Biosynthesis of Starch in Proplastids of Germinating *Ricinus communis* Endosperm Tissue

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**ABSTRACT**

Electron photomicrographs of endosperm tissue from germinating seed of *Ricinus communis* L. cv. Hale show proplastids which contain prominent starch grains. The content of starch in endosperm tissue increased from 500 micrograms per seed, in imbibed seed, to 1,100 micrograms per seed in 5-day-old seedlings. The maximum net rate of starch deposition was 1.1 nanomoles glucose incorporated per minute per seed. About 200 micrograms of starch remained in the endosperm 9 days after imbibition. Starch content followed the same developmental pattern as the content of sucrose, free reducing sugars, and other metabolic processes found in this tissue. Two key enzymes of starch synthesis, adenosine diphosphoglucose (ADPG) pyrophosphorylase and ADPG-starch glucosyl transferase (starch synthetase) exhibited maximum activities at 4 and 5 days after germination, respectively. The maximum activity of ADPG pyrophosphorylase was 8.17 nanomoles ADPG formed per minute per seed, whereas starch synthetase exhibited an activity of 125 nanomoles glucose incorporated per minute per seed. These levels of enzyme activity are sufficient to account for the starch synthesis observed. Other enzymes which may be involved in starch synthesis include 3-phosphoglycerate kinase which showed an activity of 8.76 units per seed, triose-P isomerase (2.56 units per seed), fructose-1,6-bisphosphate aldolase (0.99 units per seed), fructose-1,6-bisphosphatase (0.23 units per seed), phosphoglucomutase (12.6 units per seed), and phosphoglucose isomerase (9.72 units per seed). The activities of these enzymes were similar to previously reported values.

Starch synthetase was found in association with the fraction containing proplastids isolated from endosperm tissue. Of the total starch synthetase activity in the endosperm, 38% was particulate. Forty-four percent of the total particulate activity of starch synthetase placed on sucrose gradients was associated with the band containing proplastids. The proplastids contained 98% of the ribulose 1,5-bisphosphate carboxylase carboxylase activity placed on the gradient.

The metabolism of the germinating seed of *Ricinus communis* has been studied extensively during the last 100 years (9, 10, 13, 23). The major emphasis has focused on the utilization of the stored lipid reserves, found in the endosperm portion of the seed (3, 23). These lipids are the source of carbon for the synthesis of sucrose (3, 4, 23), which is ultimately transported to the growing embryo (19). Early attempts to establish the localization of the biochemical processes involved led to the discovery of a new organelle which was termed the glyoxysome (8). These same experiments also demonstrated the presence of another organelle which was called the proplastid. Proplastids have received a great deal of attention in recent years. Early work suggested that proplastids may be the location of glucogonegenic sucrose production (18). This hypothesis was based on the observation that these organelles possessed several enzymes which are involved in glucogonegenesis (18). These enzymes are also common to both glycolysis and the reductive pentose phosphate pathway found in mature chloroplasts. Similarly glucogonegenic enzymes were detected in proplastids from cultured tobacco cells (28). It was suggested that these organelles could manufacture starch, even though no starch data was reported (28). Recently Nishimura and Beevers (21) showed that the activities of the glucogonegenic enzymes in the proplastids of *Ricinus communis* are not sufficient to account for the rates of sucrose synthesis by the endosperm tissue, and concluded that proplastid enzymes were not involved with sucrose production (21).

Electron photomicrographs of proplastids from *Ricinus communis* endosperm (12) and a variety of tissues (2) show starch grains. Thus, it appears that the proplastids of *Ricinus communis* may have metabolic functions similar to proplastids from other tissues. We report here evidence which shows that *Ricinus communis* endosperm contains the enzymatic machinery to convert organic acids to starch during development, and this machinery is localized in proplastids.

**MATERIALS AND METHODS**

**Plant Material.** Dry seed of *Ricinus communis* L. cv. Hale were soaked overnight in distilled H2O with Thiram (bis(dimethylthiocarbamoyl]) to inhibit fungal growth. Seeds were germinated in moist vermiculite at 32°C in the dark, in a humidified incubator. In all experiments the endosperm was separated from the cotyledons and radicle tissue.

**Starch Determinations.** Fifty seed of the appropriate age (imbibed to 9-d-old seeds) were weighed, and the endosperm was extracted with 95% ethanol in a Soxhlet extractor. The residue was dried and weighed. For starch determinations, 50 mg residue were ground with a pinch of sand to a fine powder in a 30-ml Corex test tube. After grinding, 5.0 ml distilled H2O was added to each tube, and the tubes were capped with foil and boiled for 1 h in a water bath. After cooling, 1.0 ml a-amylase solution (10 mg/ml Sigma-type IVA) was added. The a-amylase solution was centrifuged prior to use, to remove insoluble materials. The samples were incubated at 50°C for 24 h. The release of reducing sugars was determined with the procedure of Nelson (20).

**Protein Determinations.** Protein was determined by the Coomassie Brilliant Blue G-250 assay as described by Bradford (7), with BSA (Sigma Fraction V powder) as the standard.

**Organelle Isolation.** Discontinuous sucrose density gradients were used to isolate organelles for the localization of starch synthesis (24). All sucrose solutions were weight/volume and were buffered with 0.1 M Tris (pH 7.5). The gradient steps consisted of 5.0 ml 60% sucrose, 7.0 ml 51% sucrose, 7.0 ml 43% sucrose, 7.0 ml 35% sucrose, and 5.0 ml 24% sucrose. The sample (1–2 ml) was

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contained in grinding buffer. The 24, 35, and 43% solutions contained 0.5 mM MgSO₄, whereas the 51% solution contained 1.0 mM MgSO₄. Twenty seeds of the appropriate age were chopped with a razor blade in 10.0 ml grinding buffer which consisted of 0.5 mM sucrose, 150 mM Tricine (pH 7.5), and 1.0 mM EDTA. After 15 min of chopping, the homogenate was filtered through several layers of glass wool, and the trapped material was washed twice with small volumes of grinding buffer. The extract was centrifuged at 500g for 10 min. The supernatant fraction was collected and recentrifuged at 15,000g for 20 min. The pellet was gently resuspended in 4.0 ml grinding buffer, and layered onto the gradients which were centrifuged for 3 h at 12,500 rpm in a SW 25.1 rotor (Beckman Instrument Co.). The gradients were removed with an ISCO density gradient fractionator and the effluent passed through a UV monitor. The A at 280 nm was recorded.

**Enzyme Assays.** The assay of ADP-glucose pyrophosphorylase (EC 2.7.7.6) was based on the procedure of Ghosh and Preiss (11). Five endosperm pairs were ground with a mortar and pestle in 2.0 ml 0.01 M Tris (pH 7.5) with 0.1 mM GSH, and the homogenate centrifuged at 15,000g for 30 min. The supernatant fraction was used as the source of enzyme. Each assay contained 20 mM Hepes (pH 8.0), 20 mM MgCl₂, 2.0 mM glucose-1-phosphate [U-¹³C] glucose with a specific radioactivity of 58 cpm/nmol), 20 mM PGA, 50 μg BSA, 1 unit inorganic pyrophosphatase, and 100 μl enzyme in a final volume of 200 μl. The reaction vessel was incubated at 37 °C and the reaction terminated by boiling the tubes for 45 s. The tubes were cooled and 3 mg alkaline phosphatase added to each tube. The tubes were reincubated for 6 h at 37 °C. The ADP-glucose was isolated by spotting 50 μl of the reaction mixture onto DE-81 filter paper (Whatman DEAE, 15-cm discs). The paper was washed in a Büchner funnel with 4 liters H₂O (pH 8.5), at a flow rate of 16 L/h. The radioactivity at each spot was then determined. Due to the low activity of ADP-glucose pyrophosphorylase in the endosperm, it was necessary to demonstrate that this low activity was not due to a problem with the assay system. Corn leaves, known to contain appreciable activity of ADP-glucose pyrophosphorylase were chosen as an alternate tissue source. The activity for crude extracts from corn was 134 nmol/min·mg protein, demonstrating that the assay system was not responsible for the low endosperm activity.

Starch synthetase (EC 2.4.1.1b) activity was determined according to the procedure of Hawker et al. (15) with several modifications. The assay is based on the incorporation of glucose from ADP-glucose into a starch primer. Soluble starch synthetase was isolated by grinding the tissue in 0.1 M Tris-acetate buffer (pH 8.5) which contained 5 mM EDTA, 2 mM DTT, and 0.1% (w/v) Triton X-100. The 15,000g supernatant was used as the source of enzyme. The assay required the preparation of a water soluble starch primer which was also insoluble in cold 70% (v/v) methanol with 1% KCl. The reaction mixture contained 100 mM Bicine (pH 8.5), 5.0 mM EDTA, 10 mM DTT, 5 mg soluble starch primer, 25 mM K-acetate, 0.5 mM ADP-glucose (uniformly labeled glucose with 560 cpm/nmol), and 250 μg protein in a total volume of 200 μl. The reaction was initiated with the addition of 100 μl reaction mixture and incubated at 37 °C for 15 min in a shaker water bath. The reaction was terminated with the addition of 2.0 ml cold 70% methanol with 1% KCl. The precipitated starch primer was pelleted by centrifugation, and washed 3 times with 2.0 ml portions of methanol solution. After removal of the methanol, the starch was dissolved in 1.0 ml H₂O and the radioactivity in 100 μl was determined. The assay for starch synthetase was linear with respect to time and protein concentration.

Ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), phosphoglyceric kinase (EC 2.7.2.3), fructose-1,6-bisphosphatase (EC 3.1.3.1), and triose-phosphate isomerase (EC 5.3.1.1) were assayed according to the procedures of Benedict (5). Phosphoglucoisom-
erase (EC 5.3.1.9) was assayed according to the procedure of Noltmann (22). Phosphoglucomutase (EC 2.7.5.1) was assayed according to the procedure suggested by Boehringer-Mannheim (6). Phosphohexokinase (EC 2.7.1.11) was assayed according to the procedure of Walker and Perry (27). All coupling enzymes and nucleotides were purchased from Sigma.

**Liquid Scintillation Spectrometry.** All radioactivity determinations were performed with a Beckmann LS-200 liquid scintillation system. The samples were counted in 10.0 ml Beckmann cocktail D prepared from 5.0 g 2,5-diphenyloxazole, 100 g naphthalene, 10 ml H2O and dioxane to 1 L final volume.

**Electron Microscopy.** Electron photomicrographs of endosperm tissue were prepared as previously described (12).

**RESULTS**

The development of starch content is shown in Figure 1. A maximum of 1.1 mg starch/seed was found at 4 to 5 d. The pattern for starch accumulation is similar to the accumulation of sucrose and free reducing sugars from the same tissue, which showed a maximum of 75 mg sucrose and 20 mg free reducing sugars/seed at 5 to 6 days. Imbibed seed and 9-d-old endosperm also contain some starch. The 9-d endosperm was judged to be nonmetabolically active based on its paper consistency and low water content. Thus, all of the starch produced by the endosperm is not fully utilized. The developmental patterns we observed agree well with other processes reported for germinating *Ricinus communis* seed (10, 23).

Two key enzymes of starch synthesis in other tissues, ADP-glucose pyrophosphorylase and ADP-glucose-starch glucosyl transferase (starch synthetase), were examined for activity. The developmental patterns for the activity of these enzymes are depicted in Figures 2 and 3. It must be emphasized that these activities must be considered minimal, since losses due to extraction, assay conditions, and enzyme instability may reduce the activity. The maximal activity observed for ADP-glucose pyrophosphorylase (8.12 nmol ADP formed/min-seed), was found in 4-d-old endosperm, whereas starch synthetase (125 nmol glucose incorporated/min-seed) activity peaked in 5-d-old endosperm. From the starch analysis, a rate of 1.1 nmol glucose incorporated/min-seed can be estimated. Thus, these minimal activities of ADP-glucose pyrophosphorylase and starch synthetase measured *in vitro* are adequate to sustain starch synthesis. The activities of other enzymes suspected of being involved in starch synthesis are shown in Table I. The minimal activities of these enzymes are also adequate to sustain the level of starch synthesis observed. The developmental profiles for these enzymes were not established.

When crude extracts were fractionated by discontinuous sucrose gradient centrifugation, starch synthetase was found to co-migrate to the 51/60% sucrose interface with RuBP carboxylase, a marker enzyme for proplastids (Fig. 4). While 98% of the RuBP carboxylase activity placed in the gradient was recovered in the proplastid fraction, only 44% of the starch synthetase activity migrated to this point. Other enzymes which also showed activity in the band containing proplastids include PGA kinase, fructose-1,6-bisphosphate aldolase, triose-Pisomerase and catalase.

**DISCUSSION**

Based on electron micrographs from a variety of tissues, it is apparent that proplastids contain starch (2). The discovery of proplastids in the endosperm of *R. communis* has allowed the biochemistry of these organelles to be studied. Soon after the discovery of proplastids in *R. communis* endosperm, it was proposed that these organelles may be the site of gluconeogenic sucrose production (18). Recent evidence has led several researchers to suggest that the proplastids from developing endosperm (26, 29) as well as endosperm from germinating seed (24) are responsible for the synthesis of some fatty acids. Recent evidence of Nishimura and Beevers (21), however, demonstrates that although most of the enzymes required for sucrose production are present in the proplastids, these enzymes do not possess sufficient activity to account for the amount of sucrose produced. The activities of the necessary enzymes are sufficient in the cytoplasm, leading to the conclusion that sucrose production is cytoplasmic. Sucrose synthesis in photosynthetic cells has also been shown to be cytoplasmic (25).

Using the rate of starch accumulation in Figure 1, starch deposition was estimated to be maximal at about 1.1 nmol glucose incorporated/min-seed. The enzymatic activities for proplastids reported by Nishimura and Beevers (21), and others studying the carbohydrate metabolism of *R. communis* endosperm (26), as well as the enzymatic activities reported here, are sufficient to synthesize starch at the rate of starch accumulation observed. The lowest enzymatic activity measured *in vitro* was ADP-glucose pyrophosphorylase which exhibited a rate of 8.17 nmol ADP formed/min-seed. Activities of other enzymes necessary for starch synthesis are given in Table I. Whereas the activities given in Table I are total activities of endosperm tissue, a certain portion of these total activities have been shown to be found in the proplastids by Nishimura and Beevers (21) and others (26). The lowest endosperm activity observed by Nishimura and Beevers (21) was for NAD-glyceraldehyde-3-P dehydrogenase which exhibited an activity of 145.5 nmol/min-endosperm. They reported that 7% of this activity was recovered in the proplastids fraction which still

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
<th>nmol/min-seed</th>
<th>nmol/min mg protein</th>
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<tr>
<td>3-Phosphoglycerate kinase</td>
<td></td>
<td>8,760</td>
<td>438</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate</td>
<td></td>
<td>2,560</td>
<td>128</td>
</tr>
<tr>
<td>dehydrogenase (NAD+)</td>
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<td>None detected</td>
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<tr>
<td>Glyceraldehyde-3-phosphate</td>
<td></td>
<td>29,260</td>
<td>1,463</td>
</tr>
<tr>
<td>dehydrogenase (NADP+)</td>
<td></td>
<td>990</td>
<td>49.5</td>
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<tr>
<td>Triose-phosphate isomerase</td>
<td></td>
<td>230</td>
<td>11.5</td>
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<tr>
<td>Fructose-1,6-bisphosphate</td>
<td></td>
<td>12,600</td>
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<td>aldolase</td>
<td></td>
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</tr>
<tr>
<td>ADP pyrophosphorylase</td>
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<td>228</td>
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<td>(21)</td>
<td></td>
<td>125</td>
<td>6.25</td>
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<tr>
<td>Starch synthetase</td>
<td></td>
<td>280</td>
<td>14.0</td>
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<tr>
<td>(21)</td>
<td></td>
<td>1.1</td>
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</table>
would be approximately 10 times the activity required for starch synthesis.

Because of the low activity of ADP-glucose pyrophosphorylase in this tissue, it was not possible to study the distribution of this enzyme on the sucrose gradients. It is probable that this enzyme is located within the plastids, inasmuch as in higher plant tissues ADP-glucose pyrophosphorylase is a plastid enzyme. Thus, the minimum activity of the plastid enzymes examined is sufficient to account for a starch production rate of 1.1 nmol/min.

The data in Figure 4 demonstrate the presence of starch synthetase in a particulate fraction. Probably the plastid membranes are easily ruptured by the starch grains during isolation, inasmuch as a low percentage of the total activity was associated with the organelles after gradient centrifugation. Catalase was also detected at the 51/60% interface. The presence of catalase demonstrates the contamination of the plastid fraction by glyoxysonomes. However, the localization of starch synthetase in the plastid is supported by the electron micrograph of plastids containing starch grains (Figure 5), since starch synthetase has been shown to be associated with starch grains (1). In higher plants, starch grains have not been observed outside the plastid membranes (3). Thus, the presence of starch in these organelles suggests that starch synthetase would also be located within these organelles.

Starch granules are known to be stored carbon for sucrose synthesis during germination (3, 23). Assuming that starch synthesis in this tissue is being sustained by acetate derived from β-oxidation and organic acids provided by the glyoxylate cycle, the question arises as to which compound is supplied to the plastid by the cytoplasm. The uptake of metabolites by plastids has been investigated, but references can be drawn from work with etioplasts and chloroplasts. Assuming that plastid membranes are at least as permeable as chloroplast membranes, at least seven compounds generated by the cytoplasmic gluconeogenic process should be able to enter the plastids and support starch synthesis (14, 16, 17). The enzymes present in the plastids would be capable of handling any compound from 3-PGA to free glucose. It is not known which of these compounds enters the plastid, or if entry is limited to one or more compounds. It is possible, however, that starch synthesis is sustained by the pool of gluconeogenic intermediates from the cytoplasm. When comparing the rates of starch production to sucrose production in R. communis endosperm tissue it becomes obvious that starch synthesis does not represent a major portion of the carbon metabolism of this tissue, nor does starch represent a large portion of the carbon content of the endosperm. Developmentally, the fact that the enzymes for starch metabolism are present is of great interest. The plastids in this tissue are at an arrested stage of development with respect to the light harvesting systems. These organelles have never been shown to contain prolamellar bodies or any other features normally associated with the development of chloroplasts. In this respect, they have features similar to amyloplasts from other tissues. It is thus probable that the control mechanisms for light harvesting systems and starch metabolism are separate and different. Whereas the development of chloroplasts is usually dependent upon a light stimulus, the enzymes for starch metabolism do not require any light stimulus. The presence of active RuBP carboxylase (5) also demonstrates that these plastids contain the enzymatic machinery necessary for protein synthesis, inasmuch as this enzyme is partially synthesized on plastid ribosomes. It is possible that several enzymes which appear to have no metabolic function may be synthesized when the genes for other essential enzyme systems are derepressed. Since starch does not represent a major portion of the carbon in this tissue, and a need for starch synthesis is not immediately apparent, the enzymes for starch synthesis may fall into this category.

LITERATURE CITED

23. Pierce HB, DE Sheldon, JR Murlin 1933 The conversion of fat to carbohydrate in the germinating castor bean. J Gen Physiol 17: 311-325