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Measurement of Metabolites Associated with Nonaqueously Isolated Starch Granules from Immature *Zea mays* L. Endosperm[1]

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ABSTRACT

Starch granules with associated metabolites were isolated from immature *Zea mays* L. endosperm by a nonaqueous procedure using glycerol and 3-chloro-1,2-propanediol. The soluble extract of the granule preparation contained varying amounts of neutral sugars, inorganic phosphate, hexose and triose phosphates, organic acids, adenosine and uridine nucleotides, sugar nucleotides, and amino acids. Based on the metabolites present and on information about translocators in chloroplast membranes, which function in transferring metabolites from the chloroplast stroma into the cytoplasm, it is suggested that sucrose is degraded in the cytoplasm, via glycolysis, to triose phosphates which cross the amyloplast membrane by means of a phosphate translocator. It is further postulated that hexose phosphates and sugars are produced from the triose phosphates in the amyloplast stroma by gluconeogenesis with starch being formed from glucose 1-phosphate via pyrophosphorylase and starch synthase enzymes. The glucose 1-phosphate to inorganic phosphate ratio in the granule preparation was such that starch synthesis by phosphorylase is highly unlikely in maize endosperm.

In maize endosperm cells, as in other storage tissues, starch accumulates in amyloplasts. Amyloplasts consist of starch granules surrounded by plastid stroma and enclosed by a double membrane (1, 22). It is assumed that some, or all, of the enzymes of starch synthesis are located within the amyloplasts (2), but this has been difficult to demonstrate becaue intact amyloplasts containing the soluble stroma enzymes and/or metabolites have not been isolated. Various enzymes presumably associated with starch synthesis have been measured *in vitro* and biosynthetic pathways have been proposed (31). We are interested in determining what enzyme reactions of starch synthesis are occurring within the amyloplast organelle. Since the isolation of intact enzymically active amyloplasts was not possible, an indirect approach was to measure the metabolite composition of the organelle. This has recently been made possible by the development of a procedure for the nonaqueous isolation of starch granules containing associated metabolites which appear to be essentially free of nuclear and cytoplasmic contaminants (22). We now report on the measurement of sugars, intermediates of starch synthesis, and metabolites, which possibly could regulate starch synthesis, in the starch granule preparation.

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MATERIALS AND METHODS

Plant Material. The *normal* maize (*Zea mays* L.) inbred, W64A, was grown in 1977 at The Pennsylvania State University Agricultural Research Center. All plants were self-pollinated. Twenty days after pollination, uniform kernels from the central portion of 10 maize ears were removed from the cob and cut in half longitudinally, and the pericarp and embryo were removed and discarded.

Tissue Lyophilization and Starch Granule Isolation. The endosperm slices were quick-frozen in Freon-12 cooled in liquid N_2 and freeze-dried. An equal weight of freeze-dried endosperm from the 10 maize ears was combined and pulverized 30 s in a Spex Mixer-Mill (Spex Industries, Inc., Metuchen, NJ). Starch granules were isolated from 12 subsamples (0.5 g each) of the pulverized tissue by the glycerol-propanediol procedure (22) using the Sorvall (Ivan Sorvall, Inc., Newtown, CT) HB-4 rotor. Once isolated, starch granules from the 12 preparations were randomly pooled to give three replications, each containing the starch granules obtained from 2 g dry endosperm tissue.

Tissue and Starch Granule Extraction and Analysis. Prior to metabolite extraction, the residual glycerol and 3-Cl-1,2-propanediol were removed by suspending the starch granules in cold (0–4 C) dry absolute methanol and by centrifuging (4000*g*, 10 min). The methanol wash was repeated a second time. The methanol wash does not remove neutral sugars from the starch granules and we assume that the other metabolites remain with the starch granules also. The methanol-washed starch granule preparations and 0.1-g samples of the freeze-dried endosperm tissue were extracted with methanol-chloroform-0.2 M formic acid (13:4:3, v/v), 10% (v/v) ethanol, and 90% (v/v) dimethyl sulfoxide as previously described (22, 30). The quantities of water-soluble polysaccharides and starch in the 10% ethanol and dimethylsulfoxide extracts, respectively, were measured as reducing sugars following complete hydrolysis with glucoamylase (21).

The methanol-chloroform-0.2 M formic acid extract was purified by established procedures (30). Fructose, glucose, and sucrose were quantitatively analyzed using a Waters Associates (Milford, MA) HPLC[2] system fitted with a μBondapak/carbohydrate column (3.9 mm × 30 cm) using acetonitrile-H_2O (85:15, v/v) as solvent with a flow rate of 2.0 ml/min. Carbohydrate peaks were detected with the differential refractometer (model R401) and quantity was determined by comparison to standards.

Separation and quantitative analyses of all nucleotides and sugar nucleotides were made using a Waters Associates HPLC system fitted with a Partisil-10-SAX column (4.6 mm × 25 cm) (Whatman, Inc., Clifton, NJ). Dual pumps (model 6000A) and

[2] Abbreviations: HPLC, high-performance liquid chromatography; G-6-P, glucose 6-phosphate; G-1-P, glucose 1-phosphate; F-6-P, fructose 6-phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde ≠3-phosphate; F-1,6-DP, fructose 1,6-diphosphate; 3-PGA, 3-phosphoglycerate; OAA, oxaloacetic acid.

solvent programmer (model 660) were operated in such a way that one pump delivered 10 mM $NH_4H_2PO_4$ (pH 3.0) and the other pump delivered 450 mM $NH_4H_2PO_4$ (pH 4.3). The flow rate was set at 0.8 ml/min, which gave an operating pressure of about 600 p.s.i. Samples were injected into a U6K injector and eluted by a concave gradient (curve No. 9 on the programmer) for 40 min, followed by 50 min with the high-concentration buffer. This procedure was suggested by E. Sharps (personal communication). The column was prepared for the next sample by reversing the gradient and then washing with the low concentration eluent for at least 20 min. All peaks were detected by the UV detector (model 440), and the unknowns were identified by comparing their retention times and their ratio of A at 280 and 254 nm to those of standards. Quantity was determined by comparison to standards.

Free amino acids in the extract from the whole-cell homogenate and amyloplast preparation were analyzed on a Beckman model 120C amino acid analyzer (Beckman Instruments, Inc., Fullertown, CA).

Pi, hexose phosphates, triose phosphates, and organic acids were determined by enzymic analyses using a Zeis PMQII spectrophotometer connected to an ISCO model 170 chart recorder. All enzymes and standards were purchased from Sigma. The concentration of a particular compound was calculated according to Bergmeyer *et al.* (3). All assay procedures were tested by preparing standard curves for each component.

Pi was assayed by following the reduction of NADP at A_{340} by the combined action of phosphorylase a, phosphoglucomutase, and G-6-P dehydrogenase (11). G-6-P, G-1-P, and F-6-P were sequentially assayed by following the reduction of NADP at A_{340} after adding G-6-P dehydrogenase, P-glucomutase, and P-glucose isomerase, respectively (18, 20).

DHAP, G-3-P, and F-1,6-DP were sequentially assayed according to Michal and Beutler (23), by following the oxidation of NADH at A_{340} after adding α-glyceroP dehydrogenase, trioseP isomerase, and aldolase, respectively. 3-PGA was assayed by following the oxidation of NADH at A_{340} resulting from the action of 3-P-glyceric phosphokinase and Gl-3-P dehydrogenase (7).

Pyruvate and phosphoenolpyruvate were sequentially assayed by following the oxidation of NADH at A_{340} after adding lactate dehydrogenase and pyruvate kinase, respectively (8). Citrate was assayed by following the oxidation of NADH at A_{340} as a result of the combined action of citrate lyase, malate dehydrogenase, and lactate dehydrogenase (9). Malate was measured by following the oxidation of NADH at A_{340} in the presence of malate dehydrogenase (13).

RESULTS

Neutral Sugars. Standard neutral sugars separated by HPLC were eluted in the following order: fructose, glucose, sucrose, and maltose. Maltose was not detected in any sample. Sucrose was the major sugar in the whole cell homogenate as well as in the starch granule preparation (Table I). However, only 13% of the total cellular sucrose was associated with the starch granules, which was the lowest percentage measured among the three sugars. The quantities of glucose and fructose were similar in the whole homogenate, but the amount of glucose was about 2 times higher than fructose in the isolated starch granule preparation.

Sugar Phosphates and Pi. Among the hexose phosphates in both the whole-cell homogenate and in the glycerol-isolated starch-granule preparation, G-6-P was highest and G-1-P was lowest (Table II). The percentage of total cellular hexose phosphates associated with the starch ranged from 16 to 27%. In the starch-granule preparation, the amount of G-6-P was over 44 times higher than that of G-1-P and about 5 times higher than F-6-P, whereas F-6-P was about 4 times higher than F-1,6-DP. Among the triose phosphates measured in the whole-cell homogenate and in the glycerol-isolated starch granules, 3-PGA was highest and G-3-P was lowest (Table II). However, the percentages of total 3-PGA and G-3-P associated with the starch granules were lowest among the triose phosphates measured. In contrast, 27% of the total cellular DHAP content was associated with the isolated starch granules. Compared to the quantity of neutral sugars associated with isolated starch granules, the amounts of hexose phosphates and triose phosphates were much lower. The amount of Pi was comparable to that of neutral sugars with over 30% of the total cellular Pi being recovered with the isolated starch-granule preparation. There was over 150 times more Pi than G-1-P in the isolated starch granules.

Organic Acids. Malate content was much higher than citrate or pyruvate in both the whole-cell homogenate and in the isolated starch-granule preparation (Table III). OAA could not be detected in either the whole cell or starch granule extracts. This may have been due to the nonenzymic conversion of OAA to pyruvate (9) in the extract prior to analysis. If this occurs, the pyruvate value given would actually be pyruvate plus OAA. Over 30% of the

Table I. *Levels of Sugars in Whole Cell Homogenate and in Glycerol-isolated Starch Granule Preparation*

Whole cell homogenate (WH) was prepared from freeze-dried endosperm material. Starch content for whole cell homogenate was 560 ± 11 mg/g dry weight and that for the starch granule preparation was 516 ± 8 mg/g dry weight.

Sugars	WH	Starch Granule Preparation	
	nmol/mg starch	*nmol/mg starch*	*% WH*
Fructose	69.5 ± 0.6[a]	11.4 ± 0.1[a]	16
Glucose	79.9 ± 1.3	21.3 ± 0.3	27
Sucrose	489.4 ± 3.0	62.5 ± 0.2	13

[a] Values are ± SE.

Table II. *Levels of Phosphorylated Intermediates and Pi in Whole Cell Homogenate and in Glycerol-isolated Starch Granule Preparation*

Whole cell homogenate (WH) was prepared from freeze-dried endosperm material.

Chemical Components	WH	Starch Granule Preparation	
	nmol/mg starch	*nmol/mg starch*	*% WH*
G-6-P	21.34 ± 0.84[a]	4.44 ± 0.20[a]	21
G-1-P	0.40 ± 0.04	0.10 ± 0.01	25
F-6-P	3.31 ± 0.18	0.93 ± 0.03	27
F-1,6-DP	1.52 ± 0.05	0.24 ± 0.02	16
DHAP	0.73 ± 0.02	0.20 ± 0.02	27
G-3-P	0.42 ± 0.02	0.03 ± 0.01	7
3-PGA	11.57 ± 0.03	0.82 ± 0.02	7
PEP[b]	1.80 ± 0.03	0.34 ± 0.01	19
Pi	44.26 ± 0.81	15.15 ± 0.68	34

[a] Values are ± SE.
[b] PEP, phosphoenolpyruvate.

Table III. *Levels of Organic Acids in Whole Cell Homogenate and in Glycerol-isolated Starch Granule Preparation*

Whole cell homogenate (WH) was prepared from freeze-dried endosperm material.

Organic Acids	WH	Starch Granule Preparation	
	nmol/mg starch	*nmol/mg starch*	*% WH*
Citrate	13.54 ± 0.62[a]	0.85 ± 0.02[a]	6
Malate	52.47 ± 1.60	17.39 ± 0.87	34
Pyruvate	0.81 ± 0.06	0.11 ± 0.01	14

[a] Values are ± SE.

cellular malate was associated with the isolated starch granules, whereas only 6% of the total cellular citrate was associated with the starch granules. The amount of total cellular pyruvate was much lower than malate or citrate, and only 14% of that present in the whole homogenate was associated with the starch-granule preparation.

Nucleotides and Sugar Nucleotides. The elution order of nucleotides and sugar nucleotides shown in Figure 1 was identified by injecting individual nucleotides or sugar nucleotides and comparing the retention times as well as the ratio of *A* at 280 and 254 nm (Table IV, A_{280}/A_{254} ratio). There was no separation between CTP and UTP. The resolution between NADP and CDP standards was not complete, but the amounts of these in the extracts were below the limits which could be accurately measured. Nucleotides and sugar nucleotides in the samples were identified by comparing the retention times and the A_{280}/A_{254} ratio as summarized in Table IV. The 280 to 260 nm *A* ratios reported in Pabst Research Chemicals Circular OR-17 (27) are also given (Table IV). In the extract obtained from the whole cell homogenate, AMP apparently co-eluted with another compound(s) inasmuch as the A_{280}/A_{254} ratio was higher than that of the standard. Thus, the amount of AMP in the whole cell homogenate would be lower than the amount determined by the peak area and, inasmuch as the AMP from the starch granules was not similarly contaminated, the percentage of cellular AMP associated with the isolated starch granules would be higher than the 29% reported in Table V. NAD from the HPLC separation of both the whole cell homogenate and the isolated starch-granule preparation was also contaminated with an unknown component which caused a higher A_{280}/A_{254} ratio than the standard. The NAD data are not presented. The single peak where UTP and CTP eluted had an A_{280}/A_{254} ratio similar to standard UTP rather than CTP (Table IV) and is reported as UTP. Thus, only the amounts of UMP, UDP, UTP, AMP, ADP, ATP, ADP-sugar, and UDP-sugar were computed and are presented in Table V.

In the whole cell homogenate, the quantity of uridine phosphates was higher than the adenosine phosphates (Table V). Similarly, the quantity of UDP-sugar was over twice as high as ADP-sugar. In the isolated starch-granule preparation, the quantity of nucleotides and sugar nucleotides ranged from 0.68 to 3.83

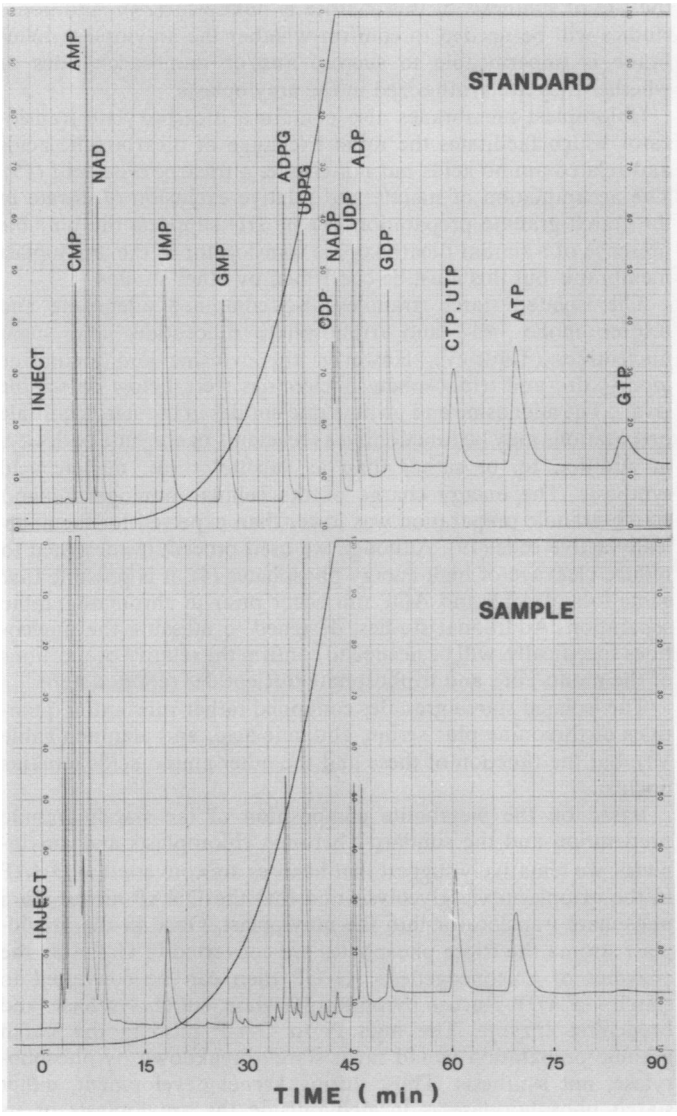

FIG. 1. Chromatographs of standard and sample nucleotides and sugar nucleotides separated by HPLC using a Partisil-10-SAX column. Sample extract was from whole cell homogenate. ADPG, ADP-glucose; UDPG, UDP-glucose.

Table IV. *Retention and Ratio between 280- and 254-nm Absorbance of Nucleotides and Sugar Nucleotides Separated on Partisil-10-SAX Column*

Whole-cell homogenate (WH) was prepared from freeze-dried endosperm material.

Chemical Components	Absorption Maximum	Retention Volume[a]	A_{280}/A_{254}		Starch Granule Preparation (A_{280}/A_{254})	Reference[b] (A_{280}/A_{260})
			Standard	WH		
	nm	*ml*	*ratio*		*ratio*	*ratio*
AMP	259	5.76	0.18	0.30	0.18	0.15
NAD	259	6.96	0.20	0.29	0.30	0.23
UMP	262	14.88	0.32	0.33	0.32	0.38
ADP sugar	259	28.56	0.19	0.19	0.20	0.15
UDP sugar	262	30.72	0.34	0.36	0.36	0.38
CDP	280	33.94	—[c]	—	—	2.10
NADP	259	34.56	0.22	T[d]	T	0.22
UDP	262	36.48	0.31	0.33	0.32	0.38
ADP	259	37.44	0.17	0.18	0.17	0.15
UTP	262	48.24	0.32	0.34	0.33	0.38
CTP	280	48.24	—	—	—	2.10
ATP	259	56.16	0.17	0.19	0.17	0.15

[a] Retention time was converted to volume by multiplying flow rate by time.

[b] Taken from Pabst Research Chemicals Circular OR-17 (27).

[c] —, not measured.

[d] T, trace amount.

Table V. *Levels of Nucleotides and Sugar Nucleotides in Whole Cell Homogenate and in Glycerol-isolated Starch Granule Preparation*

Whole cell homogenate (WH) was prepared from freeze-dried endosperm material.

Chemical Components	WH	Starch Granule Preparation	
	nmol/mg starch	*nmol/mg starch*	*% WH*
UMP	11.48 ± 0.35[a]	2.79 ± 0.08[a]	24
UDP	12.04 ± 0.22	1.45 ± 0.04	12
UTP	22.98 ± 2.08	3.83 ± 0.04	17
AMP	9.10 ± 0.12	2.66 ± 0.03	29
ADP	7.32 ± 0.48	1.09 ± 0.02	15
ATP	10.89 ± 1.43	2.43 ± 0.01	22
ADP sugar	7.48 ± 0.20	0.68 ± 0.01	9
UDP sugar	17.32 ± 0.21	1.91 ± 0.02	11

[a] Values are ± SE.

nmol/mg starch, where ADP-sugar had the lowest and UTP had the highest amounts measured. The percentage recovered with the starch granule preparation ranged from 9% (ADP-sugar) to 29% (AMP).

Amino Acids. Over 20% of all cellular free amino acids measured were recovered in the isolated starch granule preparation. The relative amounts of alanine, glutamic acid, and threonine plus serine were high in the starch-granule preparation compared to the other free amino acids. The percentage of total free amino acids associated with the starch granules ranged from 21 to 51% (Table VI). Asparagine and glutamine were not detected with the system used and the amounts of arginine and cysteine in the extracts were below detectable limits. An unknown compound eluted at the leading edge of aspartic acid and, when a more concentrated extract was used, little separation was observed. Although the amount of the unknown compound was much higher than aspartic acid, the amount of aspartic acid plus unknown was estimated using aspartic acid as the standard. Threonine and serine in the extracts were not separated, and thus the combined amount of threonine and serine was estimated based on the average constant of equal quantities of standard threonine and serine.

DISCUSSION

The starch granule preparations contained numerous metabolites and not simply those thought to be directly used in starch synthesis. This is not surprising inasmuch as other cellular organelles contain complex mixtures of metabolites (25). The low mol wt components associated with the starch granule preparations apparently are not a random contamination with cytoplasmic components. Evidence for this is the major differences in the distribution of certain components between the starch preparation and the whole cell extract, *i.e.* 7% 3-PGA *versus* 27% DHAP (Table II) or 6% citrate *versus* 34% malate (Table III). Also, similar starch granule preparations were shown to be relatively free of cytoplasmic and nuclear contamination, as evidenced by the low amounts of RNA and DNA associated with the granules (22).

Because starch synthesis occurs in amyloplasts, it is necessary for the carbohydrate substrates to enter the amyloplasts. Because intact amyloplasts have not been isolated, it has not been possible to characterize the metabolite transport systems in amyloplasts. However, amyloplasts and chloroplasts both develop from proplastids and under certain conditions amyloplasts develop into chloroplasts (24) and vice versa (1). Thus, it is possible that both organelles have similar mechanisms for metabolite transfer across the membrane. For example, DHAP and 3-PGA, products of photosynthesis, move out of chloroplasts in exchange with Pi via the phosphate translocator (15). By analogy, Jenner (16) suggested that DHAP and/or 3-PGA are transported into the amyloplast stroma in exchange for Pi by a similar phosphate translocator. The recovery of these triose phosphates in the starch granule preparation (Table II) supports this suggestion. The isolated starch-granule preparation contained sucrose, glucose, and fructose. The chloroplast membrane appears to be impermeable to sucrose and only very slowly permeable to monosaccharides (14, 32). By analogy, if the amyloplast membrane is also relatively impermeable to sucrose, glucose, and fructose, then they are probably synthesized within the amyloplast. Although chloroplasts often accumulate little sucrose (14), chloroplasts from cold-hardened cabbage contained up to 20% of the cellular sucrose, but the site of synthesis of this sucrose is unknown (29). Additional studies will be needed to confirm whether the amyloplast membrane is impermeable to sucrose and/or monosaccharides or whether they are synthesized in the amyloplasts.

Chloroplast membranes also contain a dicarboxylate translocator which facilitates the rapid exchange of dicarboxylic acids and related amino acids but not citrate, a tricarboxylic acid (15). The accumulation of malate and relative exclusion of citrate in the starch-granule preparation (Table III) supports the possible presence of a similar dicarboxylate translocator in the amyloplast membrane, but this must be confirmed by other studies.

The isolated starch granules also contained adenosine and uridine mono-, di-, and triphosphate nulceotides and sugar nucleotides (Table V). However, the cytosine and guanosine mono-, di-, and triphosphate nucleotides were below detectable levels. The adenosine and uridine nucleotides in the starch granule preparations may be functioning as reactants in enzymic pathways in addition to, or rather than as substrates for, nucleic acid synthesis. The energy charge of the cellular homogenate and starch granule preparation was lower than expected for metabolically active cells (19). Although we used procedures designed to reduce cleavage of high-energy phosphates (4), it is possible that some loss of ATP and ADP did occur prior to chromatographic separation. Additional studies, designed to measure the nucleotides specifically, will be needed to confirm the relative proportions of the mono-, di-, and triphosphate nucleotides reported here.

The isolated starch granules contained rather substantial quantities of threonine plus serine, glutamic acid, and alanine (Table VI), but the function of these and the other amino acids remains unclear.

Based on the metabolite composition of the starch granule preparation and the similarity between chloroplasts and amyloplasts, we tentatively suggest that hexoses are converted to DHAP in the cytoplasm via glycolysis and that the DHAP moves via a phosphate translocator into the amyloplast. Once in the amyloplast stroma the triose phosphates are converted to G-1-P by the enzymes of gluconeogenesis. G-1-P then can be converted to starch via ADP-glucose pyrophosphorylase, starch synthase, and branching enzyme. The high Pi to G-1-P ratio in the starch granule preparation would favor starch breakdown by phosphorylase, not synthesis. Thus, during kernel development, either starch phosphorylase is located outside the amyloplasts or its activity *in vivo* is inhibited.

Starch synthesis in chloroplasts appears to be regulated by the activity of ADP-glucose pyrophosphorylase. Activity of this allosteric enzyme varies considerably in response to changes in the 3-

Table VI. *Levels of Amino Acids in Whole Cell Homogenate and in Glycerol-isolated Starch Granule Preparation*

Whole cell homogenate (WH) was prepared from freeze-dried endosperm material.

Amino Acids	WH	Starch Granule Preparation	
	nmol/mg starch	*nmol/mg starch*	*% WH*
Lysine	5.87	1.21	21
Histidine	1.00	0.25	25
Arginine	T[a]	T	T
Aspartic acid + unknown	23.47	8.96	38
Threonine + serine	148.53	47.50	32
Glutamic acid	37.60	14.91	40
Proline	10.78	4.17	39
Cysteine	T	T	T
Glycine	5.17	2.63	51
Alanine	65.51	28.11	43
Valine	7.65	2.48	32
Methionine	3.02	1.07	35
Isoleucine	1.49	0.39	26
Leucine	2.08	0.57	27
Tyrosine	1.67	0.48	29
Phenylalanine	1.46	0.42	29

[a] T, trace amount.

PGA to Pi ratio (12, 28). *In vitro* activity of maize kernel ADP-glucose pyrophosphorylase is much less affected by changes in the 3-PGA to Pi ratio (10). Because of the relatively low quantity of 3-PGA and high Pi in the starch granule preparation isolated from kernels actively synthesizing starch, we suggest that the 3-PGA to Pi ratio has little effect on the *in vivo* activity of maize kernel ADP-glucose pyrophosphorylase.

Citrate has been shown to stimulate the *in vitro* activity of unprimed starch synthase (26), and Boyer *et al.* (6) suggested that citrate may stabilize a starch synthase-branching enzyme complex *in situ*. Malate is 80% as effective as citrate in stimulating the *in vitro* activity of unprimed starch synthase (5). Thus, inasmuch as malate rather than citrate accumulated in the amyloplasts, it is possible that malate may be stimulating starch synthase *in vivo* by stabilizing a starch synthase-branching enzyme complex.

Based on the metabolite composition of the starch granule preparation and the similarities between chloroplasts and amyloplasts, we have suggested a possible mechanism of carbohydrate transfer and metabolism in amyloplasts. However, we must stress that confirmation of these suggestions must await direct measurement and characterization of the various enzymes in isolated starch granule preparations. Kirsch *et al.* (17) measured the activity of several glycolytic enzymes in glycerol-propanediol-isolated nuclei. Thus, it is possible that several of the enzymes of gluconeogenesis and starch synthesis thought to be present in amyloplasts will be stable to the amyloplast isolation procedure used here. However, confirmation of the suggested membrane translocators can only be accomplished using aqueously isolated amyloplasts.

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