Fluoride-Induced Inhibition of Starch Biosynthesis in Developing Potato, *Solanum tuberosum* L., Tubers Is Associated with Pyrophosphate Accumulation

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**ABSTRACT**

Pretreatment of discs excised from developing tubers of potato (*Solanum tuberosum* L.) with 10 millimolar sodium fluoride induced a transient increase in 3-phosphoglycerate content. This was followed by increases in triose-phosphate, fructose 1,6-bisphosphate and hexose-phosphate (glucose 6-phosphate + fructose 6-phosphate + glucose 1-phosphate). The effect of fluoride is attributed to an inhibition of glycolysis and a stimulation of triose-phosphate recycling (the latter confirmed by the pattern of ^13^C-labeling [NMR] in sucrose when tissue was supplied with [2-^13^C]glucose). Fluoride inhibited the incorporation of [U-^14^C]glucose, [U-^14^C]sucrose, [U-^14^C]glucose 1-phosphate, and [U-^14^C]glycerol into starch. The incorporation of [U-^14^C]ADPglucose was unaffected. Inhibition of starch biosynthesis was accompanied by an almost proportional increase in the incorporation of ^14^C into sucrose. The inhibition of starch synthesis was accompanied by a 10-fold increase in tissue pyrophosphate (PPI) content. Although the subcellular localization of PPI was not determined, a hypothesis is presented that argues that the PPI accumulates in the amyloplast due to inhibition of alkaline inorganic pyrophosphatase by fluoride ions.

The exact metabolic pathway involved in the conversion of sucrose into starch in potato tubers is not fully understood, despite substantial progress in this area in recent years. In particular, using different technical approaches, Hatzfeld and Stitt (10) and Viola et al. (29) have provided independent evidence that carbon flow into the potato amyloplast does not occur in the form of three carbon molecules but, most likely, as hexose-P. Evidence for a similar pathway has been provided for other nonphotosynthetically active starch storing organs (12). However, the mechanisms that regulate carbon partitioning between starch and other products in these tissues are still not known. One important mechanism could be the control of PPI concentration in the cytosol. Although PPI is generated in several biosynthetic pathways, including starch formation, its concentration in plant cells is usually low due to the presence of inorganic PPases (13). It has been hypothesized that hydrolysis of the PPI produced is required to drive fluxes in the synthetic direction (13). Furthermore, it has been proposed that soluble PPases are restricted to the plastidic compartment in plant cells, allowing cytosolic PPI to be used as an energy donor (30). Indeed, in cells actively synthesizing starch, PPI is required to sustain sucrose conversion into starch precursors in the cytosol. This is believed to occur via the PPI-dependent sucrose synthase pathway described by several authors (11, 32, see also Fig. 1). No unequivocal evidence has been presented as to how PPI is produced in the cytosol during active starch biosynthesis.

Cycling of Fru 6-P through PFK and PFP could, however, be involved by converting indirectly ATP into PPI (6, 11, 18). A very tight control of PPI production and utilization seems to exist in the cytosol of higher plant cells, and large fluctuations of PPI concentrations are not usually observed (2, 6, 30). How this regulation is achieved is not known.

In a preliminary report, we showed that sodium fluoride inhibits starch biosynthesis and induces a substantial increase in the PPI content of discs isolated from developing potato tubers (28). Independently, Quick et al. (23) reported that fluoride also induces an accumulation of PPI in discs from spinach leaves. This accumulation was associated with an inhibition of photosynthetic carbon flux into sucrose. However, the mechanisms of fluoride-induced PPI accumulation were not elucidated.

The present study examines in detail the relation between PPI accumulation and the capacity for sucrose-starch interconversion in the starch-storing parenchyma of potato tubers.

**MATERIALS AND METHODS**

**Plant Material**

Potato (*Solanum tuberosum* L., cv Record) plants were grown in compost in an unheated glasshouse. Tubers approximately 4 cm in diameter were selected and used within 1 h after excision from the mother plant.

**Incubation Conditions for Tuber Discs**

Potato tubers were cut transversely into 3 mm thick slices and discs 5 mm in diameter were excised with a cork borer from the phloem-rich perimedulla. Discs were washed four times in 50 mM Mes-KOH buffer, pH 6.5, to remove debris. Prior to supplying radiolabeled precursors, replicates of 20
discs were pretreated by gently shaking (1 h at 20°C) in 650 µL 50 mM Mes-KOH, pH 6.5, in the presence or absence of 10 mM NaF. Discs were subsequently washed three times for 3 min in 3 mL of 50 mM Mes-KOH, pH 6.5, to remove apoplastic inhibitor. In 13C experiments, discs were then incubated for 180 min in 650 µL of 50 mM Mes-KOH, pH 6.5, containing 50 µM [2-13C]glucose (Aldrich Chemical Company Ltd, Dorset, Great Britain). In 14C experiments, samples were incubated for 120 min in 650 µL of 50 mM Mes-KOH, pH 6.5, containing 14C isotopes. [U-14C]sucrose was used at a specific activity of 9.25 GBq·mmol⁻¹. [U-14C]Glc 1-P was used at 6.59 GBq·mmol⁻¹ and [U-14C]ADP-Glc (ammonium salt) at 11.3 GBq·mmol⁻¹. [U-14C]glycerol at 1.99 GBq·mmol⁻¹. All 14C isotopes were used at a final concentration of 37 kBq mL⁻¹. [U-14C]sucrose, [U-14C]Glc 1-P and [U-14C]ADP-Glc were obtained from Amersham International, (Bucks., UK). [U-14C]glucose and [U-14C]glycerol were from ICN Biomedicals Inc. (Irving, CA).

**Extraction and Analysis of Labeled Compounds**

At the end of the incubation period, discs were washed as described previously (20) and subsequently immersed in boiling 80% (v/v) ethanol. Tissue was exhaustively extracted in ethanol to quantify label in the soluble fraction. 14C incorporated into starch was determined as described earlier (8). In all cases, more than 90% of the radiolabel released after digesting starch with amyloglucosidase was recovered as glucose following HPLC separation (see below). Ethanol extracts were concentrated on a rotary film evaporator. Acidic and basic components were then removed by ion exchange (7).

Individual sugars and glycerol in the neutral fraction were separated by HPLC on a 15 cm reverse phase amino column. The mobile phase (2 mL min⁻¹) was acetonitrile:water (85:15, v/v). Compounds of interest were collected and radioactivity determined by liquid scintillation counting.

In 13C experiments, discs were also extracted in ethanol and the sucrose purified by HPLC. NMR operating conditions have been described elsewhere (29). Where 14CO2 was collected and quantified, the method of Mohabir and John (17) was used.

**Extraction of Glycolytic Intermediates and PPI**

Tuber discs were prepared and incubated in 10 mM NaF as described above. At various intervals, the discs were removed from the solution containing the inhibitor, botted to remove excess moisture, and rapidly frozen in liquid N2. Samples were stored under liquid N2 until processed. Frozen slices were ground to a fine powder under liquid N2 in mortars prechilled to −20°C. Prior to complete evaporation of the liquid N2, 5 mL of 1.41 M perchloric acid was added. The frozen mass was ground further with repeated addition of liquid N2 to assist complete homogenization and extraction. After thawing on ice, the samples were centrifuged at 15,000g for 5 min at 3°C. The pellet was washed with 2 mL of perchloric acid and supernatants combined. Supernatants were neutralized (pH 7–8) with 5 M KOH containing 100 mM Hepes. The resulting pellet was removed by centrifugation and the supernatant used for metabolite determinations.

**Metabolite Measurements**

Sequential measurement of Glc 6-P, Glc 1-P and Fru 6-P and assay for 3-PGA were carried out according to Liu and Shannon (14). Triose-P, Fru 1,6-P2, and PPI were assayed, sequentially, in 50 mM Tris-acetate, pH 8.0, containing 2 mM Mg-acetate, 1 mM Fru 6-P, 20 µM Fru 2,6-P2, 0.2 mM NADH, and up to 0.4 mL of extract in a total volume of 0.5 mL. Triose-P was measured following the addition of 2.5 units of triose-P isomerase and 0.85 unit of glyceraldehydrogenase. Once the reaction was complete, Fru 1,6-P2 was quantified by adding 0.23 unit aldolase. Finally, PPI was measured following the addition of 0.5 µmol Fru 6-P and 0.07 unit of PFP. In some experiments, the commercial reagent for the measurement of PPI (Sigma Chemical Co., P-7275) was used (24). However, it should be noted that the reagent is not specific for PPI but measures, simultaneously, triose-P and Fru 1,6-P2, present in the sample. Nevertheless, the reagent was used as a rapid, routine check of PPI content by monitoring NADH oxidation before and after treating extracts with yeast inorganic PPase (Sigma).

The validity of the techniques used for metabolite extraction and analysis was established by quantifying the recovery of pure metabolites added to frozen discs. The poorest recovery was obtained with DHAP (77%). Recoveries for all other metabolites were greater than 83%. Values quoted do not take account of any losses.
Determination of Enzyme Activities

Enzyme extraction was carried out in developing tubers as previously described (20) with the exception that polyethylene glycol was omitted. PFK and PFP were assayed as described by Morrell and ap Rees (18).

Hexose-P isomerase assay contained 0.1 M Tris-HCl (pH 7.5), 5 mM MgCl₂, 5 mM Fru 6-P, 1 mM NAD, and 1 unit mL⁻¹ Glc 6-P dehydrogenase. Pyruvate kinase assay contained 50 mM Hepes-NaOH (pH 7.2), 3 mM MgSO₄, 5 mM KCl, 0.2 mM NADH, 2 mM phosphoenolpyruvic acid, 1 mM ATP, and 2 units mL⁻¹ lactate dehydrogenase. Hexokinase (glucose substrate) assay contained 0.1 M Tris-HCl (pH 8), 3 mM MgSO₄, 2 mM ATP, 0.2 mM NAD, 2 mM glucose, and 1 unit mL⁻¹ Glc 6-P dehydrogenase. For the assay of hexokinase (fructose substrate), the conditions were identical except that 2 mM fructose replaced the glucose. Enolase activity was assayed in 0.1 M Tris-HCl (pH 7.5), 3 mM MgSO₄, 1 mM KCl, 1 mM ADP, 0.2 mM NADH, 1 mM 2-PGA, 1 unit mL⁻¹ pyruvate kinase, and 1 unit mL⁻¹ lactate dehydrogenase. Phosphoglucomutase assay contained 0.1 M Tris-HCl (pH 7.5), 5 mM MgCl₂, 2 mM Glc 1-P (Glc 1,6-P₂-free [Sigma]), 0.3 mM NAD, 1 unit mL⁻¹ Glc 6-P dehydrogenase, and Glc 1,6-P₂ at various concentrations.

The effects of NaF on inorganic alkaline PPase activity was determined with a partially purified enzyme preparation. This was necessary to eliminate interference by acid phosphatase. One kilogram of tubers was homogenized in a juicer (ACME, New Hartford CT) and the fluid combined with a concentrated solution of Tris-HCl, pH 6.5, sodium sulfite, and PMSF. Final molarities were 100 mm, 10 mm, and 1 mm, respectively. The extract was centrifuged at 10,000 rpm for 30 min to remove insoluble material. Protein precipitated from the supernatant between 20 and 80% ammonium sulfate was resuspended in 20 mM Tris-HCl, pH 6.5, and dialyzed for 18 h against two changes of buffer. The extract was made 0.1 m with NaCl and loaded onto a 20 x 2.6 cm column of concanavalin-A Sepharose 4B preequilibrated with the same buffer. Inorganic alkaline PPase eluted with the unbound fraction. Acid phosphatase was subsequently eluted with buffer including 250 mm sodium molybdate. Inorganic alkaline PPase was assayed in 50 mm 1,3-bis[tris(hydroxymethyl)-methylamino]propane-KOH, pH 8.3, containing 5 mm MgCl₂ and 0.85 mm Na₂H₂P₂O₇. The release of Pi was determined with the malachite green reagent described by Baykov et al. (3). After addition of the color reagent, samples were kept on ice for 30 min prior to recording absorbance at 620 nm.

All extractions were carried out at 4°C and assays at 25°C.

RESULTS

Effect of NaF on the Content of Glycolytic Intermediates and PPI

Incubation of tuber discs in 10 mM NaF resulted in a substantial increase in the concentration of triose-P, Fru 1,6-P₂, and PPI (Fig. 2). In particular, the PPI content increased 10-fold during the first 60 min, the level stabilizing at approximately 50 nmol g⁻¹ fresh weight. 3-PGA content increased markedly in the first 30 min, but subsequently declined to approximate the control values by 180 min. Similar changes

![Figure 2. Changes in glycolytic intermediates and PPI in discs from developing tubers incubated for up to 3 h in Mes buffer (50 mM) pH 6.5 ( ), or in Mes buffer containing 10 mM NaF ( ). Results are means ± SE (n = 5).](image-url)
Table II. Metabolism of Radiolabeled Compounds by Discs from Developing Tuber. Preincubated for 1 h in either Mes-KOH (50 mM), pH 6.5, or Mes Buffer Containing NaF (10 mM Final Concentration) Prior to Transfer to 14C Substrate

Values are percentages of total 14C incorporated by discs and partitioned to various components. Values in brackets represent data for discs preincubated in NaF. SEMs (n = 3) have been omitted for clarity, but errors do not exceed 15% of the values given.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>14C incorporated as % of Total Incorporated</th>
</tr>
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<tbody>
<tr>
<td>CO2</td>
<td>1.9</td>
</tr>
<tr>
<td>Ethanol insoluble</td>
<td>13.4</td>
</tr>
<tr>
<td>Starch</td>
<td>10.8</td>
</tr>
<tr>
<td>Other ethanol insoluble</td>
<td>2.6</td>
</tr>
<tr>
<td>Ethanol soluble</td>
<td>84.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>61.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.3</td>
</tr>
<tr>
<td>Fructose</td>
<td>2.7</td>
</tr>
<tr>
<td>Glycerol</td>
<td>31.9</td>
</tr>
<tr>
<td>Other ethanol soluble</td>
<td>16.9</td>
</tr>
<tr>
<td>14C incorporated as %</td>
<td>3.1</td>
</tr>
</tbody>
</table>

in 3-PGA levels following NaF treatment have been observed in slices of Jerusalem artichokes (15). Hexose-P levels almost doubled during the first 30 min incubation in NaF and then decreased (Fig. 2). Hexose-P concentration was, however, always higher in NaF-treated discs compared with the control. Fluoride did not affect the Glc 6-P:Fru 6-P:Glc 1-P ratio (approximately 10:2.5:1).

Effect of NaF on Enzyme Activities in Vitro

As reported for other systems (16, 23) NaF is a potent inhibitor of enolase from potato tubers (Table I). Partially purified inorganic alkaline PPase activity (free of any PPase activity at acid pH) was almost completely inhibited by 10 mM NaF (Table I). With 1mM NaF activity was inhibited by 70% (not shown). Substantial inhibition of hexokinase (fructose substrate) but not hexokinase (glucose substrate) was observed with 10 mM NaF. Fluoride also inhibited phosphoglucomutase, acting as a competitive inhibitor for Glu 1,6-P2 and increasing the KAapp for this metabolite from 0.35 to 3.2 μM at 10 mM NaF (data not shown). There was only slight inhibition of activity at the highest Glu 1,6-P2 concentration used (20 μM). For this reason, we routinely incubate 20 μM Glu 1,6-P2 in assays that use phosphoglucomutase as a coupling enzyme. No detectable inhibition of either PFK or PFP (glycolytic or gluconeogenic direction) was observed under the conditions used. Fluoride, at 10 mM, did not inhibit the other coupling enzymes used in the assay of the potato extracts.

Effect of NaF on the Partitioning of 14C-Labeled Precursors

Table II shows the effect of a 1 h pretreatment in 10 mM NaF on the subsequent partitioning of 14C in tuber discs. Uptake of labeled precursors was not affected by the preincubation period in fluoride. Because fluoride inhibits energy-mediated uptake mechanisms (4), the washing procedure used to deplete the apoplastic inhibitor was clearly effective.

In general, fluoride caused a reduction in the percentage of label released as CO2, the decrease ranging between 20 and 40%. With [U-14C]ADP-Glc, the decrease in 14CO2 output was not significant. With the exception of [U-14C]ADP-Glc, fluoride inhibited the conversion of all precursors into starch and other insoluble components (Table II). In all cases, an almost proportional increase in the percentage of label recovered in sucrose was observed. In general, labeling of hexoses was also increased by fluoride.

Experiments in which fluoride and 14C-labeled precursors were supplied to detached tubers through stolons confirmed these effects (not presented). They are not, therefore, related to a substantial wound response in isolated discs.


Table III shows the percentage redistribution of 13C from C2 to C5 in the hexosyl moieties of sucrose after incubation of tuber discs with 50 mM [2-13C]glucose. Fluoride increased the percentage redistribution in both the glucosyl and fructosyl moieties of sucrose. This implies that in both the control and fluoride-treated tissue, a substantial proportion of the glucose is converted to triose-P and recycled to hexose-P (12, 29). The partitioning of enrichment between the hexosyl moieties of sucrose was not affected by fluoride.

**DISCUSSION**

The substantial increase in 3-PGA content induced by fluoride can be explained by the adverse effect of the inhibitor...
on enolase activity (Fig. 2). Fluoride-induced accumulation of 3-PGA has been reported in a range of tissues (15, 23). The decrease in 3-PGA after 30 min incubation in fluoride cannot be completely explained, although conversion into 2,3-PGA via 3-PGA kinase is possible. This enzyme catalyzes a readily reversible reaction and the net flux depends on the relative concentrations of adenine nucleotides (27). In favor of this hypothesis is the large increase in triose-P and Fru 1,6-P2 after NaF treatment (Table II). Conversion of triose-P into hexose-P could then occur in the cytosol, most likely via PFP because potato tubers apparently lack fructose 1,6-bisphosphatase (9) (Fig. 1). An enhanced rate of triose-P recycling could explain the large increase in PPI content if PPI scavenging mechanisms were altered by fluoride in the same way as proposed for the spinach leaf tissue (23). Indeed, in fluoride-treated discs, the extent of label redistribution between C2 and C5 in the hexosyl moieties of sucrose was increased when [2-13C] glucose was supplied as a precursor (Table III). This suggests that fluoride increased the rate of triose-P recycling. However, by the time the [2-13C]glucose was supplied (60 min), a 10-fold increase in PPI content had occurred in fluoride-treated discs (Fig. 2). Because PPI is a competitive inhibitor with respect to Fru 1,6-P2, for the reaction catalyzed by PFP (23), a decrease in the extent of triose-P recycling would be expected if PPI accumulated in the cytosol.

Preliminary NMR experiments showed that fluoride increased total 13C enrichment in sucrose when tuber discs were incubated with [2-13C]glucose (data not shown). This contrasts with published data on spinach leaf discs in which PPI accumulation induced by fluoride is associated with an inhibition of sucrose biosynthesis (23). The effect of preincubating tuber discs for 1 h in 10 mM NaF on the metabolism of a range of 14C-labeled precursors was subsequently examined. The general reduction of 14CO2 release associated with the fluoride pretreatment confirms the partial inhibition of respiration noted by others (21). Furthermore, with the exception of [U-14C]ADP-Glc, starch synthesis from all 14C-labeled precursors was almost completely inhibited by fluoride. An increase in the labeling of the sucrose pool, almost proportional to the decrease in starch labeling, occurred (Table II), thus confirming the initial NMR observation. The results confirm that the pathways of sucrose and starch biosynthesis are intimately related in the potato tuber (Fig. 1). Detachment of developing tubers from the mother plant also leads to a reduced capacity for incorporating [U-14C]glucose into starch and an increase in the partitioning of 14C into the sucrose pool (20). The incorporation of [U-14C]glycerol into compounds derived from hexose-P (starch + sucrose + hexoses) was increased 20% by pretreatment with fluoride (Table II). This provides additional evidence that triose-P conversion into hexose-P is enhanced by the inhibitor.

In spinach leaf discs, the inhibition of sucrose biosynthesis by fluoride is attributed to the inhibition of UDP-Glc production by elevated PPI concentration (23). In our NMR studies, however, both total enrichment and the degree of 13C redistribution was similar in the glucosyl and fructosyl moieties of sucrose after the supply of [2-13C]glucose (Table III). This indicates isotopic equilibrium between UDP-Glc and Fru 6-P (12, 29, 31) and that UDP-Glc formation is not inhibited by fluoride in potato tuber storage parenchyma. This result also implies that fluoride does not inhibit phosphoglucomutase activity in vivo and that the Glc 1,6-P2 concentration in fluoride-treated discs is high enough to counteract the competitive inhibition by fluoride ions (Table I). Whereas fluoride induces a substantial accumulation of PPI in both spinach leaf (23) and potato tuber tissues (Fig. 2), the effects on carbon partitioning are different between the two tissues. This may be explained if the PPI accumulates in different subcellular compartments.

During starch biosynthesis, conversion of Glc 1-P into ADP-Glc via ADP-Glc pyrophosphorylase occurs in the amyloplast and is accompanied by PPI production (Fig. 1). Rapid PPI hydrolysis in this compartment is required to drive the equilibrium toward ADP-Glc production (PPI strongly inhibits the conversion of Glc 1-P into ADP-Glc [1]). Inhibition of the inorganic alkaline Pase by fluoride (Table I) will result in an accumulation of PPI in tuber amyloplasts. This will decrease the rate of ADP-Glc formation and, hence, the rate of starch synthesis. This hypothesis was confirmed by the results obtained with [U-14C]Glc 1-P and [U-14C]ADP-Glc (Table II). Fluoride inhibited conversion of [U-14C]Glc 1-P into starch substantially, but had no effect on the utilization of [U-14C]ADP-Glc. This indicates that the conversion of Glc 1-P to ADP-Glc in the amyloplast is the point in the starch biosynthetic pathway which is inhibited by fluoride. To hold firm, this hypothesis obviously requires that [U-14C]Glc 1-P and [U-14C]ADP-Glc are not dephosphorylated or converted to other products prior to uptake, and that [U-14C]ADP-Glc is not metabolized significantly in the cytosol. As far as Glc 1-P is concerned, the possibility of metabolism prior to uptake cannot be eliminated. However, in fluoride-treated yeast cells, Glc 1-P uptake occurs without dephosphorylation due to the efficient inhibition of non-specific phosphatases by the ion (19). Fluoride is also known to inhibit plant phosphatases (5). The very low percentage of label recovered in the ethanol-soluble fraction after supplying [U-14C]ADP-Glc indicates that this compound is not readily metabolized in the cytosol but seems to enter the amyloplast freely under our experimental conditions (see also ref. 22). The hypothesis is further validated by the fact that [U-14C]ADP-Glc was the only precursor incorporated into starch equally effectively in fluoride-treated and untreated discs.

CONCLUSIONS
Kornberg (13) has proposed that biosynthetic pathways that involve a release of PPI are driven to completion by inorganic Pase-mediated PPI hydrolysis. Data in support of this theory are scarce, however (26, and references therein). In this report, we provide evidence that validates the hypothesis for the pathway of starch biosynthesis. The data presented confirm that the apparent “waste” of the energy of the PPI bond in the plastid is the price that the higher plant cell has to pay to make starch synthesis, mediated by ADP-Glc pyrophosphorylase, thermodynamically irreversible.

We are unable to explain fully how fluoride can bring about an activation of both triose phosphate recycling and net sucrose synthesis (both processes generating PPI) without a resulting PPI accumulation in the cytosol and an inevitable
inhibition of one or both of these fluxes. More investigations are clearly required to elucidate the mechanisms that regulate PPI concentration in the cytosol of plant cells.

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LITERATURE CITED