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FRUCTOSAN METABOLISM IN *CICHORIUM INTYBUS* ROOTS

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Key Word Index—*Cichorium*; Compositae; chicory; glucofructosan; fructosyl transferase; fructosan hydrolysis; glucose utilization.

Abstract—Incorporation of [^{14}C]sucrose into difructosyl glucose (F_2G), trifructosyl glucose (F_3G) and tetrafructosyl glucose (F_4G) in the presence of various nucleoside triphosphates revealed that formation of F_4G and F_3G is retarded in the presence of ATP, and formation of F_3G and F_2G is significantly enhanced in the presence of CTP, whereas UTP has no effect on the synthesis of these oligosaccharides. Different fructosyl transferases seem to be responsible for the different fructosylation steps and self transfer seems to be the major pathway for fructosan synthesis. Utilization of added glucose, which is formed by sucrose sucrose fructosyl transferase action *in vivo*, is completely inhibited in acetate buffer whereas in phosphate, citrate and citrate–phosphate buffers glucose is actively utilized. In the presence of fluoride ions both glucose utilization and its conversion to CO_2 is inhibited by *ca* 50%. CO_2 production from [^{14}C]glucose is completely inhibited in acetate ions. No evidence for the incorporation of ^{14}C from [^{14}C]glucose into [^{14}C]sucrose is observed. The ratio of bound fructose to bound glucose is the same in the entire length of the root indicating that there is no preferential zone for fructosan synthesis.

INTRODUCTION

Fructosan metabolism has been studied in various plants [1–11]. Sucrose sucrose fructosyl transferase, which forms difructosyl glucose from two molecules of sucrose, has been isolated and purified from many sources [12–22]. Further addition of fructose is achieved either with the help of fructan fructan fructosyl transferase which utilizes difructosyl glucose but not sucrose as the donor of fructosyl residues [23–25] or by self transfer giving rise to a series of polymers ranging in degree of polymerization both above and below that of the original substrate [15, 26]. The possibility that fructan fructan fructosyl transferase is not a single enzyme but a mixture of a number of fructosyl transferases, which differ slightly from each other and hence may be difficult to separate by traditional chromatographic techniques, cannot be ruled out. It has been seen earlier that glucose, which is formed by sucrose sucrose fructosyl transferase and invertase, is not accumulated in *Cichorium intybus* roots during the whole growth period [18]. The pertinent question whether glucose is converted back to sucrose and then utilized in fructosan biosynthesis or oxidized partly or fully to meet the cellular energy demand still remains unanswered. The regulatory factors responsible for fructosan hydrolysis during the post-flowering stage are still not clear. The present study aims to fill some of the above mentioned gaps in the area of fructosan metabolism.

RESULTS AND DISCUSSION

Effect of nucleotides on the incorporation of ^{14}C *from* [^{14}C]*sucrose in oligofructosaccharides*

In the presence of CTP the ratio of ^{14}C incorporation from [^{14}C]sucrose into F_3G/F_2G and F_2G/sucrose was 0.69 and 0.77, respectively, which was considerably higher than the normal ratio of 0.48 and 0.66 (Table 1). However, the F_4G/F_3G ratio was not affected. F_4G can be formed either by self transfer of fructose from F_3G to another F_3G molecule or by fructose transfer from F_2G to F_3G by the action of fructan fructan fructosyl transferase. If the same enzyme is responsible for the synthesis of F_3G and F_4G then the synthesis of F_4G should also have been enhanced along with F_3G synthesis. Therefore, it appears that the enzyme catalysing the synthesis of F_4G is different from that responsible for the synthesis of F_3G. In the presence of ATP, ^{14}C incorporation ratio of F_2G/sucrose remained at 0.66 which was almost the same as in the presence of UTP and the control experiment. However, ^{14}C incorporation ratios of F_3G/F_2G and F_4G/F_3G were drastically reduced to 0.39 and 0.21, respectively. The above effect could be due to either nucleoside triphosphates or their hydrolytic products

Table 1. Effect of nucleotides on ^{14}C incorporation from [^{14}C]sucrose on F_4G/F_3G, F_3G/F_2G and F_2G/sucrose ratios

	*	ATP	UTP	CTP	Sucrose
F_4G/F_3G	0.35	0.21	0.30	0.30	0.31
F_3G/F_2G	0.48	0.39	0.53	0.69	0.50
F_2G/sucrose	0.66	0.66	0.64	0.77	0.66

The roots were crushed in a pestle and mortar at 0–4° and 1 g of this material was incubated with 1 ml of hot [^{14}C]sucrose (1 μCi), 1 ml of 0.1 M acetate buffer (pH 5.4) at 30° for 24 hr under toluene. The concentration of nucleotide was 30 mg each. Cold sucrose (166 μmol) was added in one assay system only.

*No nucleotide or cold sucrose was added. The experiment was performed in March. After developing the chromatogram, 0.5 cm strips were cut from the base and radioactivity determined.

because of the unstable nature of nucleoside triphosphates under the assay conditions of pH 5.4.

Incorporation studies with [^{14}C]sucrose

When crushed roots were incubated with [^{14}C]sucrose, the label was observed in F_2G, F_3G and F_4G only and no radioactivity could be detected in higher oligosaccharides, indicating that smaller oligosaccharides were unable to serve as fructose donors for the higher oligosaccharides (Fig. 1). Obviously, high M_r fructosans in *Cichorium intybus* are not synthesized by fructan fructan fructosyl transferase as low M_r fructosans are unable to donate fructose to high M_r fructosans and thus self transfer may be the major pathway for fructosan synthesis. It is not unreasonable to assume that with an abundant supply of sucrose from source to sink the overall direction of self transferring fructosyl transferases will be in the direction of fructosan synthesis. Self transfer has also been observed to be the major pathway for fructosan synthesis in *Agave* species [15, 26]. The activity of 1F-fructosyl sucrose:sucrose transferase [27] or any fructosyl transfer reaction where high M_r fructosans act as fructose donors to sucrose or low M_r fructosans will either be of insignificant value during active fructosan synthesis or will be active only during the post flowering stage in *Cichorium intybus* when fructosans are actively hydrolysed to meet the energy demands of seed formation [18].

In *Helianthus tuberosus*, fructosan synthesis is suggested to occur *via* a transfer system involving a sucrose surcrose fructosyl transferase and fructan fructan fructosyl transferase. Sucrose sucrose fructosyl transferase specifically synthesizes difructosyl glucose from sucrose while fructan fructan fructosyl transferase utilizes difructosyl glucose as a substrate to produce higher polymers [23]. However, fructan fructan fructosyl transferase had a preference for accepting high M_r fructosans for fructose transfer from difructosyl glucose [6]. Though sucrose sucrose fructosyl transferase and fructan fructan fructosyl transferase are also present in *Allium cepa* but with F_2G as a substrate, the predominant reaction was self transfer to form F_3G and sucrose and further addition of low concentrations of high M_r fructosans (DP > 20) did not alter this reaction [24, 25]. Clearly these enzymes in *Allium cepa*, in contrast to those of *Helianthus tuberosus*, do not have a strong preference for high M_r fructosans.

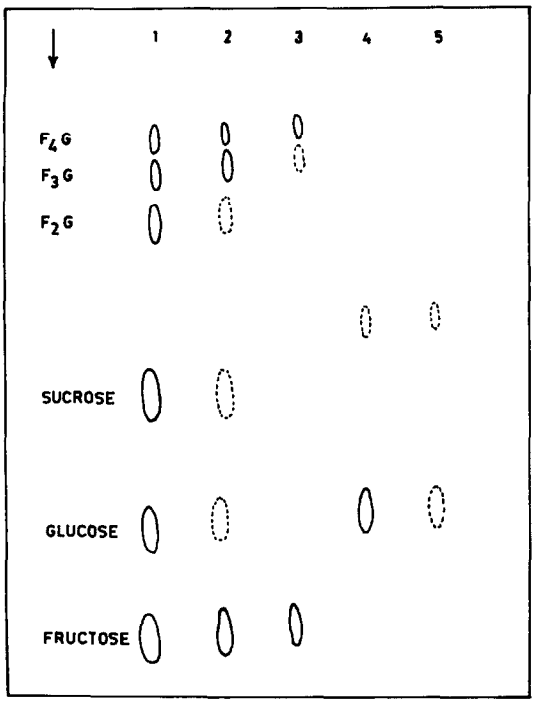

Fig. 1. Diagram of an autoradiograph showing the fate of [^{14}C]glucose and [^{14}C]sucrose. In the five experiments (1–5), 1 g crushed roots were incubated with 1 ml of 2% [^{14}C]sugar solution (3 μCi) and 1 ml of 0.1 M buffer (pH 5.4) and incubated for a specified time under a layer of toluene at 30°. Soluble sugars were extracted and separated by PC. X-Ray film was kept in contact with the chromatogram for 30 days and then developed. Incubation: (1) with [^{14}C]sucrose in sodium acetate buffer for 24 hr, (2) with [^{14}C]sucrose in sodium phosphate buffer for 16 hr, (3) with [^{14}C]sucrose in phosphate buffer for 24 hr, (4) with [^{14}C]glucose in phosphate buffer for 16 hr and (5) with [^{14}C]glucose in phosphate buffer for 24 hr.

Glucose utilization in the root

When roots were incubated with [^{14}C]sucrose in the presence of 0.1 M phosphate buffer (pH 5.4) for 24 hr, no spot corresponding to [^{14}C]glucose after paper chromatography and autoradiography could be detected (Fig. 1). However, a spot corresponding to fructose was quite conspicuous. Theoretically, the level of glucose should have been higher as compared to fructose after the action of sucrose sucrose fructosyl transferase and invertase. Obviously, glucose is preferentially utilized as compared to fructose.

Effect of acetate, phosphate and citrate–phosphate buffers on glucose utilization

Acetate ions were strong inhibitors of glucose utilization whereas glucose was actively utilized in citrate, phosphate and citrate–phosphate buffers. The greater the concentration of acetate ions in the phosphate buffered assay system, the greater the inhibition of glucose utilization (Table 2). The level of acetate, which may be in the form of acetyl CoA in the cell, may play a crucial role in the regulation of glucose utilization by inhibiting the phosphorylation of glucose.

Effect of inhibitors on the utilization of glucose

In the presence of sodium fluoride, a specific inhibitor of glycolysis, only 50% of the glucose supplied was utilized by the roots whereas *p*-chloromercuribenzoate, an inhibitor of sulphydryl group containing enzymes, like hexokinase and glucose-6-phosphate dehydrogenase, completely inhibited glucose utilization (Table 3). These results indicate that at least 50% of the glucose is utilized by glycolysis. Glucose in the above experiment was estimated by glucose oxidase and the inhibitors tried above have no effect on a glucose oxidase preparation of BDH.

Fate of [^{14}C]glucose in Cichorium intybus *roots*

No $^{14}CO_2$ was produced on incubation of crushed roots with [^{14}C]glucose in presence of acetate buffer

Table 2. Effect of acetate, phosphate, citrate and citrate–phosphate buffers on glucose utilization

Composition of buffering system (pH 5.4)	Percentage glucose utilized
100 μmol of phosphate buffer	80
100 μmol of phosphate buffer + 20 μmol of acetate buffer	40
100 μmol of phosphate buffer + 40 μmol of acetate buffer	30
100 μmol of phosphate buffer + 60 μmol of acetate buffer	20
100 μmol of phosphate buffer + 80 μmol of acetate buffer	10
100 μmol of acetate buffer	0
50 μmol of citrate buffer	85
50 μmol of citrate phosphate buffer	85
100 μmol of phosphate buffer (pH 7.0)	85

Crushed roots (1 g) were incubated with 111 μmol glucose in 2 ml required buffer for 16 hr at 30°. Glucose was estimated by glucose oxidase. The experiment was performed in March.

Table 3. Effect of sodium fluoride and *p*-chloromercuribenzoate on glucose utilization

Inhibitor used	Glucose (μmol) left after 24 hr incubation
Control (no inhibitor)	10
Sodium fluoride	55
p-Chloromercuribenzoate	111

Assay system consisting of 1 g of crushed roots and 1 ml of inhibitor solution (10^{-2} M) in 0.1 M phosphate buffer (pH 7.0) was incubated at 30°. The experiment was performed in March. Glucose (111 μmol was added).

Table 4. Effect of phosphate buffer, acetate buffer and fluoride ions on $^{14}CO_2$ production from [^{14}C]glucose

Buffering composition (pH 5.4)	Counts per minute of $^{14}CO_2$ trapped in NaOH
0.1 M acetate buffer	40 ± 10
0.1 M phosphate buffer	6800 ± 700
0.1 M phosphate buffer + sodium fluoride	2900 ± 500

Crushed roots (2 g), 2 ml of 4% glucose, 6 ml of buffer and 1 ml of [^{14}C]glucose (2 μCi) were incubated for 24 hr at 25° in the outer compartment of a conway diffusion cell. CO_2 was trapped in 2 ml of 2 M NaOH in the inner compartment of the cell. It was dried over $CaCl_2$ in vacuum at 50° and radioactivity was measured in toluene-based scintillation fluid. Sodium fluoride (120 μmol) was added. ± S.d. from mean of three experiments.

whereas fluoride ions in phosphate buffer inhibited $^{14}CO_2$ production by 60% (Table 4). These results are in line with earlier results where glucose utilization was inhibited by 100 and 50% in the presence of acetate and fluoride ions, respectively (Table 3).

No radioactive incorporation was observed in sucrose or in any other oligofructosaccharide when [^{14}C]glucose was incubated with crushed roots in the presence of phosphate buffer for 16 hr. However, a faint spot between sucrose and F_2G was seen which possibly may be due to some intermediate of glycolysis. After 24 hr incubation, glucose was completely utilized and again no spot corresponding to sucrose was observed. Evidently, glucose is not converted back to sucrose. In the presence of acetate buffer exogenous glucose was unable to accept fructose from any of the oligofructosaccharides to form sucrose [18]. However, leaf discs of *Cichorium intybus*, though they did not contain any oligofructosaccharides like F_2G, F_3G and F_4G, when incubated in phosphate buffered 5% glucose or fructose for a period of 72 hr were able to synthesize a homologous series of inulin type fructosans. This is probably due to the availability of an enzyme system in leaf discs for the conversion of glucose and fructose into sucrose [9]. Explants from mature dormant tubers of *Helianthus tuberosus* when grown for 2–8 days in a sterile culture of [^{14}C]glucose were also able to synthesize fructosans [28, 29].

A comparison of oligofructosaccharide synthesis in phosphate and acetate buffer

Crushed roots were incubated with [^{14}C]sucrose in phosphate and acetate buffers. After 16 hr incubation in phosphate buffer weak spots corresponding to sucrose, glucose and F_2G and dense spots corresponding to F_3G and F_4G were observed. However, after 24 hr incubation in this buffer no radioactive spots corresponding to glucose, sucrose and F_2G were detected, while spots corresponding to [^{14}C]F_3G and F_4G were observed (Fig. 1). Possibly, when glucose, one of the products of sucrose sucrose fructosyl transferase, is actively consumed (Table 2) the balance of the assay system will be in favour of glucose formation. However, when all of the sucrose is utilized then glucose can be obtained only from F_2G, and possibly from higher oligomers, by the action of invertase or fructosan hydrolase. The presence of invertase along

with sucrose sucrose fructosyl transferase has been shown during the active formation of fructosans [18].

Distribution pattern of fructosans in the root

The ratios of bound fructose to bound glucose vary from 8 to 10 in the entire length of the root indicating that there is no localized zone in the root for fructosan accumulation (Table 5).

Table 5. Distribution pattern of fructosans in the roots

Sr. no.	Percentage bound glucose	Percentage bound fructose	Ratio (bound fructose/bound glucose)
1	8.0	64.0	8.0
2	7.4	67.0	9.0
3	6.9	70.4	10.2
4	7.0	67.2	9.6
5	6.8	65.3	9.6
6	6.8	65.3	9.6
7	7.2	65.5	9.1
8	8.0	71.2	8.9
9	7.5	69.7	9.3
10	8.2	65.6	8.0

A root was cut into 10 equal parts. 1 represents the part nearest to the reduced stem. Serial numbers 1–10 are in sequence from above to below. The experiment was performed in March.

EXPERIMENTAL

Materials. Uniformly labelled [^{14}C]glucose and [^{14}C]sucrose were obtained from BARC, Trombay. ATP and glucose oxidase were purchased from Cal. Biochem. and BDH, respectively. UTP and CTP were from Boehringer. *Cichorium intybus* (chicory var. Kalpa No. 1) was sown in the first week of November in the fields of the Department of Biochemistry, Punjab Agricultural University, Ludhiana and samples were taken when required.

Extraction and estimation of carbohydrates. The carbohydrates were extracted repeatedly with boiling H_2O as described in ref. [30]. Free fructose, bound fructose, free glucose and bound glucose was estimated as described earlier [18]. *n*-BuOH–HOAc–H_2O (4:1:5) was used for PC [31].

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