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SUGAR LEVELS IN THE PEA EPICOTYL: REGULATION BY INVERTASE AND SUCROSE SYNTHETASE

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Abstract—In meristematic regions of the pea epicotyl (plumule and hook), fructose was the predominant sugar (about 1 per cent fr. wt.), sucrose was also present (0·4 per cent) but glucose was barely detectable. In adjacent regions of internode where cell expansion occurred, glucose became the main sugar (> 1 per cent) and levels of fructose and sucrose declined. None of these sugars was absorbed or utilized for metabolism preferentially by excised sections of any one region of the epicotyl. The distribution of various glucosidase, exo-glucanase and sugar phosphatase activities bore no clear relationship to actual hexose concentrations. However, the distribution of invertase activity was parallel to that of glucose, and sucrose synthetase was most active in regions where fructose and sucrose were concentrated. It is suggested, therefore, that the principal factors controlling sugar levels in the pea epicotyl are location and relative activities of these two enzymes, both utilizing translocated sucrose as substrate.

INTRODUCTION

CURIOUS differences were observed to occur between levels of free glucose and fructose in growing regions of the pea epicotyl at successive stages of development. In order for this to happen, there must have been major change(s) in the rate of transport, use or formation of one or the other hexose. The aim of the present study was to establish which of these processes was mainly responsible for controlling sugar levels during morphogenesis. To this end, measurements were made of the uptake and utilization of radioactive sugars by epicotyl sections, and assays were performed for various enzymes which produce or use free sugars in order to determine the distribution of their maximum potential activities.

RESULTS AND DISCUSSION

Pea epicotyls were cut into sections as illustrated in Fig. 1, and values for fresh weight, protein and DNA contents of the sections are given in Table 1. The non-vacuolated meristematic tissue near the apex (plumule and hook) contains far more protein and DNA per unit fresh weight than the basal tissues which develop from them. On the assumption that the DNA content per cell is constant, the average volume per cell increased about 25-fold and the total protein per cell 2-fold during development from a location in the plumule to one in fully vacuolated tissue.

Distribution of Free Sugars

As shown in Fig. 1, fructose was the predominant sugar in meristematic tissue of the pea epicotyl. Its concentration (about 1 per cent of fr. wt.) was at least five times that of free glucose. The only other plant sources which have been reported to yield fructose as the main

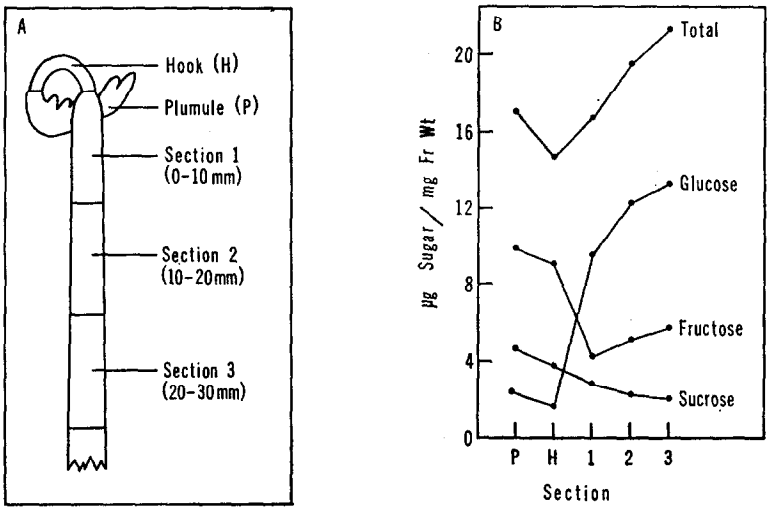

FIG. 1. CONCENTRATIONS OF FREE SUGARS IN THE PLUMULE (P), HOOK (H) AND SUCCESSIVE 10-mm SECTIONS OF THE THIRD INTERNODE OF THE PEA EPICOTYL.

(A) Diagram of sections used to separate regions where cells divide (P and H), elongate (0–10 mm) and mature (10–20, 20–30 mm). Growth and the microscopic appearance of tissues in such sections have been examined often.[1,2] (B) Sugar estimations were made on eluates from chromatographs of 80% ethanol-soluble fractions. Since some fructose trailed into the area occupied by glucose on the papers, the true fructose: glucose ratios were higher than above values indicate. In fact, for example, glucose was hardly detectable with the benzidine spray in extracts of plumule and hook.

TABLE 1. FRESH WEIGHT AND THE PROTEIN AND DNA CONTENTS OF PEA EPICOTYL SECTIONS EXCISED AS IN FIG. 1

Component*	Plumule	Hook	0–10 mm	10–20 mm	20–30 mm
Fr. wt. (mg/section)	10·8	3·5	24·8	28·6	30·3
Protein (µg/mg fr. wt.)	56·5	54·3	13·9	5·4	3·7
DNA (µg/mg fr. wt.)	2·7	1·8	0·33	0·11	0·09
Fr. wt./DNA (mg/µg)†	0·4	0·5	3·0	9·1	11·1
Protein/DNA (µg/µg)†	21	30	42	49	41

* Fifty sections were weighed, homogenized and extracted with hot 80% ethanol and warm (70°) perchloric acid. DNA was estimated in the perchloric acid-soluble fraction with diphenylamine; protein was calculated from values for nitrogen which were determined by Nesslerization in digests of the final insoluble residue.

† These ratios provide a means of comparing the average volume and protein levels per cell at different stages of morphogenesis in the internode.

sugar are woody xylem exudates[3] and storage tissues in which the major reserve polysaccharide is fructosan.[4] The pea epicotyl apex has very few and immature xylem elements[1] and it contains no fructosan.[5] Accordingly, one part of the objective of this investigation was to explain the origin of excess fructose in the region of cell division.

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[3] A. R. SHELDRAKE and D. H. NORTHCOTE, *J. Expt. Botany* **19**, 681 (1968); D. SINGH and E. B. SMALLEY *Can. J. Botany* **47**, 335 (1969).
[4] T. G. JEFFORD and J. EDELMAN, *J. Expt. Botany* **12**, 177 (1961).
[5] G. A. MACLACHLAN and C. T. DUDA, *Biochim. Biophys. Acta* **97**, 288 (1965).

In tissue which contained elongating cells (0–10 mm from hook), as well as in more basal tissue, glucose was the main sugar with a concentration (1·0–1·3 per cent of fr. wt.) about double that of fructose. Thus, fructose levels decreased suddenly and glucose levels increased even more markedly in the narrow region of epicotyl where new cells began their lateral expansion and elongation. Clearly, the maintenance of sugar concentrations during vacuolation was due entirely to accumulation of glucose; hence a second aim of this study was to define the factors governing selective retention of this sugar.

No such abrupt changes occurred in sucrose or in total hexose concentrations at any particular stage of growth in the epicotyl. Sucrose levels were highest where total hexose levels were lowest (at the apex), and the sum of all three sugar concentrations was highest in the most basal tissue (closest to cotyledon reserves).

Cotyledonary starch is the ultimate source of carbohydrate for the developing pea embryo, and the fact that young seedlings contain all of the enzymes needed for complete hydrolysis of starch to glucose has been taken to indicate that oligodextrin or glucose itself is the form in which carbohydrate is exported.[6] However, sucrose is a more probable candidate for translocation because it is the main free sugar in cotyledons[7] and also the main product which is synthesized there from supplied glucose.[8] Invertase is present in the epicotyl[6,9] but its activity alone can hardly account for the grossly unequal glucose and fructose levels which accumulate in different regions.

Utilization of Supplied Sugars

Tables 2 and 3 show the amounts of carbon incorporated in 2 hr from different radioactive sugars into CO_2, polysaccharides (wall materials), ethanol-soluble fractions, and free sugars

TABLE 2. UPTAKE AND INCORPORATION OF SUPPLIED SUGARS INTO 80% ETHANOL-SOLUBLE MATERIALS, RESIDUE (WALL) AND RESPIRED CO_2 BY EXCISED PEA EPICOTYL SECTIONS

Supplied sugar*	Fraction†	Incorporation* (μg/mg fr. wt. tissue/2 hr)		
		Hook	0–10 mm	10–20 mm
Sucrose	Soluble	2·52	0·70	1·00
	Wall	0·35	0·12	0·08
	CO_2	0·33	0·06	0·03
Glucose	Soluble	1·91	0·82	0·98
	Wall	0·36	0·12	0·11
	CO_2	0·34	0·07	0·03
Fructose	Soluble	2·12	0·65	0·80
	Wall	0·44	0·09	0·06
	CO_2	0·36	0·06	0·03

* Supplied sugars were labelled uniformly with ^{14}C and incorporation was calculated from values for label distribution measured after 2 hr incubation.

† The values for CO_2 output from supplied sugars represent approx. 2·5%, 0·5% and 0·4% of the total CO_2 released during the test from hook and the first two sections respectively.

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TABLE 3. CONVERSION OF SUPPLIED SUGARS INTO OTHER SUGARS IN EXCISED PEA EPICOTYL SECTIONS

Supplied sugar	Extracted sugar*	Incorporation (μg/mg fr. wt. tissue/2 hr)		
		Hook	0–10 mm	10–20 mm
Sucrose	Sucrose	1·08	0·08	0·15
	Glucose	0·31	0·16	0·31
	Fructose	0·45	0·22	0·35
Glucose	Sucrose	0·45	0·09	0·07
	Glucose	0·34	0·36	0·53
	Fructose	0·37	0·06	0·08
Fructose	Sucrose	0·51	0·06	0·06
	Glucose	0·25	0·05	0·07
	Fructose	0·50	0·37	0·56

* Sugars soluble in 80% ethanol were isolated by chromatography; total incorporation into this fraction is recorded in Table 2.

in those fractions. By the end of the incubation, only a small part (6–13 per cent) of the supplied sugars had been absorbed. Concentrations of labelled free sugars that accumulated inside sections (0·01–0·1 per cent of fr. wt.) were much lower than those outside (0·625 per cent initially). Very little of the total respired CO_2 was labelled. Evidently the tissues were only beginning to take up and incorporate the supplied sugars into other products. Accordingly, values in these tables would be expected to reflect any major differences that existed between tissues in permeability or in the activities of key enzymes (e.g., kinases) which might control entry of specific sugars into metabolic circulation.

The most striking feature of these data (Tables 2 and 3) is the clear indication that, within any one section of tissue, the three supplied sugars were equally available for uptake and conversion to other products. In particular, sucrose was not absorbed or metabolized by any section more rapidly than either hexose, and the two hexoses were used equally for sucrose synthesis and interconverted to approximately the same extent.

In all of these capacities, meristematic tissue was more active on a fresh weight basis than elongating or maturing tissue, as might be expected from the relatively high concentration of protein in the former (Table 1). Also there can be no doubt that activities of different enzymes and whole metabolic pathways must have altered in relation to one another during development, e.g. the ratio of sugar respired: sugar anabolized decreased with age. The important point for present purposes, however, was that growth and maturation in the epicotyl did not appear to be accompanied by preferential or suppressed rates of utilization of any one of the free sugars.

Distribution of Enzyme Activities

Higher plants can generate hexoses by the action of: *a.* glycosidases on phenolic glycosides or disaccharides, *b.* exo-hydrolases on polysaccharides, *c.* phosphatases on sugar phosphates, or *d.* sucrose synthetase on sucrose (producing UDPG and free fructose). Several of these enzymes were assayed in extracts of the epicotyl sections and results are summarized in Tables 4–6 and Fig. 2.

TABLE 4. DISTRIBUTION OF ACTIVITIES OF α- AND β-GLUCOSIDASES, DISACCHARIDASES AND GLUCANASES IN PEA EPICOTYL EXTRACTS

Substrate	μg glucose or glucose equivalents formed/mg fr. wt./hr*				
	Plumule	Hook	0–10 mm	10–20 mm	20–30 mm
α-Glucoside	0·049	0·043	0·006	0·003	0·003
β-Glucoside	13·1	12·8	0·4	0·1	0·1
Maltose	3·8	1·9	0·7	0·2	0·3
Cellobiose	2·8	1·2	0·4	0·2	0·2
Starch	26·9	7·5	4·8	2·2	2·5
Laminarin	0·21	0·10	0·04	0·02	—
CMC	0·043	0·034	0·007	0·003	0·002

* Enzyme extracts were added to substrates and activities were measured by the initial rates of production of *p*-nitrophenol (from glucosides), free glucose (from disaccharides) or reducing groups in maltose (from starch) or oligosaccharides (from laminarin and CMC, i.e. carboxymethylcellulose).

TABLE 5. DISTRIBUTION OF ACTIVITIES OF VARIOUS PHOSPHATASES IN PEA EPICOTYL EXTRACTS

Substrate	μg Pi released/mg fr. wt./hr*				
	Plumule	Hook	0–10 mm	10–20 mm	20–30 mm
p-Nitrophenylphosphate	27·8	17·7	3·4	2·0	1·9
Glucose-1-phosphate	1·2	0·7	0·3	0·2	0·1
Glucose-6-phosphate	7·6	6·1	1·2	0·6	0·4
Fructose-1-phosphate	8·7	7·9	2·1	1·7	1·8
Fructose-6-phosphate	2·6	1·3	0·8	1·0	0·5
Fructose-1,6-diphosphate	9·0	6·3	1·8	1·4	1·6

* Enzyme levels in extracts were measured by the initial rate of production of inorganic phosphate (Pi) from sugar phosphates at pH 5·4, the optimum for acid phosphatase activity using *p*-nitrophenylphosphate as substrate. 1·0 μg Pi released from a hexose ester is equivalent to the formation of 0·55 μg free hexose.

TABLE 6. DISTRIBUTION OF ENZYME ACTIVITIES WHICH METABOLIZE SUCROSE IN PEA EPICOTYL EXTRACTS

Enzyme	μg sucrose metabolized/mg fr. wt./hr*				
	Plumule	Hook	0–10 mm	10–20 mm	20–30 mm
Invertase	2·0	7·5	35·5	19·1	9·6
Sucrose synthetase	5·5	5·5	1·7	1·4	1·2
Sucrose-P synthetase	0·7	0·5	0·2	0·1	0·1

* Invertase activity of enzyme extracts was measured by the initial rate of production of reducing power from sucrose, and synthetase activities by the initial rate of formation of sucrose from UDP-^{14}C-glucose plus either fructose or fructose-6-phosphate.

Calculated on a fresh weight basis, activities of all the glucosidases (Table 4), the phosphatases (Table 5) and sucrose and sucrosephosphate synthetase (Table 6) were highest in meristematic and lowest in mature vacuolated tissue. Most activities, like total protein levels (Table 1), fell by a factor of about 10 during development of tissue from the plumule to the epicotyl 10 to 30 mm below. Calculated per unit DNA (i.e., on a cellular basis), such activities increased

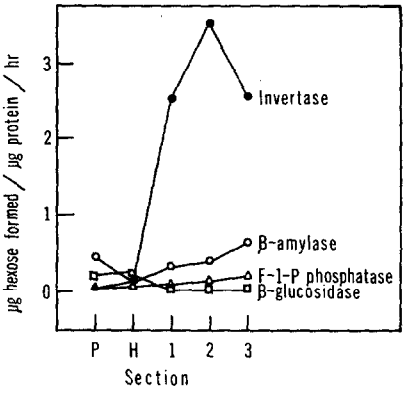

FIG. 2. SPECIFIC ACTIVITIES PER UNIT PROTEIN OF INVERTASE AND THE MOST ACTIVE OF THE GLUCANASES, GLUCOSIDASES AND PHOSPHATASES WHICH WERE ASSAYED IN THIS STUDY (CF. TABLES 1 VS. 4–6).

about 2-fold during growth and maturation. Thus, there was no very marked difference between tissues at successive stages of growth in specific activities of any one of these enzymes (see Fig. 2), and all appeared to be synthesized slowly during development.

One exception to this generalization was β-glucosidase acting on *p*-nitrophenylglucoside. This activity was virtually confined to the youngest meristematic tissues (Table 4). Further studies on its development in the epicotyl in response to hormone treatment[10] led to the conclusion that it was formed in dividing cells only, selectively inactivated in expanding cells and not replaced during maturation. Although this would imply a special role for β-glucosidase in very young cells, its activity obviously failed to lead to detectable accumulation of glucose in the region containing such cells (Fig. 1).

The distribution of invertase activity differed from that of all the above enzymes. On a fresh weight basis, its concentration was lowest in meristematic tissue, much higher in the region of elongation, and lower again in more mature tissue (Table 6). Expressed on a protein (or DNA) basis, as illustrated in Fig. 2, maximum invertase activity was reached in tissue which was just completing elongation. The increase over corresponding values in the plumule was dramatic (300-fold).

Strikingly similar distribution patterns have been described for invertase in various regions of young roots, e.g. in pea,[11] broad bean[12] and corn.[13] There is also growing evidence[9,10,14] that the biosynthesis of this particular enzyme is carefully regulated in plant tissues by mechanisms which include intervention by growth hormones. It is not surprising, therefore, that invertase activity has often been proposed as essential for plant growth.

Control of Sugar Concentrations

From the uniqueness and magnitude of the rise in extractable invertase activity in the regions of cell expansion and maturation (Fig. 2), and the relative ease with which supplied sucrose was inverted there (Table 3), it can hardly be doubted that this enzyme had prime responsibility for the high reducing sugar : sucrose ratio that occurred in these tissues (Fig. 1).

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The difficulty is to understand how glucose levels came to exceed fructose levels. The obvious explanations were not supported by further investigation: neither hexose was preferentially absorbed or utilized by excised sections (Table 2 and 3); potential activities of the common glucosidases and exo-glucanases were relatively low (Table 4); and phosphatases for glucose phosphate were no more active than those for fructose phosphate (Table 5).

To accommodate these results, there seems to be no alternative but to invoke compartmentation of invertase at a locus in growing and maturing tissue where its action results in segregation of the two hexoses of incoming sucrose. A mechanism for such an action can only be speculated about at the present time. Perhaps invertase functions as a hydrolytic permease, utilizing energy in the β-2,1-bond of sucrose to force accumulation of glucose during vacuolation, and leaving fructose susceptible to continued translocation.

In meristematic regions, invertase activity was relatively low and exceeded by that of sucrose synthetase (assayed by measuring the rate of sucrose formation, Table 6). The equilibrium which the latter enzyme catalyses can, under appropriate conditions, favor the formation of fructose and UDPG, and the rate of sucrose cleavage can actually exceed the V_{max} for sucrose synthesis.[15] Thus, any sucrose which survived translocation through the epicotyl to reach plumule and hook might well have escaped inversion there and accumulated instead to the point where it was used as a substrate for sucrose synthetase. In this event, the UDPG so produced could have been used for synthesis of polysaccharide (Table 2), thereby conserving energy of the sucrose bond in chemical form. Fructose could accumulate as observed (Fig. 1). It is possible, therefore, that sucrose synthetase rather than invertase was the key enzyme which governed final sugar concentrations, as well as availability of substrate for wall synthesis, in the plumule and hook.

EXPERIMENTAL

Plant Material

Seeds of *Pisum sativum* L. var. Alaska were surface-sterilized with NaOCl and grown in vermiculite in darkness until the epicotyl developed third internodes 3 to 5 cm long (about 8 days). The internodes were cut with razor blades into sections as shown in Fig. 1.

Sugar Extraction and Estimation

Tissue sections were homogenized three times in hot 80% EtOH and soluble extracts were separated from residues by centrifugation. Aliquots and standard sugars were chromatographed in parallel on Whatman No. 1 filter paper using as solvent *n*-PrOH:EtAc:H_2O (7:1:2, by vol.). Sugars were located with the benzidine reagent or by radioautography. They were eluted from the paper with H_2O or, in the case of sucrose, with dilute invertase solution. Reducing power was estimated with the Nelson modification of the Somogyi reagent.[16]

Labelled Sugar Uptake and Metabolism

Sections from fifty epicotyls were shaken in flasks attached to manometers at 30° in 2·0 ml 0·625% (wt./vol.) sugar uniformly labelled with 1·25 μc ^{14}C. Respired CO_2 was absorbed in alkali in center wells and O_2 uptake was measured manometrically. Respiration rates were linear for at least 2 hr of incubation and they were not affected by the kind of sugar supplied.

At 2 hr, the total amount of respired CO_2 was measured by titration and collected as $BaCO_3$. Sections were washed in H_2O and then homogenized and extracted with hot 80% EtOH. Total radioactivity in these fractions was measured in dried aliquots with a gas-flow detector and counter which registered 70 cpm per μg hexose at the specific activity used in these tests. The main labelled products in the EtOH-soluble fraction were glucose, fructose and sucrose plus several benzidine-positive substances, presumably sugar esters, which travelled on chromatographs with relatively low R_F values. The amounts of label in the free sugars were measured by cutting out the areas they occupied and counting by standard liquid scintillation techniques.

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[16] J. E. HODGE and B. T. HOFREITER, in *Methods in Carbohydrate Chemistry* (edited by R. L. WHISTLER and M. L. WOLFROM), Vol. I, p. 380, Academic Press, New York (1962).

The efficiency was such that 150 cpm were registered per μg hexose. The EtOH-insoluble fraction contained virtually all of its label in polysaccharides[5] and therefore it is referred to here as the wall fraction.

Assays for Enzymic Activities

For routine preparation of enzymic protein, sections were homogenized twice in a total of 4 vol. of cold 20 mM Na phosphate, pH 7·0, and centrifuged at 35,000 *g*. The supernatant was dialysed vs. H_2O overnight. The soluble fraction contained 70 to 85% of the total protein in all sections and essentially all (95%+) of the enzyme activities which were assayed in this study (exceptions noted below).

Reaction conditions for measuring various enzyme activities are summarized below. Unless indicated otherwise, during every assay boiled controls and at least three aliquots of reaction mixture were removed routinely at intervals after zero time in order to record the progress of the reaction and ensure that initial rates were being measured. The appropriate tests established for each assay that these rates were proportional to enzyme concentration.

Invertase (E.C. 3.2.1.26). Reaction was initiated by adding enzyme to sucrose at a final concentration of 0·1 M in 50 mM sodium succinate buffer, pH 5·0, at 30°. Aliquots were boiled 3 min to stop reaction and reducing power was measured with the Somogyi reagent.[16] Virtually all of the invertase activity in the epicotyl was recovered in the soluble enzyme fraction and had a pH optimum of 5·0.[9,10]

α- and β-Glucosidases (E.C. 3.2.1.20–21). Reaction mixtures contained the enzyme present in total (uncentrifuged) homogenate plus 1·1 mM *p*-nitrophenylglucoside in 60 mM Na acetate buffer, pH 5·0. After incubation at 30°, reaction was terminated in aliquots by making them alkaline with Na_2CO_3. Free nitrophenol was estimated colorimetrically at 420 nm.[17] Maltase and cellobiase activities were measured under these same conditions except that initial disaccharide concentrations were 10 mM and production of free glucose was estimated with glucose oxidase ("Glucostat" reagent, Worthington Biochemicals).

Glucanases. Reaction mixtures contained soluble enzyme plus 1·0% (wt./vol.) dialysed suspension of polysaccharide and 50 mM Na acetate, pH 5·8. After incubation at 30°, increment in reducing power was measured with the Somogyi reagent. With starch as substrate, chromatography of reaction mixtures showed that all of the reducing power which was produced was present as maltose, thus confirming the finding[6] that pea epicotyl amylase is an exo-hydrolase (β-amylase, E.C. 3.2.1.2.). Using laminarin (largely β-1,3-glucan) as substrate, however, chromatographs showed that several reducing dextrins were produced by the enzyme which therefore was an endo-hydrolase (E.C. 3.2.1.6). Using long-chain cellulose derivative (CMC) as substrate, viscosity measurements[18] also indicated endo-hydrolase activity (i.e. cellulase, E.C. 3.2.1.4).

Phosphatases acting on sugar phosphates (E.C. 3.1.3.9–11). Reaction mixtures contained enzyme and 10 mM substrates in 40 mM phthalate buffer, pH 5·4, at 30°. Production of inorganic P was measured by a modification of the Fiske-Subba Row method.[19] Acid phosphatase activity (E.C. 3.1.3.2) was assayed using *p*-nitrophenylphosphate as substrate under the above reaction conditions and measuring production of *p*-nitrophenol.[17]

Sucrose synthetase (E.C. 2.4.1.13). Reaction mixtures[20] contained enzyme (concentrated by dialysis 2- to 20-fold with polyethylene glycol), 1 μmole uridine diphospho-^{14}C-glucose (UDPG, 10^6dpm/μmole), 2 μmoles fructose, 0·1 μmole $MgCl_2$, 10 μmoles tris buffer, pH 8·0, in a total volume of 0·1 ml. After 30 min at 30°, the mixture was transferred and dried quickly at the origin of a Whatman No. 1 paper chromatograph. "Control" preparations incubated in the absence of fructose were added to adjacent spots. After chromatography, radioactive sugars were located by radioautography and amounts were calculated from counts measured by liquid scintillation. Negligible amounts of sucrose were synthesized by these preparations unless fructose was added. No glucose was formed from the sucrose indicating that invertase was not active under conditions of these tests (cf. pH optima).

Sucrose phosphate synthetase (E.C. 2.4.1.14). The assay measured production of sucrose under conditions identical to those for sucrose synthetase except that fructose-6-phosphate replaced fructose in the reaction mixture.

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