Starch Synthesis Studies in Zea mays

II. MOLECULAR DISTRIBUTION OF RADIOACTIVITY IN STARCH

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ABSTRACT

The amylase and amyllopectin fractions from kernel starch synthesized shortly after exposure of intact Zea mays L. plants to 14C02 had similar specific radioactivities (counts per min per mg of carbohydrate). In both fractions the radioactivity was distributed throughout the molecules. These data are consistent with a model in which the polysaccharides are synthesized in the matrix of the amyloplast followed by crystallization of the completed molecules onto the starch granule.

Starch from dent corn (Zea mays L.) is composed of amylase (straight chain polymers of glucose linked by α-D-(1 → 4)-glucosidic bonds) and amyllopectin (a branched polymer of glucose containing α-D-(1 → 6) branches in addition to the α-D-(1 → 4) linkages). The enzyme phosphorylase is capable of producing amylase-like molecules in vitro (10), and Q-enzyme can convert amylase to amyllopectin-like molecules in vitro (11). Starch is localized in cellular organelles called amyloplasts. Badenhuizen (2) suggested that starch is synthesized in the liquid matrix of the amyloplast by the combined action of phosphorylase and Q-enzyme. Furthermore, he suggested that once the polysaccharides attain a certain size they are added to the growing starch granule by apposition.

The discovery of the sugar nucleotide (UDPG and ADPG) involvement in polysaccharide biosynthesis (8, 13, 17), has led to many studies in vitro with the starch granule-bound enzyme, UDPG (ADPG):starch glucosyltransferase (4). In studies in vitro glucose molecules are added to the nonreducing ends of the amylase and amyllopectin molecules of the granule (5, 9). Several workers have concluded from these studies that starch synthesis in vivo proceeds by a similar mechanism (15).

Both phosphorylase and UDPG (ADPG):starch glucosyltransferase produce linear α-D-(1 → 4) segments which must then be branched by Q-enzyme or possibly some other branching enzyme. Erlander (6, 7) suggested that amylase and amyllopectin are derived from a water-soluble precursor, phytoamylacein.

We have undertaken a study of starch synthesis in vivo to examine and distinguish between the various suggested mechanisms of starch synthesis in maize (18). The distribution of radioactivity within the amylase and amyllopectin molecules isolated from maize kernels collected at various times following 14CO2 treatment of intact plants is reported.

MATERIALS AND METHODS

Administration of 14CO2 and Isolation of Starch. Z. mays L. plants of inbred lines Oh 43E bearing ears 18 days after pollination were treated with 14CO2 for 1 hr as described previously (18). The starch samples fractionated and reported in this paper are the same as those presented in Figure 3 of Reference 18. Kernel samples were collected immediately after treatment with 14CO2 for 1 hr and at intervals up to 6 hr. The alcohol-soluble sugars, water-soluble polysaccharides, and starch were extracted as described earlier (18, 19). The starch extract was stored in 90% DMSO prior to fractionation into amylase and amyllopectin.

Separation of Starch Components. Amylose was separated from amyllopectin by differential precipitation with thymol and n-butanol as described below. A 0.5 to 1.0% solution of starch in 90% DMSO was centrifuged at 10,000g for 15 min to remove any remaining insoluble protein contaminant. Forty milliliters of the supernatant solution were precipitated overnight with 2 volumes of n-butanol plus 2 ml of 20% NaCl. Two volumes of n-butanol effectively precipitate amylase and amyllopectin out of DMSO and form a precipitate which is readily soluble in hot water. The precipitate was collected by centrifugation at 2,000g for 10 min and was dissolved in 25 ml of boiling water. The starch suspension was filtered through a course grade sintered glass filter. The filter was rinsed with a total of 25 ml of hot water. The filtered starch was adjusted to 50 ml, and aliquots were removed for total carbohydrate and radioactivity determinations. An aliquot (48 ml) was added to an Erlenmeyer flask and placed in a 60° water bath. One-half milliliter of thymol solution (10% solution in 95% ethanol) was added with swirling. Following complete stirring the flask was stoppered and placed in an insulated 60° sand bath and

1 Cooperative investigations of the Crops Research Division, Agricultural Research Service, United States Department of Agriculture; the Purdue Agricultural Experimental Station, Lafayette, Indiana; and the Pennsylvania Agricultural Experiment Station, University Park, Pennsylvania. This work was begun when J. C. Shannon was a visiting Professor in Horticulture at The Pennsylvania State University. This is Purdue Agricultural Experiment Station Journal Paper No. 3727.

2 The paper given in Reference 18 is considered no. 1 in this series.

3 Abbreviations: UDPG: uridine diphosphoglucose; ADPG: adenosine diphosphoglucose; DMSO: dimethylsulfoxide.
allowed to cool slowly. The sand temperature normally dropped to about 30° in 18 hr. The flask was left in the sand bath for approximately 60 hr, and then it was transferred to a cold room (2°) for a final 10 to 12 hr. The amylose-thymol complex was collected by centrifugation at 10,000g for 20 min. The supernatant fraction containing amylopectin was saved. The precipitated thymol-amylose complex was suspended in 25 ml of boiling water, the suspension was again filtered through a sintered glass filter, and the filter was washed with a total of 25 ml of water. The filtered solution was heated to 100°, and 10 ml of n-butanol were added to it. The solution was stirred, loosely stoppered, and placed in a 100° sand bath. The sand bath was allowed to cool slowly to room temperature. After slow cooling for 24 hr the flask was moved to the cold room for 6 hr. The n-butanol-amylose complex was collected by centrifugation at 20,000g for 20 min. The supernatant containing branched polysaccharides either was saved separately or was added to the thymol supernatant. If the final amylose pellet was to be used immediately, it was suspended in 10 ml of 10⁻³ m sodium acetate buffer, pH 5.0. If it was to be stored prior to use, it was suspended in 90% DMSO to inhibit retrogradation of the amylose. When needed, the amylose was precipitated from the DMSO by the addition of 2 volumes of n-butanol, and the resulting pellet was washed in the sodium acetate buffer. The branched fractions were heated on a hot plate to drive off the excess n-butanol and thymol. The remaining concentrated fractions were freeze-dried.

β-Amylase Degradation of Starch Fractions. The β-amylase degradation procedure was the same for both amylose and amylopectin. An aliquot (4 to 5 ml containing 10 to 25 mg) of a polysaccharide fraction was added to a short section of 24 mm wide dialysis tubing which was tied at one end. The tubing was lowered into 20 ml of water in a 50-ml beaker and dialyzed for 30 min to remove any small contaminating carbohydrates and solvents. The dialysis bag containing the polysaccharides was transferred to 20 ml of fresh water in 50-ml beakers, and 0.1 mg of β-amylase (0.1 mg; 0.1 ml of 10⁻³ M Na acetate buffer, pH 5.0) was added to the dialysis tubing. The β-amylase, lot 9416, was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. These and the following incubations as well as their associated dialyses were carried out with continuous shaking in a 30° water bath. The first incubation (degradation A) was terminated at the end of 5 min by transferring the dialysis bag and its contents to a 50-ml beaker containing 30 ml of boiling water. The solution was boiled for 5 min to inactivate the enzyme. The beaker and dialysis bag were then moved to the shaker, and dialysis was continued for 30 min. Preliminary studies indicated that the bulk of the released maltose dialyzed out of the sack within 30 min. The released maltose was added to the maltose, which dialyzed out of the bag during the original incubation. The resulting maltose fraction contains only the glucose residues located nearest the nonreducing ends of the polysaccharides treated and is designated as A in Figure 1. The residue remaining in the dialysis sack was further degraded by adding more β-amylase and incubating 10 min before stopping the reaction and dialyzing out the released maltose (degradation B). Additional degradative steps of 20 min, 60 min, 4 hr, and 11 hr (degradations C through F) were carried out on each polysaccharide fraction studied. The various maltose fractions were placed in beakers and dried under heat lamps. The dried maltose was dissolved in a minimum of water and quantitatively transferred to Whatman No. 3MM chromatography paper or to Whatman GF/B glass paper. The dried papers were then placed into counting vials containing 10 or 20 ml of scintillation fluid (5 g of 2,5-diphenyloxazole (PPO) and 0.3 g of p-bis-2-(5-phenyloxazolyl)benzene (POPPO) per liter of toluene). The radioactivity was determined using either a Beckman model S-100B or Packard model 3350 scintillation counter. After radioactivity determination the papers were removed, dipped successively into two beakers oftoluene to remove the bulk of the scintillation fluors, and dried. The maltose was eluted from the papers with water and the amount estimated by the phenol-H₂SO₄ procedure (12).

Based on the mode of action of β-amylase (16), each successive degradative step released maltose further removed from the original nonreducing end. β-Amylase will degrade the outer chains of amylopectin to within 2 to 3 glucose moieties of any α-D-(1 → 6) branch point (16). To investigate the labeling pattern interior to these branch points in the amylopectin molecules, the amylopectin β-limit dextrins were degraded with pullulanase (obtained from Wallerstein Company, Wallerstein Sq., Mariners Harbor, Staten Island, New York) an enzyme which specifically cleaves the α-D-(1 → 6) linkages (1).

Pullulanase Degradation of the Amylopectin β-Limit Dextrin. The amylopectin β-limit dextrin solution was adjusted to 10 ml, and the total carbohydrate and radioactivity content was determined. An aliquot (usually 9 ml) was placed in a 15 × 150-mm test tube, and 0.5 mg of pullulanase was added. Following incubation for 4 or 8 hr at 30° the reaction was terminated by transferring the tubes to a boiling water bath for 5 min. The total mixture was concentrated to dryness. The hydrolyzed solution was suspended in 0.5 ml of 90% DMSO. The malto-oligosaccharides produced were separated by thin layer chromatography using plates (20 cm × 46 cm) coated with a 0.25-mm layer of Kieselguhr G adsorbent (Brinkman Instruments, Waterbury, New York). The enzyme digest (0.4 ml) was applied as a narrow band 2 cm from the lower edge of the plate. The chromatogram was serially developed in three solvents containing the following proportions of n-butanol: pyridine:water; A = 5:4:2; B = 6:4:3; and C = 13:4:3, v/v, as described elsewhere (20). The plate was first developed approximately 25 cm with solvent A, dried, next developed 35 cm with solvent B, dried, and finally developed 44 cm in solvent C. The carbohydrate zones were located by placing the plate, adsorbent side down, over a 17- × 28-cm Pyrex tray containing iodine crystals. The carbohydrate zones absorbed the iodine vapors more rapidly than the Kieselguhr and appeared as yellow bands across the plate. The bands were marked on the glass with a felt tip pen and the Kieselguhr was scored between each zone. The radioactivity and carbohydrate content of the malto-oligosaccharides were determined by scraping the respective bands into scintillation vials, adding 10 ml of scintillation fluid, and counting. Correction for self-adsorption caused by the Kieselguhr was made by referring the weight of the Kieselguhr in the sample to a predetermined self-adsorption curve and finding the counting efficiency at that weight. Following counting, the vials were centrifuged, the scintillation fluid was siphoned off, and the Kieselguhr plus the malto-oligosaccharides were dried. Ten milliliters of water were added, and an aliquot was taken for carbohydrate determination by the phenol-H₂SO₄ procedure (12). In a second method, the carbohydrates were chromatographed off the plate and into Whatman No. 1 paper strips 1 cm × 4 cm with water as described elsewhere (20). These strips were placed into vials containing 20 ml of scintillation fluid and were counted. The papers were removed and dried, and the sugars were eluted with water. The carbohydrate contents were determined as above.

**RESULTS**

This study was limited to an investigation of the amylose and amylopectin of the starch granules. It did not include soluble

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polysaccharides, which were extracted with the 10% ethanol prior to granule solubilization.

**Radioactivity Distribution between Amylose and Amylopectin.**
Amylose comprised approximately 20% of the total starch obtained from the granule (Table I). At all sampling times the specific radioactivity (cpm/mg of carbohydrate) of amylose and amylopectin was similar, and the ratio of the specific radioactivities did not change.

**Table I. The Distribution of Radioactivity between Amylose and Amylopectin**

 Intact Oh 43 plants with ears 18 days after pollination were exposed to $^{14}$CO$_2$ for 1 hr. Kernel samples were taken 3 hr, 6 hr, etc., after termination of the $^{14}$CO$_2$ treatment.

<table>
<thead>
<tr>
<th>Sampling Time (Time after Treatment)</th>
<th>Amylose$^1$</th>
<th>Amylopectin$^1$</th>
<th>Specific Radioactivity Ratio, Amylose/Amylopectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td>Weight (%)</td>
<td>Specific radioactivity (cpm/mg)</td>
<td>Weight (%)</td>
</tr>
<tr>
<td>3</td>
<td>22.0</td>
<td>3.6</td>
<td>78.0</td>
</tr>
<tr>
<td>6</td>
<td>17.5</td>
<td>150.2</td>
<td>82.5</td>
</tr>
<tr>
<td>12</td>
<td>21.4</td>
<td>1635.7</td>
<td>78.6</td>
</tr>
<tr>
<td>24</td>
<td>24.0</td>
<td>5221.6</td>
<td>76.0</td>
</tr>
<tr>
<td>36</td>
<td>20.5</td>
<td>7663.1</td>
<td>79.5</td>
</tr>
</tbody>
</table>

$^1$ The amylose and amylopectin data are the average of three fractionations.

**Distribution of Radioactive Glucose in Amylose.** Both phosphorylase and UDPG (ADPG):starch glucosyltransferase add glucose molecules to the nonreducing ends of polysaccharides in vitro. We were interested in knowing if polysaccharides of the starch granule were being lengthened by a similar mechanism. If so, we would expect the glucose moieties at or near the nonreducing ends to have a specific activity higher than those interior to the molecule. Likewise, a regular decline in specific radioactivity at regions deeper within the molecule would be expected. Figure 1 shows that radioactive glucose is scattered throughout the amylose molecule. This is true as early as 6 hr after treatment of the plant with $^{14}$CO$_2$ as well as at all other sampling times. The Tr + 3 hr amylose was also degraded with the result that even this early the radioactivity was scattered throughout the polysaccharide molecules, but the amount of activity per fraction was too near background for the data to be considered reliable. The higher specific radioactivity of fractions A and B 6 hr after treatment may have been due to the low levels of radioactivity observed in these samples with the resultant higher error expected in counting. It is clear, however, that the radioactivity is not confined to the nonreducing ends.

**Distribution of Radioactive Glucose in Amylopectin.** Radioactive glucose was distributed throughout the entire length of the outer chains of amylopectin (Fig. 2). Also the $\beta$-amylase limit

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**Fig. 1.** The specific radioactivity (dpm/mg) of maltose fractions released from amylose by stepwise $\beta$-amylase degradation. Data in the figure identified as A through F represent maltose fractions released by the sequential $\beta$-amylase treatments as described under "Materials and Methods". R is the final nondialyzable residue. The percentage of the initial carbohydrate recovered in each fraction is given above each bar. The designations Tr + 6 hr, etc., indicate the sampling times after treatment of the plants with $^{14}$CO$_2$. 

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The dashed carbohydrate recovered treatments. Cleaved linkages hydrate, which dextrins, as with pullulanase, malto-oligosaccharides

![Diagram](https://example.com/diagram.png)

**Fig. 2.** The specific radioactivity (dpm/mg) of maltose fractions released from the outer chains of amylopectin by stepwise β-amylase degradation. Data identified as A through F are as in Figure 1 and R is the nondialyzable β-amylase limit dextrin. The percentage of the initial carbohydrate recovered in each fraction is given above each bar.

![Graph](https://example.com/graph.png)

**Fig. 3.** A diagrammatic representation of an amylopectin molecule. The dashed outer chains represent the portion removed by the β-amylase treatments. The point of the arrows represent the α-D-(1 → 6) linkages cleaved by pullulanase.

dextrins, which contained 55 to 60% of the amylopectin carbohydrate, had approximately as much radioactivity per mg of carbohydrate as the outer chains (Fig. 2, bar R).

When the amylopectin β-amylase limit dextrins were degraded with pullulanase, malto-oligosaccharides of varying lengths were obtained. From an examination of the diagrammatic representation of an amylopectin molecule (Fig. 3) it can be seen that the shortest malto-oligosaccharides would be formed from the cleavage of the outermost α-(1 → 6) linkage. Similarly, increasingly longer malto-oligosaccharides would be produced by the cleavage of α-(1 → 6) linkages located at increasingly greater depths within the molecule. Based on the specific radioactivity of the malto-oligosaccharides formed by pullulanase treatment of the β-amylase limit dextrins, it is apparent that radioactivity is distributed throughout the entire amylopectin molecule (Fig. 4). The specific radioactivity of the largest malto-oligosaccharides (greater than 20 glucose units long) was comparable to those located near the nonreducing ends of the original molecule. Although there was considerable difference in the specific radioactivities of the malto-oligosaccharide fractions obtained from the chromatographic zones in the two experiments, the over-all pattern of radioactivity was similar. The number of zones varied with the chromatogram. Consequently, there are no bars for some zones in Figure 4.

These data are from starches isolated from kernel samples in which the entire plant was treated with 14CO2 for 1 hr. Similar fractionations were made of starches isolated from kernel samples taken from plants in which only three leaves were treated with 14CO2. The treated leaves were removed at Tr + 2 hr and kernel
samples were taken at various times from Tr + 2 hr to Tr = 36 hr. The general pattern of radioactivity in the polysaccharide fractions of starch from this "in vivo chase" study was the same as that given in Figures 1, 2, and 4, and the data will not be presented here.

**DISCUSSION**

It has generally been concluded that amylopectin is derived from amylose or amylose-like molecules (2, 14). Whistler and Young (21) demonstrated that, in wheat starch, amylose was synthesized prior to amylopectin. The data for maize presented in Table I are not in agreement with the above findings. Starch from maize endosperm contains about 3 times as much radioactivity incorporated into amylopectin as into amylose. The specific radioactivities of the amylose and amylopectin fractions were approximately equal and the ratios of specific radioactivity did not change up to 36 hr after treatment. In the present study all watersoluble polysaccharides were extracted with 10% ethanol prior to solubilization of the starch. Thus the distribution reported represents only the polysaccharides of the water-insoluble granules. Whistler and Young (21) showed that after 56 hr, there was little additional conversion of amylose to amylopectin. They suggested that the conversion of amylose to amylopectin may have occurred prior to polymer deposition in the granule. McConnell et al. (14) have also concluded that amylopectin is derived from amylose. It should be pointed out, however, that there is not complete agreement on the interpretation of these data. Erlander (7), using the data from these papers (14, 21), has made additional calculations and concluded that they support his hypothesis that amylose and amylopectin are both produced from a phytoglycogen precursor. From the present study we must conclude either that amylose and amylopectin are being synthesized at the same time, as suggested by Pazur (16), or that all interconversions are completed prior to deposition onto or into the starch granule.

Based on the enzymatic degradation studies of both amylose and amylopectin (Figs. 1, 2, and 4), it is apparent that the radioactivity is scattered throughout the entire length of the molecules. This was observed even at the earliest times after treatment of the plants. These data do not support a biosynthetic mechanism in which granular starch molecules are lengthened by the starch-grain-bound UDPG (ADP)-starch glucosyltransferase. The starch granule-bound UDPG (ADP)-starch glucosyltransferase transfers glucose from the sugar nucleotides UDPG and ADPG to the nonproducing ends of pre-existing amylose and amylopectin molecules (5, 9). If, in fact, starch was synthesized primarily by the UDPG (ADP)-starch glucosyltransferase enzyme, as has been concluded by some workers (15), one would...
expect to find the bulk of the radioactivity near the nonreducing ends at the earliest sampling times. \( \beta \)-Amylase degrades starch by a stepwise removal of maltose units beginning at the nonreducing ends (16). Although \( \beta \)-Amylase has been shown to release approximately 4 maltose units per effective enzyme-substrate encounter (3), essentially all the chains are progressively shortened in the course of the reaction (16). By the stepwise release of maltose fractions from amylose and the outer branches of amylpectin with \( \beta \)-amylase we have shown that the majority of the radioactivity is not confined to the nonreducing ends. In fact, the \( \beta \)-amylase limit dextrin of amylpectin had a specific radioactivity as high as the outer chains (Fig. 2). A study of the malto-oligosaccharides derived from the pullulanase digestion of the \( \beta \)-amylase limit dextrin also demonstrated that the radioactivity was distributed throughout the entire amylpectin molecule (Fig. 4).

The data presented in this study are consistent with a model proposed by Badenhuizen (2) in which the polysaccharides are synthesized in the matrix of the amyloplast, followed by crystallization of the completed molecules onto or into the starch granule. Since this study was limited to the water-insoluble starch of the granules, we are unable to suggest which enzyme or enzymes are actually involved in the biosynthesis of amylose and amylpectin. From a study of the water-soluble polysaccharide fraction presumably derived from the amyloplast matrix we should be able to distinguish between the phytoglycogen precursor theory (6) and a mechanism in which the linear portions are produced by phosphorylase or UDPG (ADPG)-starch glycosyltransferase (or both) followed by branching to amylpectin by Q-enzyme.

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LITERATURE CITED


