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Starch Phosphorylation in Potato Tubers Proceeds Concurrently with de Novo Biosynthesis of Starch[1]

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The in vivo phosphorylation of starch was studied in *Solanum tuberosum* cv Dianella and Posmo. Small starch granules contain 25% more ester-bound phosphate per glucose residue than large starch granules. The degree of phosphorylation was found to be almost constant during tuber development. Isolated tuber discs synthesize starch from externally supplied glucose at a significant rate. Tuber discs supplied with glucose and [^{32}P]orthophosphate incorporate radiolabeled phosphorus into the starch. The level of ^{32}P incorporation is proportional to the amount of starch synthesized. The incorporation of ^{32}P from orthophosphate is correlated to de novo synthesis of starch, since the incorporation of ^{32}P is diminished upon inhibition of starch synthesis by fluoride. Based on the amount of [^{14}C]glucose phosphate isolated after hydrolysis of purified starch from tuber discs incubated in the presence of [U-^{14}C]glucose, approximately 0.5% of the glucose residues of the de novo-synthesized starch are phosphorylated. This value is in general agreement with the observed levels of phosphorus in starch accumulated during tuber development. Thus, the enzyme system responsible for starch phosphorylation is fully active in the isolated tuber discs, and the starch phosphorylation proceeds as an integrated part of de novo starch synthesis.

Starch is composed of the Glc polymers amylose and amylopectin. The Glc residues are linked together by $\alpha(1\rightarrow 4)$ bonds except for the branch points, which are $\alpha(1\rightarrow 6)$ linkages. Most of the branch points reside in amylopectin. Starch contains minor amounts of other components, such as lipid, protein, and phosphate (Morrison and Karkalas, 1990). These components may be tightly associated with the starch or covalently bound to the Glc residues. Starches from potatoes (*Solanum tuberosum*) and other tuber crops are characterized by a relatively high content of phosphorus in comparison to, for example, cereal starches (Rooke et al., 1949; Hizukuri et al., 1970; Tabata et al., 1975; Moorthy, 1991). The phosphorus in potato starch is present primarily as phosphate esterified to the Glc residues of the starch. The majority of the phosphate is bound in the amylopectin fraction of the starch, whereas phosphorylation of amylose is insignificant (Hizukuri et al., 1970). Approximately 60 to 70% of the phosphate groups are linked to C-6 of the Glc residues, the rest to C-3 (Hizukuri et al., 1970; Tabata and Hizukuri, 1971; Muhrbeck and Teiller, 1991). A small fraction (1%) may be linked to C-2 (Hizukuri et al., 1970). On average, 1 of every 200 to 500 Glc residues is phosphorylated. This level of phosphate affects the viscosity of gelatinized starch (Samec and Blinc, 1941; Schreiber, 1958) and is significant because of the diversified uses of starch for foods and industrial purposes. The level of phosphorylation may vary approximately 2-fold among different potato varieties and depends on the growth conditions (Schreiber, 1958; Nikuni et al., 1969; Palasinsky, 1980). Although the level of phosphorylation appears low, the starch-bound phosphate constitutes a major part of the total phosphate pool in the potato tubers (Quick and Li, 1976). Thus, the level of starch phosphorylation significantly affects the overall phosphate status of the tuber tissue.

Starches phosphorylated in vivo have been known for decades, but the biosynthetic reactions that are responsible for their formation have remained unknown. In the present study we report quantitative data on the level of phosphorylation in two different potato cultivars dependent on tuber size and starch granule size. Based on these data we have designed and optimized an in vivo system to study the phosphorylation process. Using ^{32}P and ^{14}C radioisotopes, we demonstrate that starch phosphorylation proceeds concurrently with starch de novo biosynthesis as an integrated part of starch biosynthesis.

MATERIALS AND METHODS

Plant Material

Tubers of different sizes from *Solanum tuberosum* cv Posmo were harvested at a commercial potato field in July. Tubers of *S. tuberosum* cv Dianella were obtained from greenhouse-grown plants in August. The seed tubers were placed in 10-L buckets filled with vermiculite (Skarrehage Molervæzrk A/S, Nykøbing Mors, Denmark) and watered daily with tap water. Two days a week, plants were watered with a 2 g L^{-1} full nutrient solution (Pioner Hornumblanding, Brøste, Den-

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Abbreviations: C-3-bound phosphate, phosphate esterified to carbon 3 of the Glc residues; C-6-bound phosphate, phosphate esterified to carbon 6 of the Glc residues.

mark). Plants were kept under natural daylength. Tubers were harvested, quickly rinsed in tap water, and immediately frozen in liquid nitrogen. The tubers were stored at −80°C.

Starch Granule Isolation

Frozen tuber tissue was finely divided and suspended in 4 volumes of ice-cold water. The material was homogenized (5 × 5 s, full speed) in a blender equipped with replaceable razor blades. After filtration through two layers of cheesecloth, the starch granules were washed (4 × 10 volumes of H_2O) with centrifugations between each wash (3000*g* for 10 min). The starch granules were subsequently washed three times in 10 volumes of acetone, dried overnight under a stream of atmospheric air, and stored at −20°C. The final starch preparation was essentially free of cell-wall material as monitored by light microscopy. Size fractionation of starch granules was achieved by filtration through a nylon net (mesh 30 μm) followed by repeated sedimentation by gravity of the aqueous suspensions.

Determination of Total Phosphorus in Starch

Total phosphorus content in the starch was determined according to the method of Morrison (1964). Dry starch (5 mg) was suspended in 0.3 mL of concentrated H_2SO_4 and completely charred over a gas burner. The solution was clarified by dropwise addition of H_2O_2 (30%, w/v) and gently boiled for 2 min. Water was added to a final volume of 4 mL followed by sequential addition of 0.1 mL of 33% (w/v) $Na_2SO_3 \cdot 7H_2O$, 1.0 mL of 2% (w/v) $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.01 g of ascorbic acid with stirring. The samples were transferred to a boiling water bath (10 min), cooled to 20°C, and diluted to a final volume of 5.0 mL, and the phosphate content was determined from the A_{822} using standards with a known KH_2PO_4 content.

Determination of Phosphate Esterified at the C-6 Position of the Glc Residues

Phosphate at the C-6 position was determined as Glc-6-P after acid hydrolysis of the starch. Glc-6-P was determined by the absorption change at 340 nm caused by the Glc-6-P dehydrogenase-mediated reduction of NAD^+. The procedure was carried out as follows. Starch (250 mg) was suspended in 1 mL of 0.7 N hydrochloric acid and kept at 100°C for 4 h. An aliquot (100 μL) was mixed with 800 μL of buffer containing 100 mM Mops-KOH (pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA in a cuvette and neutralized with 100 μL of 0.7 N KOH. NAD (final concentration 0.4 mM) and 2 units of Glc-6-P dehydrogenase from Leuconostoc (Sigma) was then successively added (final assay volume 1 mL). Standard curves demonstrated that the Glc-6-P dehydrogenase contained no interfering enzyme activities. Pi in the neutralized hydrolysates was determined by the colorimetric method described by Lanzetta et al. (1979).

Biosynthetic Radiolabeling Experiments Using Tuber Discs

^{32}P Labeling and Starch Synthesis Rate

Isolated tuber discs were used for ^{32}P and ^{14}C labeling of synthesized starch. Tuber discs were isolated by punching out rods with a 5-mm cork borer and slicing the material into 1.5-mm-thick discs with a razor blade. A specially developed hand-driven device for advancing and cutting the tuber rods ensured fast production of discs of constant thickness. The average weight of each disc was 50 mg.

The tuber discs were incubated for 0 to 4 h at 20°C in vials placed in a closed container, the bottom of which was covered with wet filter paper to restrict evaporation from the tuber discs. Each vial contained three discs supplied with 100 μL of a solution containing either 12 kBq of [U-^{14}C]Glc or 74 kBq of carrier-free ^{32}Pi and additional components as indicated in "Results" and adjusted to a final osmolyte concentration of 300 mM by addition of sorbitol. At the end of the incubation period, the discs were frozen, stored at −20°C, and analyzed the next day. The frozen tuber discs were homogenized in 5 mL of ice-cold water using a Dual glass homogenizer. The homogenate was kept on ice and processed within 15 min. The solid material (starch granules and cell-wall material) was successively washed at 20°C using 2 × 10 mL of H_2O, 5 mL of 1 M NaCl, 2 × 10 mL of H_2O, 2 × 5 mL of acetone, and 2 × 10 mL of H_2O. Excess water was removed by centrifugation and the starch was resuspended in 750 μL of 5 mM Mes (pH 6.4)/4 mM $CaCl_2$.

The starch was gelatinized by heating for 2 min in a boiling water bath under vigorous stirring. After cooling to 20°C, 60 units of α-amylase (Termamyl, Novo, Ballerup, Denmark) in 10 μL of the same buffer that had been preheated at 100°C for 3 min were added. After 1 h at 20°C, the resulting hydrolysate was transferred to a microfuge tube, diluted to 1.50 mL with water, and centrifuged (3000*g* for 5 min). An aliquot (500 μL) of the supernatant was mixed with 8 mL of Ecoscint A (National Diagnostics, Manville, NJ) and its radioactivity was determined by liquid scintillation counting. In the initial biosynthetic experiments where ^{32}Pi was used as radiolabel, it was observed that a small fraction of Pi was adsorbed strongly when mixed with isolated starch granules. Interference from residual Pi in the samples (700-μL aliquot) was avoided through precipitation with 75 μL of 0.4 N $Ba(OH)_2$ after addition of unlabeled carrier (10 μL of 3 N H_3PO_4). Control experiments using internal standards of ^{32}Pi demonstrated that Pi was precipitated within 3 min and could be removed subsequently by centrifugation (3000*g* for 5 min). ^{32}P in the supernatant (500-μL aliquot) was determined by liquid scintillation counting. The recovery of Glc-6-P was above 80% as determined by addition of authentic Glc-6-P to the starch sample before gelatinization and subsequent spectrophotometric quantitation. Vials that had been incubated with [U-^{14}C]Glc were analyzed to estimate starch synthesis from supplied Glc.

HPLC Analysis of Radiolabeled Starch

Radiolabeled starch was analyzed by HPLC to identify the structure of the phosphorylated compounds. Starch samples derived form tuber discs incubated with radioactive Pi (as above) were degraded to monomers by acid hydrolysis (0.7 N hydrochloric acid, 100°C, 4 h). The acid was removed by repeated co-evaporation with toluene. The remaining sample was neutralized with KOH and applied to a 2-mL column of DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden). All

radioactivity was retained by the column. After a wash with 6 mL of H_2O, the radioactivity was eluted with 4 mL of 0.1 N hydrochloric acid. After removal of the acid by co-evaporation with toluene, the dry sample was redissolved in water and used for HPLC analysis. The analysis was performed on a Dionex 4500i chromatographic system consisting of a GMP-2 pump and a pulsed electrochemical detector used in pulse amperometric detection mode. The CarboPac PA1 column (4 × 250 mm) (Dionex) was eluted isocratically with 100 mM NaOH/180 mM sodium acetate (flow rate, 1 mL min^{-1}, 20°C). Fractions of 1 mL were collected. To each fraction was added 5 mL of Ultima Gold (Packard), and the samples were counted on a 1214 Rackbeta liquid scintillation counter (LKB Wallac). Total radioactivity of the sample was determined by counting the volume of sample loaded on the column (5 μL) in the presence of 1 mL of eluent. Standards of Fru-6-P, Glc-1-P, Glc-6-P, Rib-5-P, and deoxy-Rib-5-P were all purchased from Sigma.

Incorporation of [^{14}C]Glc into Phosphorylated Glc Residues during de Novo Synthesis of Starch

The tuber discs were supplied with 100 μL of a solution containing 0.37 MBq [U-^{14}C]Glc (0.2 mM) and 300 mM sorbitol. To achieve sufficient incorporation for subsequent analyses, the experiments were performed without the inclusion of unlabeled Glc. Starch extraction and enzymic hydrolysis were performed as described above. After α-amylase treatment, pelleting of debris, and addition of concentrated hydrochloric acid to 1.2 mL of the supernatant (final concentration of 1.0 M), the samples were incubated in a boiling water bath for 4 h. Colored degradation products were removed by adding 15 mg of activated charcoal suspended in 100 μL of H_2O, allowing the sample to stand for 10 min, and then centrifuging (0.5 min at 20,000*g*). Acetic acid (30 μL, 1 M) was added to 1.2 mL of the supernatant and the sample was neutralized with 0.4 N $Ba(OH)_2$ (color indicator). The sample was diluted to 6 mL with H_2O, Glc-6-P (100 μL, 0.4 M) was added as a carrier, and Glc-6-P was quantitatively precipitated by adding absolute ethanol to a final volume of 50 mL and allowing it to stand for 15 min. The radioactivity of the supernatant containing the unphosphorylated Glc residues was determined by liquid scintillation counting. The Glc-6-P-containing pellet was dissolved and reprecipitated thrice. In each cycle, the pellet was dissolved in 6 mL of 0.1 M barium acetate/0.23 M Glc and precipitated by addition of ethanol to a final volume of 50 mL. The final pellet was resuspended in 2 mL of H_2O and its radioactivity was measured.

To determine the recovery of Glc-6-P, unlabeled tuber discs were treated in the same way and [U-^{14}C]Glc-6-P was added immediately before gelatinization of the starch sample. The recovery of Glc-6-P was close to 80% during enzymic and acid hydrolysis, and likewise close to 80% during the repeated precipitations. Corresponding experiments with [U-^{14}C]Glc demonstrated the removal of more than 99.8% of the tracer.

As an additional test to be certain that the label in the pellet sample represented phosphorylated sugars, samples were treated for 30 min with 8 units of alkaline phosphatase (*Escherichia coli*, type III-S, Sigma) after addition of 1 mL of 50 mM Gly/KOH (pH 9.5) to the acid hydrolysate and adjustment to pH 9.5. No Glc-6-P was detectable in the sample after this treatment. The alkaline phosphatase was obtained as an $(NH_4)_2SO_4$ suspension. Prior to use, it was precipitated by centrifugation and redissolved in 0.1 M Gly/KOH (pH 9.5). When mixed with the starch hydrolysate, residual sulfate formed a precipitate with barium ions that was removed by centrifugation (3000*g*, 5 min).

RESULTS

Two commercially important potato cultivars were selected for the experiments. Dianella was chosen because this cultivar has been used in a large number of studies and serves as a useful reference. Posmo was chosen as a high-yielding cultivar of industrial importance. During the initial characterization of the experimental material, the dependence of starch phosphorylation on tuber development was studied. Phosphorus located at the C-6 position of the Glc residue was determined as the Glc-6-P formed by acid hydrolysis of purified starch. During acid hydrolysis the liberation of Glc-6-P proceeds in a time-dependent manner and reaches a maximum after 4 h (data not shown). Glc-6-P represented about two-thirds of the total phosphate. The rest was present as free Pi in the acid hydrolysates (Table I). Internal standards of Glc-6-P revealed more than 85% recovery during the acid hydrolysis. Thus, some of the free phosphate is derived from the hydrolysis of C-6-bound phosphate, but most of the free phosphate probably arose from hydrolysis of C-3-bound phosphate, which is more acid labile than C-6-bound phosphate (Tabata and Hizukuri, 1971). Compared with Posmo, Dianella showed slightly elevated levels of total phosphate and correspondingly elevated amounts of Glc-6-P after acid hydrolysis (Table I). For both Dianella and Posmo, the starch samples from tubers of different sizes contained approximately the same amount of total phosphate and phosphate esterified at the C-6 position (Table I). This shows that the

Table I. *Total phosphate content in potato tuber starch and Glc-6-P and Pi produced by 4-h acid hydrolysis of starch*

Starch was isolated from Dianella and Posmo tubers of different sizes harvested during tuber development. The results are means ± SE of four determinations.

Tuber Diameter	Total Phosphorus	Glc-6-P	Pi
cm	*nmol (mg starch)$^{-1}$*		
Dianella			
<1	16.5 ± 0.5	11.9 ± 0.4	6.1 ± 0.2
1.5–2	19.4 ± 0.8	14.2 ± 0.1	6.7 ± 0.3
3	20.3 ± 0.5	14.4 ± 0.3	6.9 ± 0.2
5	19.5 ± 0.7	14.2 ± 0.2	6.0 ± 0.2
6	21.6 ± 0.3	15.3 ± 0.2	7.2 ± 0.1
Posmo			
<1	14.4 ± 0.3	10.3 ± 0.2	4.9 ± 0.1
1.5–2	14.1 ± 0.8	9.8 ± 0.6	5.0 ± 0.1
3	16.9 ± 0.5	11.5 ± 0.2	5.5 ± 0.4
4	16.1 ± 0.4	11.2 ± 0.2	5.4 ± 0.1
6	15.9 ± 0.1	11.3 ± 0.1	4.9 ± 0.2

degree of phosphorylation is nearly constant during tuber development. Except for the higher level of phosphorylation in Dianella, the two cultivars show great similarity. Subsequent experiments were carried out using only tubers of Dianella.

The degree of phosphorylation of starch in tubers with an average diameter of 5 cm (a new batch of tubers) was analyzed for total phosphorus and C-6-bound phosphate in relation to starch granule size (Table II). Small starch granules contained 25% more ester-bound phosphate per Glc residue than large starch granules, indicating a slight decrease in the efficiency of phosphorylation in the course of starch granule development. The small starch granules represented about one-fifth of the unfractionated starch.

The constant rate of phosphorylation during tuber development makes any tuber size suitable for studies of the phosphorylation process. Tubers with diameters between 4 and 5 cm were used because these are conveniently obtained and large enough to provide several uniform tissue slices. In these tubers the C-6-bound phosphate constituted about 70% of the total bound phosphorus (Tables I and II). In Dianella the phosphate content corresponded to a phosphorylation of 0.3% of the Glc residues.

Isolated Dianella tuber discs were incubated with 300 mM Glc in the presence of either [U-^{14}C]Glc or carrier-free ^{32}Pi. The synthesis of starch from exogenous Glc was estimated from the incorporation of ^{14}C label into the insoluble fraction, which upon gelatinization was sensitive to degradation by α-amylase. A fairly high rate of starch synthesis from supplied Glc was observed during the entire 4-h incubation period (Fig. 1). Incorporation into the starch fraction of covalently bound ^{32}P followed a similar pattern and appears to be closely correlated to the amount of starch synthesized from the supplied Glc (Fig. 2).

The incorporated radiolabeled phosphate was qualitatively analyzed by HPLC analysis of the isolated phosphorylated monomers. The radiolabeled starch was decomposed to monomers by acid hydrolysis, and the phosphorylated units were isolated from the abundant free Glc units by an ion-exchange step. Essentially all radioactivity bound to the starch fraction was recovered after this procedure. During HPLC analysis, all radioactivity that eluted from the column was proven to correspond to Glc-6-P (Fig. 3). About 20% of the radioactivity was retained by the column. This radioactivity corresponds to free Pi, which is also retained by the column over the 40-min elution period.

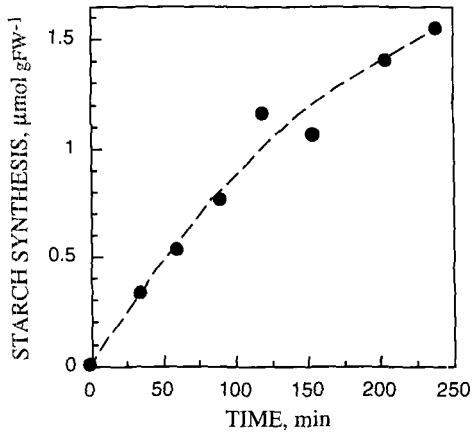

Figure 1. Starch synthesis from supplied [^{14}C]Glc by isolated tuber discs during incubation in 300 mM Glc solution. Each point represents one sample of three tuber discs (approximately 150 mg of tissue) supplied with 100 μL of 300 mM Glc. FW, Fresh weight.

Fluoride inhibits starch synthesis in potato tuber tissue (Viola and Davies, 1991). Using the tuber disc system, fluoride was found to inhibit starch synthesis from added [^{14}C]-Glc as well as the incorporation of ^{32}P into starch (Fig. 4). The inhibition was progressive with increasing fluoride concentration. This strongly indicates that the phosphorylation process is linked to de novo biosynthesis of starch.

From the experiments described above, it cannot be unambiguously concluded that the ^{32}P-labeled Glc moieties were those incorporated into the starch during the incubation

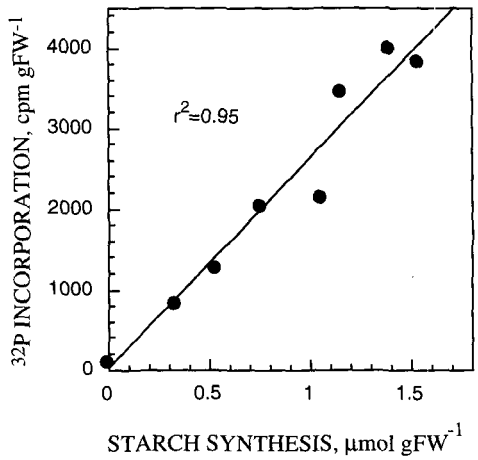

Figure 2. ^{32}P incorporation into the starch fraction and starch synthesis from supplied Glc by isolated tuber discs incubated in 300 mM Glc. The different data points were generated by increasing the length of incubation (see Fig. 1). Sets of three tuber discs were produced pairwise from proximate tuber tissue. Three discs were used for the estimation of starch synthesis, and the other three discs were used for ^{32}P labeling. The curve represents a linear regression with the indicated correlation coefficient. FW, Fresh weight.

Table II. *Total phosphate content and Glc-6-P produced by 4-h acid hydrolysis of starch isolated from Dianella tubers with an average diameter of 5 cm*

The starch preparations were divided into fractions containing small and large starch granules by differential sedimentation followed by filtration (filter mesh 30 μm). The results are means ± SE of five determinations. nd, Not determined.

	Average Granule Diameter	Total Phosphorus	Glc-6-P
	μm	nmol (mg starch)$^{-1}$	
All starch	nd	17.3 ± 2.3	12.6 ± 0.8
Small granules	20	nd	15.5 ± 0.2
Large granules	60	nd	11.6 ± 0.7

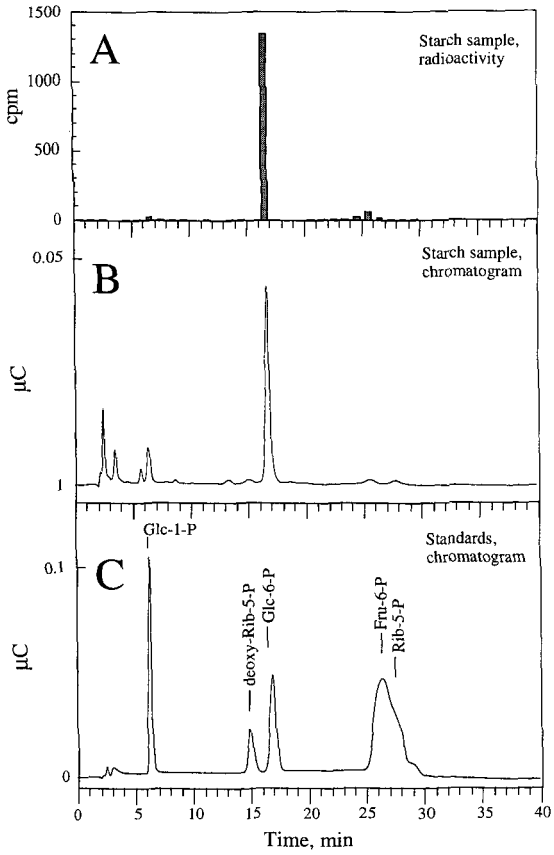

Figure 3. HPLC analysis of isolated phosphorylated monomers derived from starch synthesized in the presence of radiolabeled Pi (as in Fig. 2). A, Radioactivity in fractions collected when running a starch-derived sample. B, Chromatogram for starch-derived sample. C, Chromatogram for a mixture of standards of hexose phosphates. The unit μC in B and C indicates the electrical output from the HPLC detection system.

period. Therefore, an experiment was designed to quantitate the degree of phosphorylation of de novo-synthesized starch. This was done by determining the amount of ^{14}C-labeled Glc-6-P liberated by acid hydrolysis of starch formed in biosynthetic experiments with [^{14}C]Glc. During the experimental procedure, thoroughly washed starch preparations including cell-wall fragments were gelatinized and hydrolyzed by α-amylase. The cell-wall components remained insoluble. The soluble oligosaccharides generated by the enzyme treatment were degraded to monosaccharides by acid hydrolysis and the phosphorylated Glc units were selectively precipitated by addition of $Ba(OH)_2$ and ethanol.

The recovery of radioactivity was 60 to 70% when authentic [U-^{14}C]Glc-6-P was added to unlabeled starch samples and subjected to enzymic and acid hydrolysis and repeatedly precipitated (Fig. 5). In contrast, [U-^{14}C]Glc added during gelatinization of the starch preparation was efficiently removed, and only 0.1 to 0.2% of the radioactivity was retained in the final pellet. On average, about 0.4% of the label in the starch fraction from discs incubated with [U-^{14}C]Glc was recovered in the precipitate (Fig. 5). When corrected for the

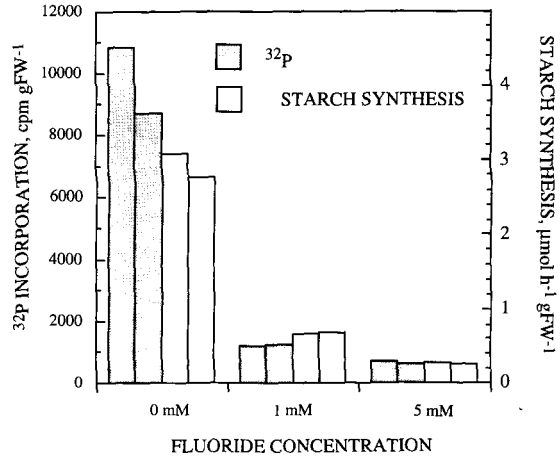

Figure 4. Effect of fluoride concentration in incubation solution on starch synthesis from supplied Glc (□) and ^{32}P incorporation into the starch fraction (□) in isolated tuber discs incubated in 100 μL of 300 mM Glc for 4 h. Each column represents one set of three discs.

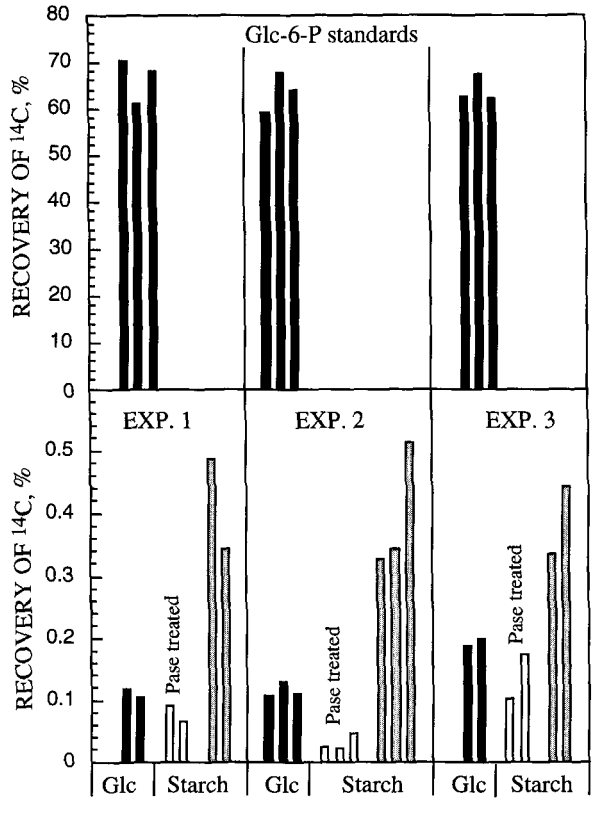

Figure 5. Recovery of radioactivity after enzymic and acid hydrolysis of a radiolabeled starch sample followed by repeated coprecipitation with unlabeled Glc-6-P. Upper panels, Recovery of an internal standard of [U-^{14}C]Glc-6-P. Lower panels, Recovery of [U-^{14}C]Glc standard (■); label in starch synthesized by tuber discs from [U-^{14}C]Glc and treated with alkaline phosphatase upon acid hydrolysis (□); and similar starch samples but not treated with alkaline phosphatase (□).

recovery of Glc-6-P and unspecific contaminating radiolabel (radiolabel in the phosphatase-treated samples), it was estimated that about 0.5% of the Glc residues of the de novo-synthesized starch were phosphorylated. Since each experiment represents tissue material from only one or two tubers, data from three separate experiments are shown in Figure 5. The method is reproducible and individual tubers yielded similar results. To verify independently that the label resides in phosphorylated units, some samples were treated with alkaline phosphatase after acid hydrolysis. This treatment abolishes the recovery of radiolabel in the final precipitate (Fig. 5). The remaining radioactivity was even lower than observed from samples to which [^{14}C]Glc was added. This difference in background radioactivity is partially caused by impurities in the [^{14}C]Glc as demonstrated by passing the [^{14}C]Glc through an anion-exchange resin. Phosphatase-treated samples, rather than the Glc standards, were therefore considered to represent "blank" values.

DISCUSSION

The present study of starch phosphorylation is based on the use of isolated potato tuber discs. Tuber discs have previously been reported to perform starch synthesis at high rates, which can be modulated by inclusion of osmotics (Oparka and Wright, 1988) or inhibitors, e.g. fluoride (Viola and Davies, 1991). Consequently, we have optimized this experimental system and used it to mimic in vivo starch synthesis in potato tubers.

The phosphorylation of potato starch proceeds during de novo starch synthesis as demonstrated here by (a) the similar incorporation patterns observed with [U-^{14}C]Glc and ^{32}Pi, (b) the formation of [^{14}C]Glc-P moieties in starch from [U-^{14}C]-Glc, and (c) the inhibition of ^{32}Pi incorporation in the presence of fluoride.

The biosynthetic data exclude the possibility of starch being phosphorylated primarily after completion of the biosynthesis of the carbohydrate chains. Likewise, the nearly constant levels of phosphorylation during tuber development (Table I) substantiates the notion that the phosphorylation process constitutes an integrated part of starch biosynthesis. The higher level of phosphorylation in small starch granules compared with large granules agrees with results presented by Jane and Shen (1993) and might reflect minor changes in substrate levels or enzyme accessibility during granule development.

The isolation of starch involved extensive washing of the starch granules in water, salt solution, and organic solvent. This essentially removed any detectable amount of free Pi from the starch (data not shown). The starch granules of the insoluble material were gelatinized and treated with an α-amylase to specifically degrade starch into water-soluble oligomers. Any solubilized radioactivity should therefore be derived from starch, as was shown by the HPLC analysis (Fig. 3). The contribution from C-3-bound phosphate was not specifically estimated. Standards of Glc-3-P are not commercially available, precluding testing of the stability of phosphate esters coupled to C-3. However, the procedure used is rather gentle toward Glc-6-P, and since Glc-3-P is not excessively labile during enzyme treatment (Tabata et al., 1975; Takeda et al., 1983), the C-3–phosphate bonds were most likely retained in our amylase-degraded starch preparations. Isolated monomers proved to be 80% Glc-6-P. The remaining 20% could be free phosphate derived from C-3 phosphate groups during the acid hydrolysis.

When [U-^{14}C]Glc was supplied to the tuber discs, a starch-derived labeled fraction was obtained that, after acid hydrolysis and neutralization with $Ba(OH)_2$, co-precipitated with Glc-6-P in 88% ethanol (Fig. 5). Since treatment with alkaline phosphatase prevented co-precipitation, the precipitated compound must be a starch-derived phosphate ester. We take this as strong evidence that the isolated tuber discs are indeed synthesizing phosphorylated starch. Glc-3-P would be expected to co-precipitate with Glc-6-P. However, the data (Table I) indicating that the phosphate groups were cleaved from the C-3 position during acid treatment and HPLC analysis (Fig. 3) support this idea, since about 80% of the ^{32}P label was found in Glc-6-P. Therefore, the recovered radioactivity represents phosphate esterified at the C-6 position. Tabata et al. (1975) report that significant amounts of maltose phosphate is produced by acid hydrolysis. If maltose phosphate is produced and recovered under our experimental conditions, the degree of phosphorylation will be overestimated, since two Glc residues are isolated for each phosphate. About 0.4% of the total radiolabel was recovered in the hydrolyzed starch fraction after repeated precipitation, which is estimated to equal a degree of C-6 phosphorylation of 0.5%. Potentially all C-3 phosphate groups are lost during acid hydrolysis, and the estimated total phosphorylation degree is then estimated to be 0.7%. Considering that the ^{14}C-labeling system operates with very low levels of exogenous Glc, this estimate is reasonably close to the value of 0.3% calculated from the measured total phosphate content of in vivo-synthesized starch (Table I), and this demonstrates the physiological relevance of the experimental system. Most likely, the presence of endogenous sugars, which decrease only slightly during the incubation period (data not shown), ensures that starch metabolism does not change dramatically at these low levels of exogenous Glc.

In conclusion, we have demonstrated that the introduction of covalently bound phosphate in potato starch proceeds concurrently with the de novo synthesis of the polysaccharide chains. To limit the hazard of measuring quantitatively important side reactions that could also contribute to starch phosphorylation, we consider it essential to have elucidated the main characteristics of the starch phosphorylation process in an in vivo system. For future, more detailed enzymological studies of the mechanism of the phosphorylation process, the availability of an in vitro system would be advantageous. Isolated intact amyloplasts could be a valuable experimental system, but a reliable method for preparation of amyloplasts from potato is not available at present (Fishwick and Wright, 1980; Mohabir and John, 1988). Currently, we are working on this approach.

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LITERATURE CITED

Fishwick MJ, Wright AJ (1980) Isolation and characterization of amyloplast envelope membranes from *Solanum tuberosum*. Phytochemistry **19:** 55–59

Hizukuri S, Tabata S, Nikuni Z (1970) Studies on starch phosphate. Part 1. Estimation of glucose-6-phosphate residues in starch and the presence of other bound phosphate(s). Staerke **22:** 338–343

Jane J-L, Shen JJ (1993) Internal structure of the potato starch granule revealed by chemical gelatinization. Carbohydr Res **247:** 279–290

Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA (1979) An improved assay for nanomole amounts of inorganic phosphate. Anal Biochem **100:** 95–97

Mohabir G, John P (1988) Effect of temperature on starch synthesis in potato tuber tissue and in amyloplasts. Plant Physiol **88:** 1222–1228

Moorthy SN (1991) Extraction of starches from tuber crops using ammonia. Carbohydr Polym **16:** 391–398

Morrison WR (1964) A fast, simple and reliable method for the microdetermination of phosphorus in biological materials. Anal Biochem **7:** 218–224

Morrison WR, Karkalas J (1990) Starch. *In* PM Dey, ed, Methods in Plant Biochemistry, Vol 2. Carbohydrates. Academic Press, London, pp 323–352

Muhrbeck P, Teiller C (1991) Determination of the phosphorylation of starch from native potato varieties by ^{31}P NMR. Staerke **43:** 25–27

Nikuni Z, Hizukuri S, Kamagai K, Hasegawa H, Moriwaki T, Fukui T, Doi K, Nara S, Maeda I (1969) The effect of temperature during maturation period on the physico-chemical properties of potato and rice starches. Memoirs of the Scientific and Industrial Research Osaka University **26:** 1–27

Oparka KJ, Wright KM (1988) Osmotic regulation of starch synthesis in potato tubers. Planta **174:** 123–126

Palasinski M (1980) Uber die phosphorseure der kartoffelstärke. Staerke **32:** 405–408

Quick WA, Li PL (1976) Phosphate balance in potato tubers. Potato Res **19:** 305–312

Rooke HS, Lampitt LH, Jackson EM (1949) The phosphorus compounds of wheat starch. Biochem J **45:** 231–236

Samec M, Blinc M (1941) Die Neuere Entwicklung der Kolloidchemie der Stärke. Verlag von Theodor Steinkopff, Dresden

Schreiber K (1958) Chemie und Biochemie unter besonderer Berücksichtigung qualitätbestimmender Faktoren. *In* R Schick, M Klinkowski, eds, Die Kartoffel, Ein Handbuch. VED Deutscher Landvirtschaftsverlag, Berlin, pp 193–352

Tabata S, Hizukuri S (1971) Studies on starch phosphate. Part 2. Isolation of glucose 3-phosphate and maltose phosphate by acid hydrolysis of potato starch. Staerke **23:** 267–272

Tabata S, Nagata K, Hizukuri S (1975) Studies on starch phosphates. Part 3. On the esterified phosphates in some cereal starches. Staerke **27:** 333–335

Takeda Y, Hizukuri S, Ozone Y, Suetake M (1983) Action of porcine pancreatic and *Bacillus subtilis* α-amylase and *Aspergillus niger* glucoamylase on phosphorylated (1→4)-α-D-glucan. Biochim Biophys Acta **749:** 302–311

Viola R, Davies HV (1991) Fluoride-induced inhibition of starch biosynthesis in developing potato, *Solanum tuberosum* L., tubers is associated with pyrophosphate accumulation. Plant Physiol **97:** 638–643