In Vivo Incorporation of Carbon-14 Into Zea Mays L. Starch Granules\textsuperscript{1,2}

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ABSTRACT

A positive linear relationship existed between starch granule size and radioactive starch per granule at 29 and 50 hr. after exposure of Zea mays L. plants to \textsuperscript{14}CO\textsubscript{2}. This relationship was observed in starch granules isolated from kernels 14 to 41 days post-pollination. The $^{14}$C per unit surface area was similar for granules in the five larger size classes, but the smallest granule class contained only half as much $^{14}$C per unit area of granule surface. Based on these data it is suggested that the smallest granules are derived from physiologically younger cells. Starch synthesis in such cells may rely on synthesis from UDP-glucose by the less efficient granule-bound UDP-glucose-starch glucosyltransferase rather than being able to utilize ADP-glucose and/or glucose-1-P as substrates. Cells containing starch granules having an average of at least 0.12 g of starch per granule are probably synthesized from ADP-glucose or glucose-1-phosphate via the action of granule-bound and soluble nucleoside diphosphate-glucose-starch glucosyltransferase and phosphorylases II and III, respectively. However, since the $^{14}$C per unit surface was similar for the five largest granule size classes, the rate of starch synthesis in these granules was probably regulated by the surface area available for starch deposition.

A developing corn kernel is composed of cells of varying physiological ages (1). There is a major gradient in cell development from the cells located in the basal endosperm region to those in the central crown region and a minor gradient from the peripheral cells toward the central interior (1,2,3). In the lower third of the endosperm from corn kernels 20 to 30 days post-pollination, development varies from cells completely void of starch, to those having tiny amyloplasts closely surrounding the nucleus, and to other cells essentially filled with granules (1, and Fig. 1). Granules within a given cell are all similar in size (3). The major gradient in endosperm development was confirmed by histochemical analyses (3) and by analysis of endosperm zones (4). The high soluble sugar and low starch contents of the lower endosperm zones (4) from 30-day-old kernels were similar to those reported in kernels 8, 10, and 12 days post-pollination and likewise the low sugar-high starch of the upper zones was similar to kernels 22 and 28 days post-pollination (5).

Tsai et al. (5) were unable to detect activity of sucrose-UDP glucosyltransferase, ADP-glucose pyrophosphorylase, soluble ADP-glucose-starch glucosyltransferase, and phosphorylases II and III in kernels younger than 12 days post-pollination. However, during the time of maximum starch deposition the activity of these enzymes increased markedly. The young kernels did contain granule-bound nucleoside diphosphate-glucose-starch glucosyltransferase and UDP-glucose pyrophosphorylase, and thus these authors (5) suggested that starch synthesis prior to 12 days post-pollination occurred via granule-bound glucosyltransferase using UDP-glucose as substrate. In contrast Ozburn et al. (6), using different procedures, detected sufficient ADP-glucose pyrophosphorylase and ADP-glucose-starch glucosyltransferase

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activity to account for the starch synthesized in maize kernels 8 to 28 days post-pollination. During the time of most rapid starch synthesis, Tsai et al. (5) and Ozburn et al. (6) reported substantial increases in activity of several enzymes thought to be significant in starch biosynthesis. Both research groups measured enzyme activities in whole kernel (6) or endosperm (5) homogenates. Since a developing kernel is composed of cells of varying physiological ages (1,2,3), the observed increases in whole kernel or endosperm enzyme activity may have been due to an increasing proportion of cells functioning in starch biosynthesis. Tsai et al. (5) suggested that "the 12-day kernel stage represents a developmental switch point" in the pathway of starch synthesis. Based on the known gradients in cell development of maize endosperm cells, it is reasonable to propose that each
individual endosperm cell might also go through a similar switch in the pathway of starch biosynthesis. For example, UDP-glucose may serve as substrate for enzymes functioning in very early starch synthesis. With increasing cellular development, the more efficient enzymes (7) responsible for producing and utilizing ADP-glucose may become active.

In kernels 8 to 12 days post-pollination, only a few cells in the central crown region of the endosperm are actively producing starch (3). Therefore, Tsai et al. (5) may have failed to detect the enzymes responsible for producing and utilizing ADP-glucose and phosphorylases II and III because of the gross dilution during extraction of these enzymes with cytoplasm from younger cells not yet synthesizing starch. Thus, in contrast to the previous suggestion, all cells may contain the complete complement of starch biosynthetic enzymes at the time starch synthesis begins.

To aid in determining which of the above suggestions depicts the correct in vivo enzymatic situation, intact *Zea mays* L. plants were exposed to $^{14}$CO$_2$ and the amount of $^{14}$C incorporated into starch granules of different sizes was determined. Assuming the latter hypothesis in which all cells synthesizing starch contain the same starch biosynthetic enzymes and the same capacity for substrate (ADP-glucose and/or G-1-P) production, radioactivity per granule or per unit area of granule surface should be the same for all size granules. Alternatively, if starch is initially produced from UDP-glucose which is utilized less efficiently than ADP-glucose (7), one might expect the smallest granules (those from cells just beginning starch synthesis) to incorporate less $^{14}$C per unit area of starch granule surface than larger granules which are being formed via a more efficient pathway.

**MATERIALS AND METHODS**

**Administration of $^{14}$CO$_2$ and Sampling**

*Zea mays* L. plants (single cross hybrid, H49 × B37) with ears 14 to 41 days after pollination were used. The ear leaf or the first leaf above the ear node of each plant was exposed to $^{14}$CO$_2$ as described earlier (8). The upper three-fourths of each ear was sampled during the first 6 hr. after treatment of the plants and these kernel samples were used for a study reported previously (4). In study 1, the basal one-fourth of a 19-day-old ear was harvested 29 hr. after $^{14}$CO$_2$ treatment of the plant. In study 2, the basal ear pieces were sampled 50 hr. after exposure of the plants to $^{14}$CO$_2$. In both studies the ear pieces were quickly frozen and freeze-dried as before (4). Prior to the final sampling the husks were tightly held around the remaining ear piece with rubber bands. Although the entire ear was not sampled, preliminary studies showed that all kernels in a row accumulated radioactivity at a similar rate. It is possible that the kernels used in this study accumulated more radioactivity per kernel due to the earlier removal of the other kernels. However, those used in this study appeared to be developing normally at the time of sampling and no change in carbohydrate metabolism would be expected.

**Starch Granule Isolation and Separation**

Endosperm free of all pericarp and embryo tissues was used for both studies. In study 1 the endosperm tissue was homogenized and extracted with MCW
(methanol:chloroform:water, 13:4:3 v./v.) and then 10% ethanol as previously described (8). The residue (containing starch) was suspended in 10 ml. of 10% ethanol. A 0.5-ml aliquot was diluted to 10 ml. with water and was filtered through a pad of glass wool to remove most of the cell debris. The starch granules were collected by centrifugation and were used for microradioautography.

In study 2, endosperms from 10 to 12 kernels 14, 18, 21, 30, and 41 days post-pollination were soaked 16 hr. at 37°C in 10 ml. of “steep” solution (0.02M sodium acetate, 0.01M HgCl₂, pH 6.5) in 16 × 150-mm. glass tubes. The soft endosperm tissue was gently mashed and disrupted in the “steep” solution with a stirring rod. The end of the stirring rod was flattened and modified to closely fit the tube (less than 1.0 mm. clearance between end of rod and side of tube). Finely ground cell-wall debris, resulting from a more vigorous homogenization, was difficult to separate from the starch granules. Therefore, the gentle mashing procedure was necessary. The homogenate was filtered through Nitex (Tobler, Ernst and Traber, Inc., New York) monofilament nylon cloth having sieve openings of 44 μ. Filtration was repeated twice. The combined filtrates were centrifuged for 5 min. at 700 × g and the starch was suspended in water and washed through Nitex nylon cloth having sieve openings of 20 μ. The filtered solution was washed through the 20-μ nylon cloth two more times. The starch was collected by centrifugation as above and was extracted three times with MCW and four times with 10% ethanol as in study 1. To remove protein and additional cell debris, the starch was suspended in 40 ml. water, and 5 ml. toluene was added. This mixture was shaken vigorously for 30 min. followed by centrifugation for 5 min. at 140 × g. The toluene layer, interface material, and

![Graph](image-url)

**Fig. 2.** The relationship between starch granule size and carbon-14 incorporation in study 1. Each bar from left to right represents the percentage of granules scored 1, 2, 3, 4, or 5, respectively, where 1 = no silver grains and 5 = very heavy silver grain density. The number of granules which were measured and scored is given above each set of bars.
half of the aqueous supernatant were removed by suction. The sedimented starch was extracted three more times as above by adding 20 ml. water and 5 ml. toluene each time.

Approximately an equal volume of wet-packed starch granules from each age kernel was separated into four to six size classes by differential settlings through glycerol-water mixtures. The size classes were designated A (smallest granules) to F (largest granules). Polypropylene centrifuge tubes (3 × 15 cm.) containing 20 ml. 70% glycerol overlaid with 20 ml. 50% glycerol and 20 ml. 30% glycerol were used for preliminary separations. Starch granules suspended in 20 ml. water were carefully layered on the glycerol solutions and were allowed to settle at room temperature for 2 hr. The contents of the settling tubes were removed by suction and four to six fractions were collected. The larger granules settled faster and were recovered lower in the tube. The smallest granules remained in the water and 30% glycerol solutions. Some small granules also settled with the larger granules, and intermediate sizes were only partially separated. To obtain relatively uniform size classes, numerous settlings through other more or less viscous glycerol-water solutions were necessary. The final fractions represent size classes and are not a quantitative recovery of all granules of a particular size but should be representative of all granules of that particular size.

**Granule Counts and Measurements**

All granules of a particular size were suspended in 10 ml. 30% glycerol and 1-

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Fig. 3. Radioautograph of freeze-dried kernel halves from plants exposed to $^{14}$CO$_2$ 14 to 41 days post-pollination. Kernels were harvested 50 hr. after $^{14}$CO$_2$ treatment. The image on the X-ray film is displaced to the right of its respective kernel section.
ml. aliquots were removed for counting. Starch samples from size B and above were suitably diluted with 1% NaCl and the number of granules was determined using an electronic particle counter (Coulter Counter, Model B, Coulter Electronics, Inc., Hialeah, Fla.). The number of size A granules was determined using a hemacytometer. Representative counts of larger granules with the hemacytometer were similar to those obtained with the Coulter Counter.

Starch granules on the slides prepared for determination of the percentage of radioactive granules in study 2 were used for granule diameter determinations. Photomicrographs of random areas of the slides were made, and the photographic negatives were projected on graph paper with a Kodak enlarger. The edge of each granule was marked at four places and the average diameter of the projected image was measured. A photomicrograph of a stage micrometer was also projected on the graph paper and this was used to convert the granule diameter to microns. Almost all granules smaller than size D appeared spherical. Some of the larger granules in kernels 30 and 41 days post-pollination were more angular in shape, and it was more difficult to obtain accurate measurements of average diameter. Granule surface area was calculated assuming that the granules were spheres.

**Determination of Starch and Carbon-14 Contents**

Starch granules in the 9 ml. of suspension left after removal of the aliquot for counting were pelleted by centrifugation for 5 min. at 1,000 × g. The granules were washed three times with water by suspension and centrifugation to remove the glycerol and were then solubilized in 8 ml. of 90% dimethyl sulfoxide (DMSO) 2 hr. at 70°C. The solubilized starch was adjusted to 10 ml. with DMSO and 2 ml. was added to a 4 × 1 cm. piece of Whatman Fiberglas paper, GF-82. The Fiberglas papers were dried under heat lamps and the amounts of radioactivity determined with a liquid scintillation spectrometer as previously described (4). Starch concentrations in the fractions were determined by measuring the reducing sugar content following complete hydrolysis by glucoamylase (9).

**Macroradioautography of Kernel Halves**

To determine the location of radioactivity or regions lacking radioactivity in different age kernels 50 hr. after 14CO2 treatment, 16 freeze-dried kernels from each age were cut in half along the central axis perpendicular to the face of the kernels. One-half of each kernel was mounted with rubber cement to a sheet of foam plastic. Kodak Royal Blue X-ray film was placed over the mounts and left for a 24-hr. exposure prior to development of the film by standard procedures (4).

**Microradioautography of Starch Granules**

Starch granules from study 1 were collected by centrifugation (1,000 × g for 5 min.) and then were suspended in 10 ml. water. A small amount of starch granule suspension (0.02 ml.) was placed on a microscope slide and was mixed with 1 drop of gelatin adhesive (0.5 g. of chromium potassium sulfate dissolved in 1 liter of warm water containing 5 g. of gelatin). The suspension was well mixed and evenly spread over the slide and dried on a 40°C. slide warmer. Three sets of slides were prepared and coated with Kodak nuclear track emulsion, type NTB-
2. Slides were coated by dipping them in the liquefied emulsion (held in a 40°C water bath) and then allowing them to dry at room temperature. The coated slides were placed in a plastic slide box and an envelope containing silica gel desiccant was added to each box. The boxes were taped light-tight and left in the dark for exposures of 1, 2, or 5 months. All photographic operations were carried out in total darkness or in the light of a Kodak safelight filter, Wratten series two. The microradioautographs were developed 2 min. in Kodak Dektol developer diluted 1:1 with water, followed by a 10-sec. dip in distilled water, a 5-min. fixation in Kodak fixing bath F-5, and a 5-min. wash in distilled water. All solutions were held at 15°C.

The diameters ±1 μ of individual starch granules from study 1 were measured with the aid of a camera lucida, and the silver grain density was scored between 1 and 5. A score of 1 was given granules having no detectable silver grains, and 5 was assigned granules surrounded by very heavy silver grain density.

The procedure for microradioautography of starch granules from study 2 was the same as that used in study 1 except that the microradioautographs were fixed 2 min. in Kodak fixer rather than 5 min. in F-5 fixer. In study 2 the percentage of granules which were sufficiently radioactive to produce silver grains was determined.

RESULTS

Based on numerous personal microscopic observations of developing normal dent corn endosperm, starch granules in a particular cell appear approximately the same size. Thus, it is likely that the amyloplasts in a cell begin accumulating starch at essentially the same time and that they all increase in size at a similar rate. These observations are in agreement with those of Lampe (3). In the lower endosperm of a developing kernel, the granules, if present, are quite tiny and closely surround the nucleus (3 and Fig. 1b and 1b'). Most of the cells in the kernel section pictured in Fig. 1 below the point of enlargement b had no amyloplasts with detectable birefringent starch granules. The cells in enlargements 1d, 1e, and 1f are filled with much larger granules. The gradient in granule size can be seen in the various cells pictured in enlargements 1b and 1c. In each case, however, granules in a given cell are similar in size. The peripheral endosperm cells are the last to be formed (1) and their starch granules remain relatively small even at maturity (10). Therefore, the smaller starch granules isolated from an immature corn kernel should have come from young peripheral cells formed late in development or from physiologically younger cells located in the lower endosperm.

Microradioautography of Starch Granules—Study 1

Smaller granules had a much higher percentage of granules with little or no detectable radioactivity (Fig. 2) while more of the intermediate and larger sizes were surrounded with a medium to high silver grain density. Thus, from this study it appeared that the larger granules had indeed accumulated more 14C starch.

14C Content of Different Size Granules—Study 2

To make a more quantitative test and to determine if the above relationship
holds for kernels of different ages, the \(^{14}\text{C}\) content of different size granules was determined. Starch granules were separated into six size classes varying in size from an average of 0.025 \(\gamma\) per granule in size A to 2.0 \(\gamma\) per granule in size F (Table I). The largest granule size class isolated from 14-day-old kernels averaged less than 0.5 \(\gamma\) per granule. Over half of all granules from kernels 14 to 30 days post-pollination contained sufficient radioactivity to be detected by microradiography (Table II). Over 90\% of size C and D granules from 14- and size E from 18-day-old kernels were radioactive (Table II). The size A granules generally had fewer granules with detectable quantities of \(^{14}\text{C}\), and the silver grain density was noticeably less intense than that of larger granules. The increasing amounts of \(^{14}\text{C}\) per granule with increasing size was confirmed following solubilization of the different size granules and quantitative measurement of \(^{14}\text{C}\) content (Table II). Size A granules from all age kernels contained only \(4.5 \times 10^{-4}\) d.p.m. per radioactive granule, and there was a progressive increase in \(^{14}\text{C}\) per granule with increasing size. The greatest increase was between size A and size B. Also size A granules contained less than half as much \(^{14}\text{C}\) per unit area of radioactive granule surface as the other sizes (Table II). The average \(^{14}\text{C}\) per granule surface was similar for sizes B through F.

The cells in the central crown region of 41-day-old kernels were maturing at the time of \(^{14}\text{CO}_2\) exposure of the plant. Therefore, these cells did not accumulate large amounts of \(^{14}\text{C}\) (Fig. 3) and nonradioactive starch from these cells probably accounts for the lower percentage of radioactive granules in older kernels (Table II). The largest starch granules are produced by cells in this central portion of the endosperm (10). Therefore, in the 41-day-old kernels the lower percentage of radioactive granules in the largest granule size class indicates that a higher proportion of these came from mature cells in the central endosperm.
A developing corn kernel is composed of cells of varying physiological ages (1,2,3 and Fig. 1). In general, the physiologically younger cells contain smaller starch granules (Fig. 1b and 1c). An in vivo study of $^{14}$C incorporation into starch granules of different sizes was made to determine whether starch was being synthesized and added to small granules as rapidly as to larger granules. The results of this study clearly demonstrate that smaller granules contain less $^{14}$C per radioactive granule or per granule surface area (Table II and Fig. 2). Possible explanations of these results are: a) the small granules were from cells which received the $^{14}$C sugar last; b) starch synthesis and deposition per granule onto or into small granules were limited by surface area of the granules; c) the small granules were isolated from physiologically younger cells which had not yet developed their maximum starch biosynthetic potential; or d) the small granules were initiated after $^{14}$CO$_2$ exposure of the plant and only shortly before sampling.

The smallest granules in immature and mature (10) kernels are located in the basal endosperm cells (Fig. 1b and 1c) and in the small peripheral cells adjacent to the aleurone layer (Fig. 1e, at arrow). In developing corn kernels all sugars enter the endosperm through the placento-chalazal zone and then diffuse throughout the endosperm (4). The $^{14}$C reaches the top of the kernel by 6 hr, after exposure of the plant to $^{14}$CO$_2$ (4) and should be in all parts of the metabolically active endosperm within 6 hr. or shortly thereafter. Since many of the cells containing the small granules are located in the basal endosperm near the site of sugar entry, these cells should have contacted the $^{14}$C sugar first. Thus, there is
little or no support for the first explanation, i.e., that the $^{14}$C sugars reached cells containing small granules later.

Geddes and Greenwood (11) suggested that starch granules are surrounded by a protein layer containing the starch-synthesizing enzymes and that starch synthesis occurs at the granule surface. According to their theory the newly synthesized starch would be immediately hydrogen-bonded to the granule. If such a mechanism were operative, the smaller granules would be expected to contain less $^{14}$C per granule due to the reduced surface area, but the amount of $^{14}$C per unit area of surface should be the same as for larger granules. Similarly, Badenuizen (12) suggested that starch polysaccharides are synthesized in the matrix of the amyloplast, followed by crystallization of the completed molecules onto or into the starch granule. The molecular distribution of radioactivity in starch following $^{14}$CO$_2$ exposure of intact plants (13) supported Badenuizen's suggestion. If amylose and amlopectin are completely synthesized in the amyloplast matrix and then added to the enlarging granule, one might suspect that the physical limitations of surface area might affect the rate of starch synthesis and deposition. In this study, there was an increase in $^{14}$C per granule with increasing size in all classes (Table II) but when expressed as $^{14}$C per unit surface area, the average differences between sizes B through F were relatively small. Thus, available surface for starch deposition may be an important factor in controlling the amount of $^{14}$C incorporation into granules of sizes B and larger.

Although limiting granule surface area may be regulating the rate of $^{14}$C incorporation into starch granules of size B and larger, this does not account for the difference between size A granules and the rest. Size A (smallest) granules, on the average, contained only half as much $^{14}$C per area of granule surface as the larger sizes (Table II). Thus it appears that limited surface area is not the primary factor responsible for the differences in $^{14}$C content of size A granules compared to the other sizes. A possible explanation for this difference is that the small granules were isolated from physiologically younger cells which had not yet developed their maximum starch biosynthetic potential. It is known that lower endosperm cells begin starch synthesis late in kernel development (3,4 and Fig. I) and that peripheral endosperm cells (which also contain small granules) are the last to be formed (1) with cell division being completed about 28 days after pollination (14). As pointed out in the introduction, Tsai et al. (5) showed that in endosperm from kernels sampled prior to 12 days post-pollination, activities of sucrose-UDP glycosyltransferase, ADP-glucose pyrophosphorylase, soluble ADP-glucose-starch glucosyltransferase, and phosphorylases II and III were not detectable. Ozburn et al. (6) measured enzyme activity from whole corn kernels and reported that although activities of ADP-glucose pyrophosphorylase and starch synthetase were low in kernels 8 to 12 days post-pollination, there was sufficient activity to account for the kernel starch content. Both groups (5,6) reported that during kernel development marked increases in the activities of enzymes implicated in starch synthesis (soluble ADP-glucose-starch glucosyltransferase, ADP-glucose pyrophosphorylase, and phosphorylase) occurred. Tsai et al. (5) suggested that starch in very young kernels (8 to 12 days post-pollination) was synthesized from UDP-glucose by granule bound UDP-glucose-starch glucosyltransferase and that at about 12 days post-pollination there was a switch to starch synthesis from the substrates ADP-glucose by the granule bound or soluble ADP-glucose-starch glucosyltransferase and/or from
glucose-1-P by phosphorylases II and III. It can be reasoned that the physiologically younger cells of developing kernels may also contain enzymes similar to those reported in kernels 8 to 12 days after pollination. Starch in such cells possibly was being synthesized from UDP-glucose by the less efficient granule-bound UDP-glucose-starch glucosyltransferase. With increasing physiological age of the cell, a switch to starch synthesis from ADP-glucose and/or glucose-1-P may have occurred. However, it is equally possible that cells containing the size A granules had the same polymerizing enzymes, i.e., the nucleoside diphosphate glucose-starch glucosyltransferase and phosphorylase II and III, as more advanced cells. In this case, the lower amount of $^{14}$C incorporated per unit of surface area of size A granules may have been due to the quantities of substrates and/or cofactors being insufficient for maximum synthesis. Since the $^{14}$C content per unit of surface area is similar for all granules of size B and larger, it seems most likely that the rate of starch synthesis and deposition in the more advanced cells is limited by the surface area for starch deposition. Although enzyme activity does not appear to be limiting the rate of starch formation in such cells, one cannot rule out the possibility of there being an increased number of starch biosynthetic enzyme molecules per cell with increasing cell development.

A final explanation of why the smallest granules contained less $^{14}$C per unit surface area may be that these granules were initiated after $^{14}$CO$_2$ exposure of the plant and only shortly before sampling. Based on the results of the present study, this possibility cannot be ruled out. A double labeling study involving a pulse with tritium-labeled sugar followed by exposure of the plant to $^{14}$CO$_2$ will be necessary to establish that the smallest granules had or had not been initiated prior to $^{14}$CO$_2$ treatment.

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**Literature Cited**


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