/g dry wt	*% WH*			
Starch	604.0 ± 13.0	83	84	86	83
WSP	3.0 ± 0.1	91	87	85	87
Total sugars	97.3 ± 0.6	7	6	6	6
RNA	8.0 ± 0.1	4	2	2	2
DNA	1.4 ± 0.0	4	1	1	1

[a] Values are ± SE.

Table III. *Purity and Metabolite Composition of Starch Granules Isolated using Sorvall HB-4 Rotor*

The whole cell homogenate (WH) and starch granules were prepared from 20-day-old freeze-dried *normal* endosperm tissue. Starch granules were isolated by the glycerol-propanediol procedure.

Chemical Components	WH	Amyloplast Preparation	
	mg/g dry wt	*mg/g dry wt*	*% WH*
Starch	560.2 ± 11.1[a]	516.6 ± 8.4[a]	92
WSP	3.3 ± 0.1	3.0 ± 0.1	90
Total sugars	91.1 ± 3.7	10.5 ± 0.5	12
RNA	5.61 ± 0.13	0.21 ± 0.04	4
DNA	1.33 ± 0.03	0.03 ± 0.01	2

[a] Values are ± SE.

[2] Abbreviation: WSP, water-soluble polysaccharide.

(17). The amounts of WSP and starch were measured as reducing sugars following complete hydrolysis with glucoamylase (14). In the preliminary study, total sugars and reducing sugars were measured by the phenol-H_2SO_4 method and Nelson's test, respectively (10). Free phosphate, 7-min phosphate, and total phosphate were assayed according to the method of Fiske and Subbarow as described by Leloir and Cardini (13).

Chemical Analyses of Starch Granule Preparations. Before extraction, the starch granules isolated by the glycerol-propanediol procedure were washed two times by suspending them in cold (0–4 C) absolute methanol followed by centrifugation (4000*g*, 10 min, 0–4 C). The procedure above was used to remove residual glycerol and 3-Cl-1,2-propanediol. Half of the starch granule preparation obtained from each isolation procedure was used for the extraction of alcohol-soluble sugars, phosphates, WSP, and starch. The other half was used for the extraction of RNA, DNA, and protein. Procedures for extraction and chemical analysis were the same as given for the whole homogenate.

Light Microscopic Examination. Starch granule preparations were examined with bright-field, plane-polarized, and phase contrast using a Zeiss WL research microscope. Samples were stained with I_2-KI solution to observe the presence of WSP and starch with bright field.

Transmission Electron Microscopic Examination. Small pieces (approximately 1 mm^3) of fresh endosperm tissue, or isolated starch granules prepared by the glycerol-propanediol procedure, were first fixed in 3% (v/v) glutaraldehyde in 50 mM K-phosphate buffer (pH 6.8) for 2 h at room temperature. Before the glutaraldehyde fixation, pulverized freeze-dried tissue and the isolated amyloplasts were jelled in 4% (w/v) agar and cut in small blocks (approximately 1 mm^3). Additional freeze-dried tissue pieces, handled without jelling in agar, were rehydrated in 0.4 M mannitol and 50 mM K-phosphate buffer (pH 6.8) for 1 h at room temperature before being fixed in 3% glutaraldehyde in the same buffer and mannitol solution or fixed in 3% glutaraldehyde in 0.4 M mannitol and K-phosphate buffer for 2 h at room temperature. Tissue or agar blocks then were rinsed in the same buffer, less glutaraldehyde, and postfixed for 2 h at room temperature in 2% (w/v) OsO_4 in the same buffer. They were dehydrated with a series of aqueous acetone of 40, 60, and 80% (v/v) at 10-min intervals. The tissue was transferred to 100% acetone. After 10 min, the acetone solution was replaced by fresh acetone. This procedure was repeated once more before infiltration. Tissue pieces or agar blocks were infiltrated with hard Spurr's epoxy embedding mixture in 25% steps at 30-min intervals (23). After being left in 100% embedding mixture overnight, tissue or agar blocks were transferred to capsules and the resin was polymerized for at least 8 h at 70 C in an oven containing a desiccant.

Pale-gold to gold sections were obtained with a Sorvall, Porter Blum Ultramicrotome, using either glass or diamond knives. The sections were either stained with 3% uranyl acetate in 15% methanol for 25 min, rinsed with water and poststained with 0.2% (w/v) lead citrate for 10 s (18), or stained with 1% (w/v) $KMnO_4$ for 5 min, rinsed with water, and poststained with 0.2% lead citrate for 1 min (3). The sections were examined with a Hitachi HU-11E-1 electron microscope (Hitachi, Ltd., Tokyo, Japan) at 75 kv.

RESULTS AND DISCUSSION

Ultrastructure of Amyloplast in Developing Maize Endosperm. The ultrastructure of fresh maize endosperm tissue was examined to establish a basis for comparison when examining freeze-dried tissue and isolated starch granules. *Normal* endosperm starch granules occupied most of the volume of the amyloplast (Fig. 1). Each amyloplast contained one starch granule as previously reported (5, 25). There was no evidence of disintegration of the amyloplast membrane structure, and no extensive internal lamellae were observed in the plastid stroma. The dark areas on the starch granules are artifacts thought to be caused by the swelling of starch when the section was floated on water during sectioning followed by folding while drying on the grid (8).

Amyloplasts in 20-day-old *ae du wx* endosperm differed from *normal*. In some cells, amyloplasts contained one or two starch granules, similar to *normal* but, in other cells, the amyloplasts were much larger and contained starch granules surrounded by a thick layer of nonbirefringent glucan (Fig. 2). The stroma between the glucan and double membrane was free of lamellar structure. The distribution of cells with these two types of amyloplasts in *ae du wx* endosperm was studied at the light microscope level (19). Cells with the amyloplasts containing the nonbirefringent glucan were generally found in the physiologically more advanced cells of the central crown region of the kernel, and cells containing amyloplasts with one or more starch granules were toward the exterior of the endosperm.

The nonbirefringent glucan stained brown with I_2-KI solution similar to phytoglycogen in *sugary* (*su*) endosperm (19). Other studies showed that *ae du wx* kernels contained a WSP extractable with 10% ethanol (9). Based on this information, we reasoned that the presence of the nonbirefringent glucan associated with isolated starch granules might indicate the presence of the amyloplast membrane and allow us to assess amyloplast integrity with the light microscope. Therefore, *ae du wx* endosperm was used for the comparison of the three isolation procedures. However, through our studies and those of Yeh (27), it became clear that the nonbirefringent glucan of *ae du wx* was not as readily extracted as phytoglycogen from *su* kernels, and freeze-drying also reduced its solubility in 10% ethanol (Table I). Therefore, presence of the glucan associated with the isolated starch granules may not indicate the presence of an intact amyloplast membrane. Thus, developing *normal* endosperm was used for the remainder of the study here.

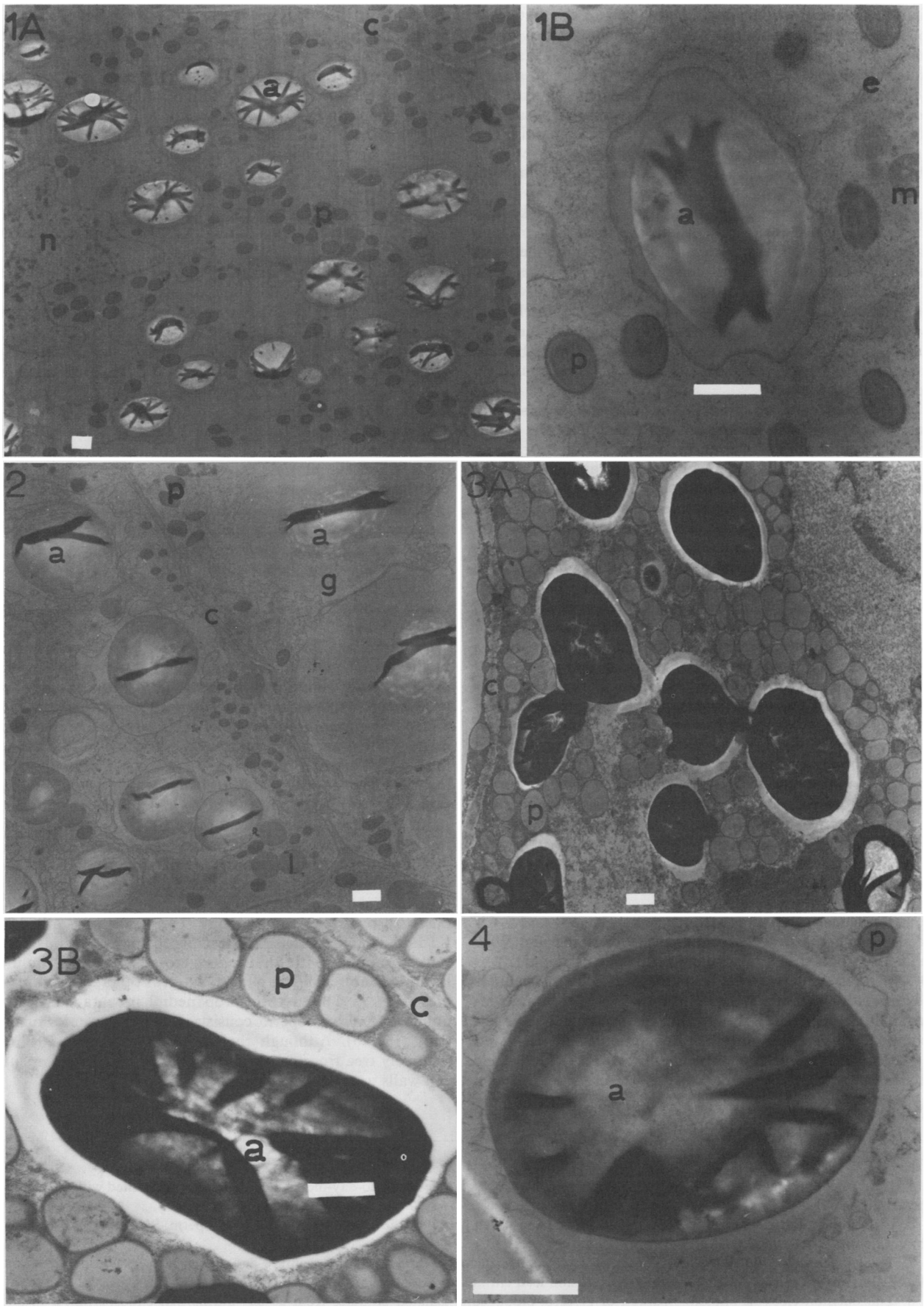
Inasmuch as freeze-dried endosperm tissue was used as the starting material for the nonaqueous isolation of starch granules, we examined the ultrastructure of this tissue prior to homogenization. In contrast to the ultrastructure of amyloplasts prepared from fresh tissue, no amyloplast membrane or any other cellular membranes were observed in the sample prepared from freeze-dried tissue (Fig. 3). The area surrounding the starch granules

FIG. 1. Ultrastructure of 20-day-old *normal* maize endosperm cell stained with uranyl acetate and lead citrate. A, an overview; B, close-up of one amyloplast. Length of bar equals 1 μm. a, amyloplast; c, cell wall; e, endoplasmic reticulum with ribosomes; m, mitochondria; n, nucleus; and p, protein body.

FIG. 2. Ultrastructure of 20-day-old *ae du wx* maize endosperm cells stained with uranyl acetate and lead citrate. Bar equals 1 μm. Identifying letters are as in Figure 1 plus: g, nonbirefringent glucan; and l, lipid.

FIG. 3. Ultrastructure of freeze-dried 20-day-old *normal* maize endopserm cell stained with uranyl acetate and lead citrate. A, an overview; B, a close-up of one amyloplast. Bar equals 1 μm. Identifying letters are as in Figure 1.

FIG. 4. Amyloplast showing lightly stained amyloplast membrane in *normal* freeze-dried endosperm tissue rehydrated in the presence of glutaraldehyde. Bar equals 1 μm. Identifying letters are as in Figure 1.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occupied by the plastid stroma and membranes in fresh tissue was void of cellular components (Fig. 3). Some membrane vesicles were observed in the cytoplasm of freeze-dried tissue that was rehydrated in a buffered 0.4 M mannitol solution prior to glutaraldehyde fixation. However, no membrane fragments appeared to be associated with the amyloplasts. When freeze-dried tissue was rehydrated in a buffered 0.4 M mannitol solution containing 3% (v/v) glutaraldehyde, lightly stained membranes were observed near the starch granule surface (Fig. 4). These are thought to be remnants of the amyloplasts' membranes.

It is possible that, during the freeze-drying process, soluble substances in the amyloplast stroma and the double membrane may have dried onto the surface of the starch granule, thus becoming indistinguishable from the starch granule. If this is so, starch granules isolated nonaqueously should retain the water soluble metabolites regardless of the integrity of the amyloplast membrane. Based on this assumption, procedures for the nonaqueous isolation of starch granules with associated metabolites, relatively free of cytoplasmic and nuclear contaminants, were studied.

Purity and Chemical Characterization of Starch Granule Preparations using Three Different Procedures. The purity and chemical characterization of starch granules isolated from developing *ae du wx* endosperm by an aqueous and two nonaqueous procedures are summarized in Table I. Here, starch recovery was highest using the aqueous procedure, but the recovery of sugars, Pi, and phosphorylated compounds in the starch granule preparation was low compared to the nonaqueous isolation procedures (Table I). Thus, soluble substances thought to be associated with the amyloplasts *in situ* are lost during isolation. In addition, the purity of the starch granule preparation from the aqueous isolation was poor, as indicated by the presence of considerable quantities of the cytoplasmic and nuclear marker compounds, RNA and DNA, respectively.

The nonaqueous procedure using hexane and carbon tetrachloride resulted in lower but acceptable recoveries of starch and WSP (Table I). The recovery of starch granules was higher than that reported by Ford (7). The proportions of cellular sugars and inorganic and organic phosphates recovered in the starch granule preparation were approximately 40% of those in the whole cell homogenate. However, cytoplasmic and nuclear contamination were excessively high, based on the quantity of RNA and DNA recovered with the starch granules. Thus, other cellular components were not satisfactorily removed from the starch granule fraction during isolation, and this procedure was judged to be unacceptable.

The nonaqueous isolation procedure using glycerol and 3-Cl-1,2-propanediol gave an acceptable recovery of starch and WSP (Table I). The isolated starch granule preparation contained 12 to 14% of the total cellular sugars and up to 25% of the cellular phosphates (Table I). Since homogenization in glycerol and centrifugation through 3-Cl-1,2-propanediol removed most other cellular components as indicated by the low quantities of RNA and DNA, we suggest that the sugars and inorganic and organic phosphates were closely associated with the starch granules *in situ* (possibly in the amyloplast stroma) and became bound to the granules during freeze-drying. Thus, this procedure appears suitable for use in a study of amyloplast metabolite compartmentation.

Improvement of the Glycerol-Propanediol Procedure. Because the glycerol-propanediol procedure appeared satisfactory for starch granule isolation from immature *ae du wx* endosperm, it was further studied and improved for use in isolating starch granule preparations from immature *normal* endosperm.

The effect of a series of centrifugations through the higher density medium 3-Cl-1,2-propanediol on starch recovery and purity of the isolated starch granule preparation was determined. Repeated centrifugations through propanediol did not reduce the recovery of starch or WSP (Table II). The per cent of WSP recovered in the starch granule fraction was comparable to that of starch, indicating that the small amount of WSP in *normal* endosperm was closely associated with the starch granules possibly in the amyloplast stroma. In contrast, over 90% of the sugars were removed by the first centrifugation, and a second centrifugation removed about 1% more. Purity of the isolated granules was improved by a second centrifugation through 3-Cl-1,2-propanediol but did not change with additional washes (Table II). Thus, two centrifugations through the higher density medium were adequate to obtain a starch granule preparation relatively free of nuclear and cytoplasmic contamination. Inasmuch as additional centrifugations did not remove all the DNA and RNA from the granule preparation, it is not clear whether the DNA and RNA measured represents contaminants or actually is a measure of the quantity of nucleic acids in amyloplasts.

After the completion of the previous study, we obtained a swinging-bucket rotor (Sorvall HB-4) and tested its suitability for use in isolating amyloplasts. With this rotor and two centrifugations through 3-Cl-1,2-propanediol, starch and WSP recovery were improved to over 90% (Table III). The percentage of sugars in the starch granule preparation was twice as high as in the previous study. This may be attributed to the different sample used here, or it may be due to higher levels of cellular contaminants. The proportion of cellular RNA and DNA increased from 2 and 1% to 4 and 2%, respectively, compared to the earlier study (Table II). Thus, cytoplasmic and nuclear contamination was still low. Because starch granule recovery was increased without appreciable sacrifice of purity, the swinging-bucket rotor was used in the isolation of starch granules for the compartmentation study reported in the accompanying paper (16).

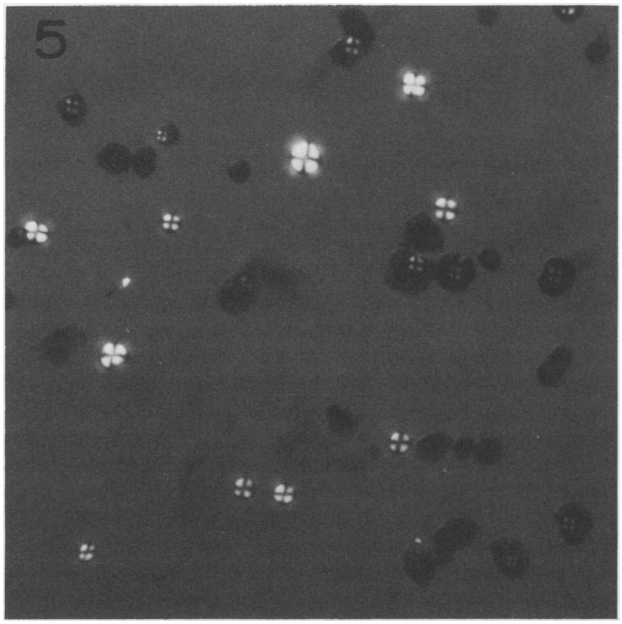
Microscopic Examination of Isolated Starch Granules. The starch granule preparation isolated from *ae du wx* by the glycerol-propanediol procedure showed the two types of plastids (Fig. 5) observed by electron microscopy (Fig. 2). When stained with I_2-KI and viewed under polarized light, many plastids contained starch granules (with birefringent cross) surrounded by the thick layer of nonbirefringent glucan, whereas others were simple granules (Fig. 5). The glycerol-propanediol preparation from developing *normal* endosperm consists of starch granules of different sizes (Fig. 6). Although the starch granule preparations were relatively free from cell wall debris, occasionally fragments from cell walls were observed.

FIG. 5. Light photomicrograph of glycerol-propanediol isolated starch granules from 20-day-old *ae du wx* endosperm. Stained with I_2-KI and viewed under polarized light. × 400.

FIG. 6. Light photomicrograph of glycerol-propanediol isolated starch granules from 20-day-old *normal* endosperm. Stained with I_2-KI and viewed under bright field. × 320.

FIG. 7. Transmission electron photomicrographs of glycerol-propanediol isolated granules from 20-day-old *ae du wx* endosperm. A and B are examples of different granules stained with uranyl acetate and lead citrate. Bar equals 1 μm.

FIG. 8. Transmission electron photomicrograph of glycerol-propanediol isolated granules from 20-day-old *normal* endosperm. A, B, and C are examples of different granules stained with uranyl acetate and lead citrate. Bar equals 1 μm.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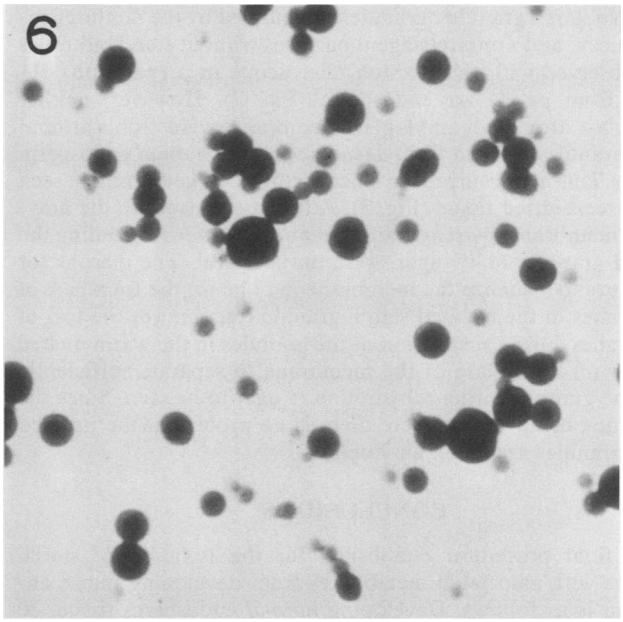
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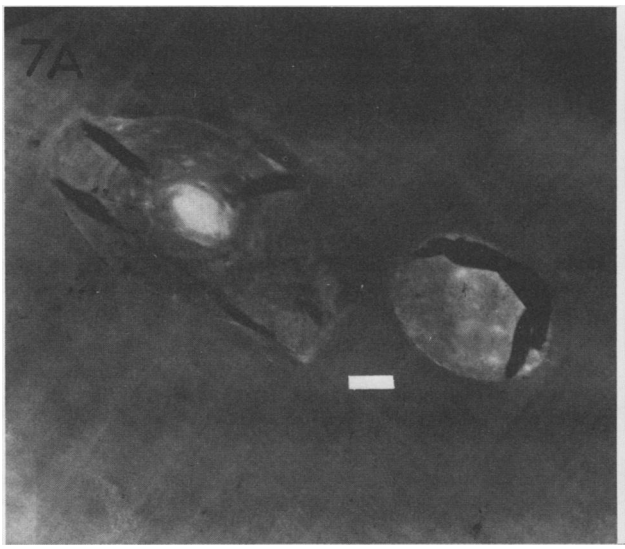
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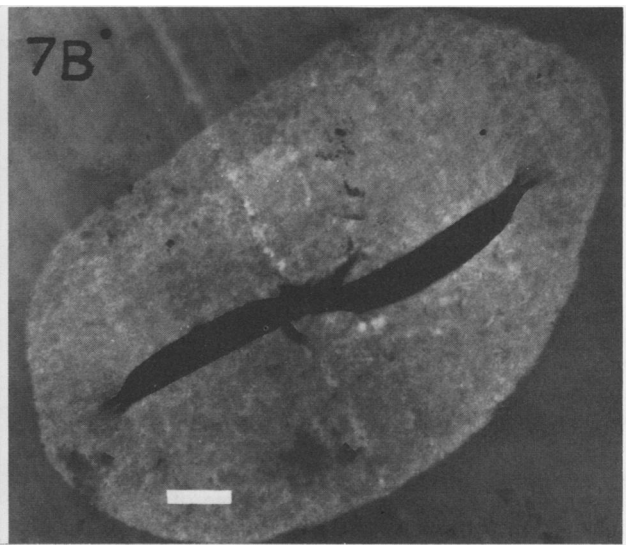
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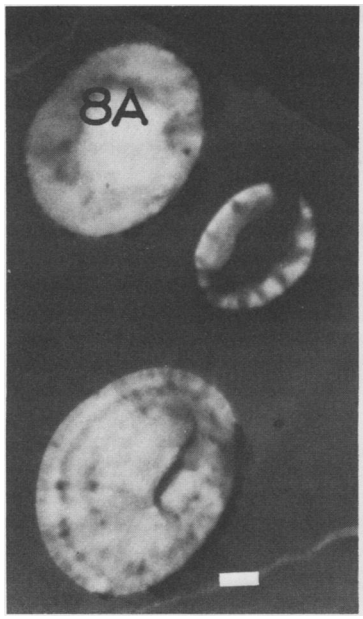
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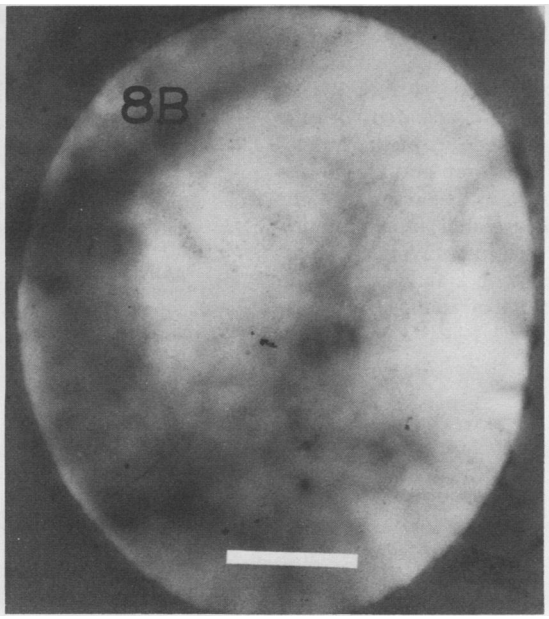
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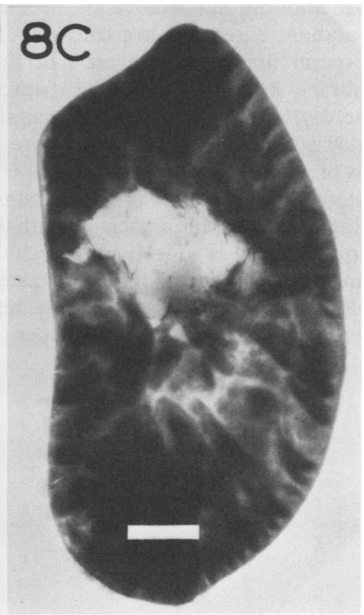
8C

Simple starch granules, granules surrounded by the nonbirefringent glucan, and nonbirefringent particles without starch granules were observed with the electron microscope in preparations obtained from *ae du wx* endosperm (Fig. 7). However, neither amyloplast stroma nor amyloplast membranes were visible around starch granules isolated from *ae du wx* or from *normal* endosperm (Fig. 8). This is not surprising since membranes could not be seen in the freeze-dried tissue (Fig. 3). Attempts to visualize the amyloplast membrane, by rehydration treatments after suspending the isolated granules in 4% agar, were unsuccessful. The reasons for the failure to visualize the membrane may be (*a*) the total lack of membranes in the isolated starch granule fraction, (*b*) the loss of membranes during suspension of the granules in the warm melted agar, or (*c*) the failure of the membrane to separate sufficiently from the granule during rehydration in agar to be seen. Since the membrane could not be seen, there is no proof that the isolated starch granules are intact amyloplasts.

CONCLUSIONS

The final procedure established for the isolation of starch granules with associated metabolites from developing maize endosperm is as follows. Developing *normal* endosperm tissue, 20 days after pollination, was quickly frozen at −156 C to minimize ice artifacts and redistribution of soluble substances and freeze-dried (12). The freeze-dried endosperm tissue was pulverized for 30 s on a Spex mixer-mill to break apart large tissue pieces. Homogenization was carried out in a cold glycerol medium using a Polytron. The homogenate was centrifuged (500*g*, 10 min) to remove unbroken cells. The supernatant was filtered through two layers of Miracloth to remove cell debris. The pellet was homogenized again and filtered. The combined filtrate was layered over 3-Cl-1,2-propanediol and centrifuged at 25,000*g* for 1 h with a swinging-bucket rotor (HB-4). The pellet was suspended in glycerol underlayered with 3-Cl-1,2-propanediol and centrifuged through the propanediol as described before. All the filtration and centrifugation procedures were carried out in the cold (0–4 C). The pellet obtained was considered as the glycerol isolated starch granule preparation.

From the results of the recovery and purity tests, the granule preparation obtained using the glycerol-propanediol procedure was high in starch content and relatively free of other cellular contamination. Further, repeated centrifugation through 3-Cl-1,2-propanediol did not remove all the soluble constituents from the preparation. Based on ultrastructural observations of freeze-dried endosperm tissue, we suggest that the amyloplast stroma and membrane become fixed to the starch granule surface during freeze drying. If this is true, the metabolites associated with the isolated starch granules represent the metabolites of the amyloplasts or at least those metabolites near the starch granules *in situ*.

Although we were unable to demonstrate the presence of the amyloplast membrane associated with the isolated starch granules, this procedure was judged suitable for use in studying the compartmentation of metabolites associated with starch granules in developing maize endosperm. The results of such a study are presented in the accompanying paper (16).

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