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FRUCTOSE 1-PHOSPHATE FORMATION IN JERUSALEM ARTICHOKE (*HELIANTHUS TUBEROSUS*) TUBERS

L. J. MORGAN* and J. B. PRIDHAM

Department of Biochemistry, Royal Holloway College, University of London, Egham Hill, Egham, Surrey, TW20 OEX, U.K.

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Abstract—Injection of D-[U-^{14}C]fructose into developing Jerusalem artichoke tubers produced labelled glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, fructans and fructose 1-phosphate. The latter ester was not produced on loading tubers with [U-^{14}C]sucrose. In contrast to the other hexose phosphates, fructose 1-phosphate did not appear to be metabolized. No evidence for the conversion of fructose, fructose 6-phosphate or fructose 1,6-bisphosphate to fructose 1-phosphate *in vitro* could be obtained.

INTRODUCTION

The tissue-specific pathways for fructose metabolism in mammals are well understood. Ketohexokinase-catalysed phosphorylation of this sugar to fructose 1-phosphate is the major reaction occurring in the liver: in extrahepatic tissues hexokinases convert fructose to fructose 6-phosphate [1].

Fructose 6-phosphate is generally considered to be the primary product of fructose metabolism in higher plants [2, 3] and since 1966, little attention has been paid to reports that fructose 1-phosphate is a component of plant tissues. Schwimmer and co-workers [4, 5] first claimed to have detected this ester in potato (*Solanum tuberosum*) tubers and an examination of data published by Cole and Ross [6] suggests that fructose 1-phosphate may have been formed when maize (*Zea mays*) roots were grown in a medium containing [^{32}P]phosphate. Graham and Ap Rees [7] presented evidence for the occurrence of fructose 1-phosphate in the roots of carrot (*Daucus carota*) and turnip (*Brassica rapa*). Identification was based on chromatographic methods and aldolase cleavage and subsequent detection of dihydroxyacetone phosphate. The tissues contained 19.5 and 21.1 nmol ester/g fr.wt, respectively.

We have now been able to demonstrate the conversion of fructose to fructose 1-phosphate *in vivo* but attempts to determine how this is achieved have failed.

RESULTS AND DISCUSSION

Initial attempts to detect endogenous fructose 1-phosphate in carrot roots and developing and germinating Jerusalem artichoke (*Helianthus tuberosus*) tubers by extraction of these tissues with perchloric acid or aqueous ethanol followed by anion-exchange fractionation [8] failed although fructose 6-phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate and other phosphate esters were present in all extracts. Similar results were obtained with broad bean (*Vicia faba*) seeds which had imbibed a 5% fructose solution, allowed to germinate and then extracted with aqueous ethanol. Finally, attempts were made to identify [^{14}C]fructose 1-phosphate in extracts of plant storage organs after injection of D-[U-^{14}C]fructose followed by an incubation period. These failed in the case of developing onion (*Allium cepa*) bulbs and potato tubers but developing Jerusalem artichoke tubers (after 8 hr incubation at 20–25°) yielded a single, labelled acidic compound distinguishable from all known glucose and fructose mono- and bis-phosphates (including fructose 2,6-bisphosphate) by ion-exchange chromatography and exhibiting an elution volume corresponding exactly to that of fructose 1-phosphate. The fractions containing the putative phosphate were pooled, freeze dried and treated with methanol to remove borate ions. Unlabelled fructose 1-phosphate was then added to a sample of the material and the mixture rechromatographed on the column: an examination of the eluate for radioactivity and ketose content (with resorcinol–HCl reagent) yielded coincident peaks. Acid and acid phosphatase hydrolyses of the material in the original pooled column fractions both yielded [^{14}C]fructose which was detected by TLC.

Developing tubers were then injected with [U-^{14}C]-fructose and the post-injection times varied from 5 min to 6 hr prior to tissue extraction. Figure 1 (study *A*) shows the changes in the labelling of the sugar phosphates during this period. Incorporation of ^{14}C into fructose 6-phosphate, fructose 1,6-bisphosphate and glucose 6-phosphate reached a maximum after 30 min. This was followed by a period (30–60 min) of rapid metabolism of all three esters and during this time radioactivity slowly appeared in the fructose 1-phosphate fraction and then, after 3 hr, at a significantly greater rate. The incorporation of label into this fraction continued to rise up to 6 hr of tuber incubation.

Total incorporation of ^{14}C into the identifiable fructose and glucose phosphates was relatively low (max *ca* 12% after 30 min) at all incubation times. Examination of the soluble carbohydrates in the aqueous ethanol extracts of

* Cyanamid of Great Britain Ltd., 154 Fareham Road, Gosport, Hants.

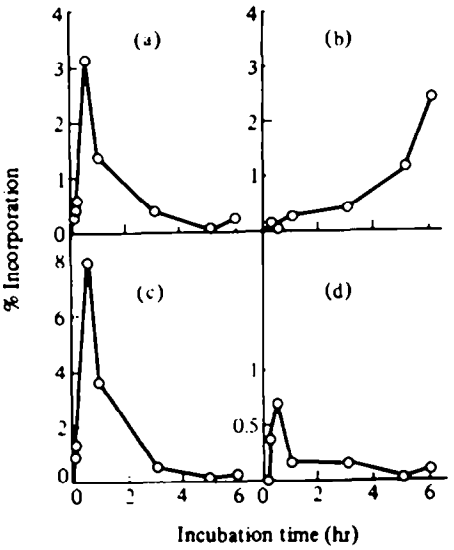

Fig. 1. Incorporation of ^{14}C into (a) fructose 6-phosphate; (b) fructose 1-phosphate; (c) glucose 6-phosphate and (d) fructose 1,6-bisphosphate following the injection of D-[U-^{14}C]fructose into developing Jerusalem artichoke tubers (study *A*).

the injected tubers and the water-soluble polysaccharides remaining in the extracted tissue showed that the bulk of the radioactivity from [^{14}C]fructose had entered the sucrose, higher oligosaccharide and polysaccharide (presumably fructan [*cf.* 9]) pools which, together, after 3 hr incubation contained about 80% of the injected label.

The results of two complementary studies (*B* and *C*) with other samples of developing artichoke tubers were similar to those in study *A* (Fig. 1): glucose 6-phosphate and fructose 6-phosphate were rapidly labelled but longer incubation periods (*ca* 2 hr) were required for maximum incorporation into these phosphates in both studies (study *B*, 2.5 and 1.6%; study *C*, 9.0 and 5.0%, respectively) and in the case of fructose 1-phosphate, the degree of labelling rose sharply after 30 min, reached a maximum (study *B*, 0.7%; study *C*, 2.1%) after 2 hr and then remained approximately constant for a further 6 hr.

In vivo, the major organic material entering developing artichoke tubers is presumably translocatory sucrose, however, when this [U-^{14}C]-labelled disaccharide was introduced into the tubers no labelled fructose 1-phosphate was detected after 4 hr incubation. Some incorporation of ^{14}C into fructose 6-phosphate (max. 0.5%) and glucose 6-phosphate (max. 1.6%) was observed but most of the label (> 60% after 4 hr) appeared in high DP (> 11) fructans. In relation to the differences observed between fructose and sucrose metabolism in the tubers, it is of interest to note that the *Aerobacter aerogenes* system which mediates the transfer of exogenous fructose into the cell, converts free fructose to fructose 1-phosphate but fructose derived from exogenous sucrose is phosphorylated at C-6 [10].

In none of the present studies where fructose 1-phosphate was produced was there any indication that the ester was further metabolized. Aldolase activity is undoubtedly widespread in plant tissues and Cardini [11] has shown that Jack bean (*Canavalia ensiformis*) aldolase can utilise fructose 1-phosphate as substrate. The rapid disappearance of labelled fructose 1,6-bisphosphate from tubers (Fig. 1) and the detection of dihydroxyacetone phosphate as a product of the incubation of fructose 1,6-bisphosphate with tuber extracts (see below) are indicative of a functional aldolase in the artichoke tuber cells. Hence, the apparent failure of these cells to metabolize fructose 1-phosphate suggests that either the ester and the enzyme are in different compartments or that the K_m for fructose 1-phosphate is high relative to that for fructose 1,6-bisphosphate which is present at a high level during the course of the incubation.

Having shown that fructose 1-phosphate was formed from fructose in developing Jerusalem artichoke tubers the possibility that the ester was produced by fructose phosphorylation catalysed by a 1-phosphotransferase was next examined. Using a DEAE cellulose disc procedure for assaying hexose kinases [12] the phosphorylation of fructose by ATP in the presence of a crude buffered extract of tuber tissue was demonstrated. The pH optimum for the reaction was approximately 7.8 (with half max velocities at pH 6.5 and 8.5) and the apparent K_m for fructose was 22 μM. The rate was increased 2-fold by 25 mM KCl. In comparison with ATP, the rates of phosphorylation with UTP and GTP were 38% and 50%, respectively. No reaction was observed using sodium pyrophosphate [*cf.* 13, 14] or phosphoenol pyruvate [*cf.* 10] as potential phosphate donors. The crude tuber extract also catalysed the phosphorylation of glucose by ATP although at 61% of the rate observed with fructose. The glucokinase and fructokinase activities in this tissue would appear to represent different enzymes as the former activity was strongly inhibited (85%) by 50 mM 2-acetamido-2'-deoxy-D-glucose, a known competetive inhibitor of animal hexokinase [15]: this concentration of inhibitor did not affect the rate of fructose phosphorylation. The hexose phosphorylating enzymes of artichoke tuber tissues may thus be similar to those in other plant storage organs such as pea (*Pisum sativum*) seeds [16–18] which possess separate hexokinase (exhibiting a low K_m and high V_{max} for glucose relative to fructose) and fructokinase activities both of which preferentially utilize ATP as a phosphoryl donor yielding glucose- and fructose-6-phosphates as products. An examination of the products obtained by incubating fructose (1 mM and 10 mM) with ATP and the tuber extract using the anion-exchange column also indicated that fructose 6-phosphate was the primary product (glucose 6-phosphate and probably glucose 1-phosphate were also present): no fructose 1-phosphate was detected.

Two other conceivable mechanisms for fructose 1-phosphate synthesis were investigated. The pattern of ^{14}C incorporation into hexose phosphates when [^{14}C]fructose was injected into tubers (Fig. 1) suggested a possible product/precursor relationship between fructose 1-phosphate and fructose 6-phosphate or fructose 1,6-bisphosphate. In the former case the transfer of phosphate from C-6 to C-1 catalysed by a phosphohexose mutase would be thermodynamically feasible and, indeed, favourable if the product underwent a change in ring size from furanoid to the expected and more stable, pyranoid form. However, when [U-^{14}C]fructose 6-phosphate was incubated with tuber extracts under a variety of conditions, including pH (4.5 and 7.5) and the presence of fructose 1,6-bisphosphate (a possible co-factor), fructose 1-phosphate formation could not be demonstrated. The

major reaction in all cases was the production of glucose 6-phosphate.

In the case of fructose 1,6-bisphosphate the possibility that a specific 6-phosphohydrolase was present in artichoke tuber tissue was considered. However, again, when fructose 1,6-bisphosphate was incubated with tuber extracts under varying conditions no fructose 1-phosphate was detectable, but the expected products of metabolism, including fructose 6-phosphate and glucose 6-phosphate and dihydroxyacetone phosphate (presumably formed by the action of fructose 1,6-bisphosphate aldolase), were detected.

At present, therefore, the immediate source of fructose 1-phosphate in artichoke tissues is not known. In some of our studies, ion-exchange fractionation of tuber extracts revealed an acidic compound (giving a positive reaction with the resorcinol–HCl reagent) with an elution volume similar to that reported for fructose 2-phosphate [8]. If this compound existed in tubers it could conceivably be converted to fructose 1-phosphate by a reaction analogous to that catalysed by phosphoglucomutase.

EXPERIMENTAL

Plant material. Jerusalem artichoke (*Helianthus tuberosus* L.) tubers were supplied by the University of London, Botanical Supply Unit. Developing tubers (weighing 2–8 g) were excised from freshly uprooted plants in the summer months and used within 1 hr of harvesting. Developing potato (*Solanum tuberosum* L.) tubers (weighing 10–20 g) were obtained from plants grown in pots in a greenhouse. Carrot (*Daucus carota* L.) roots, onion (*Allium cepa* L., a small pickling var.) bulbs and broad bean (*Vicia faba* L. var Bunyards Exhibition) seeds were obtained from local merchants.

Introduction of carbohydrates into plant tissues. Vicia faba seeds were soaked in H_2O containing 5% D-fructose for 20 hr then transferred to moist cotton wool (5 days). Testas were removed prior to extraction. Potato and artichoke tubers and onion bulbs, after surface sterilization with 0.1% NaClO, were rapidly injected at multiple sites with 5 μl vols of D-[U-^{14}C]fructose (241–301 μCi/μmol; 0.5 μCi/g fr. wt) or [U-^{14}C]sucrose (382 μCi/μmol; 0.25 μCi/g fr wt) soln. They were then wrapped in Al foil and incubated for various times up to 8 hr at ambient temps (20–25°) and finally frozen in liquid N_2 and stored at −15° prior to extraction.

Extraction of metabolites. $HClO_4$ extractions were carried out as described in ref. [19] and the procedure of ref. [20] used for extractions with 80% aq. EtOH. Concn of extracts were effected by rotary evaporation at 30° and fractions were stored at −18°. Tissue pellets remaining after 80% aq. EtOH extraction were washed twice with 80% aq. EtOH and, after centrifugation, polysaccharides were extracted by stirring the pellets with H_2O (20 min at 80°). The extracts was then cooled and treated with absolute EtOH: precipitated polysaccharides were collected by centrifugation, washed with absolute EtOH and dried in a stream of N_2.

Ion-exchange fractionation of sugar phosphates. The method of ref. [8] was used but with column dimensions of 1 × 30 cm and an elution rate of 0.2 ml/min. Fractions were analysed for total carbohydrate, with anthrone [21], ketoses, with resorcinol–HCl [22] and radioactivity (see below).

Fractionation of neutral sugars. Carbohydrates (mainly low-M_r fructans) present in 80% aq. EtOH tuber extracts were separated by PC using Whatman No. 1 paper and PrOH–H_2O–EtOAc (6:3:1) solvent. TLC on silica plates with EtOAc–pyridine–HOAc–H_2O (6:3:1:1) solvent [23] was used to examine hydrolysis products of [^{14}C]fructose 1-phosphate. For PC and TLC, *p*-aminobenzoic acid was used to locate carbohydrate [24].

Measurement of radioactivity by scintillation counting. PC strips and DEAE-cellulose discs (kinase assay [12]) were air-dried and counted in vials containing 0.5% PPO in toluene (10 ml). Radioactive bands were scraped from TLC plates, eluted with H_2O (1 ml) and the silica removed by centrifugation. Aliquots of the centrifugates were counted in 0.5% PPO (in toluene): Triton-X 100 (2:1; 10 ml). Aliquots of ion-exchange column fractions were similarly counted. Polysaccharide residues were digested with NCS (500 μl) at 37° for 18 hr then neutralized with HOAc (17 μl) and counted in 0.5% PPO in toluene (10 ml). % Incorporation values were calculated from the amount of ^{14}C supplied to the tissues.

Characterization of [^{14}C]*fructose 1-phosphate.* Ion-exchange column fractions containing the phosphate were pooled, frozen and freeze dried then MeOH was added and removed by rotary evaporation: the latter process was repeated × 2. The product dissolved in H_2O was mixed with unlabelled fructose 1-phosphate and re-examined on the ion-exchange column. A further sample of the [^{14}C]fructose 1-phosphate was hydrolysed with 2 M HCl (100°, 30 min) and the acid then removed by subjecting the soln to red. pres. over KOH pellets. The residue was dissolved in H_2O, D-fructose added and the mixture examined by TLC for hexose and radioactivity. A third sample of [^{14}C]fructose 1-phosphate was incubated with potato acid phosphatase (Sigma; 1 unit) in 0.1 M sodium citrate buffer (1 ml, pH 5.6) for 5 min at 37°. EtOH (4 vols) was added, the precipitated protein removed by centrifugation and the supernatant freeze-dried. The product was examined as in the case of the acid hydrolysate.

Tuber enzyme preparation. All operations were carried out at 4°. Tubers were washed with water then homogenized in 0.1 M Tris–HCl (pH 8.2) buffer containing 28 mM EtSH (3.9 ml/g fr. wt) and PVP (0.20g/g fr. wt). The homogenate was centrifuged (15000 *g*, 20 min) and the supernatant dialysed, with stirring, against buffer (4–51) for 2 hr. All such supernatants were prepared on the day required for enzyme assay.

Hexose kinase assay. The standard reaction mixture (total vol. 0.8 ml) contained enzyme preparation (0.5 ml), [U-^{14}C]fructose (241 μCi/μmol; 4.2 nmol) or [U-^{14}C]glucose (295 μCi/μmol; 3.4 nmol), ATP (2.5 μmol), $MgCl_2$ (3 μmol), NaF (25 μmol), KCl (50 μmol) and 0.1 M Tris–HCl buffer (pH 8.2) to vol. Incubation was at 25° for 30 min. Aliquots (25 μl) of reaction mixtures were pipetted on to Whatman DEAE-cellulose discs (2.1 cm diam) which were then bulk-washed under suction with H_2O (*ca* 100 ml/disc) (12). After drying (100°) radioactivity was measured as described above. Remaining reaction mixtures were frozen and stored at −18° prior to analysis by ion-exchange chromatography. Protein was determined by the method of ref. [25] using BSA as a standard. The pH optimum for fructokinase activity was determined using the standard assay mixture but reducing the enzyme volume to 0.1 ml and adding 0.1 M NaOAc (pH 4.5), 0.1 M sodium citrate–NaOH (pH 5.5), 0.75 M MES (pH 6.5), 0.1 M KP_i (pH 7.4) or 0.1 M sodium borate (pH 9.2) to volume (0.8 ml). Acidic digests were made alkaline with 0.1 M NaOH (0.8 ml) before application to DEAE discs. Assays using UTP, GTP, $NaPP_i$ and PEP were carried out by replacing ATP by 2.5 μmol of these phosphates.

Fructose 6-phosphate metabolism. The standard reaction mixture (total vol. 100 μl) was [U-^{14}C]fructose 6-phosphate (268 μCi/μmol; 0.89 nmol), tuber enzyme preparation (25 μl), NaF (2.5 μmol), $MgCl_2$ (1 μmol) and KP_i (5 μmol, pH 7.5) buffer or NaOAc buffer (5 μmol, pH 4.5). The effect of adding fructose

1,6-bisphosphate (1 nmol) was also investigated as well as the effects of omitting NaF and/or $MgCl_2$ from the digests. Samples of reaction mixtures were examined by PC on Whatman No. 1 paper using EtOAc–formamide–pyridine (4:4:1) and 95% aq. EtOH–1 M ammonium acetate (5:2) as solvents and visualized with *p*-aminobenzoic acid reagent [24]. Paper strips were subjected to scintillation counting as described above. The reaction mixtures were also examined on the anion-exchange column.

Fructose 1,6-bisphosphate metabolism. In the standard reaction mixture fructose 1,6-bisphosphate (1 μmol or 10 μmol) was incubated at 25° for 45 min with the tuber enzyme preparation (0.9 ml), $MgCl_2$ (10 μmol) and Tris–HCl (9.0 μmol; pH 8.2), total vol. 1 ml. In other incubations Tris–HCl was replaced by sodium citrate buffer (pH 4.5) and with both buffers the effect of omitting $MgCl_2$ was examined. Reaction products were analysed as described for fructose 6-phosphate metabolism, excluding the radiation assays.

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