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Characteristics of Sugar Uptake in Hypocotyls of Cotton[1]

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ABSTRACT

Uptake of sucrose and hexoses by cotton (*Gossypium hirsutum* L.) hypocotyl segments from free space was shown to be an active, carrier-mediated process. Separate carriers existed for hexoses and sucrose. Accumulated sugars appeared in both soluble and insoluble fractions of the tissue. At optimum temperature and pH, sucrose uptake rate *versus* concentration was fit by a rectangular hyperbola with V_{max} of 14 micromoles per gram fresh weight per hour and K_m of 8 mM. Sucrose was the principal sugar found in the free space *in vivo*, and invertase activity was essentially absent from that space except after aging.

The term "sink strength" refers to the ability of tissues of higher plants to increase in dry weight through the acquisition of assimilates. Sink strength may be in part an intrinsic property of the tissue related to its size and activity, or it may result indirectly from factors affecting assimilate availability. For the whole plant, there is considerable evidence that the most limiting factor to assimilate availability is assimilate production rather than vascular transport capacity. Investigation of the substrate dependence of growth rate in callus by Hunt and Loomis (13) suggested that the movement of assimilates within the sink tissue is also important.

Transport from the phloem to individual cells within sink tissue can be either symplastic or apoplastic. The continuity of the symplast is well established (8, 21), though its importance as a solute pathway is uncertain. The apoplast now appears to be of major importance for short distance transport (3–7, 20).

If apoplastic transport is of general significance, then the intrinsic sink strength of a tissue should be reflected in the rate of assimilate uptake from the free space solution. The use of excised tissue allows the level of free space assimilate to be varied with a bathing solution, permitting direct measurement of the characteristics of uptake. Excised tissue also permits precise manipulation of a number of other variables, such as temperature, osmotic stress, light, nutrient supply, and hormone levels, so that their influences on sink activity can be examined. From studies of sugar uptake from the free space solution, it has been concluded that sugar uptake in a number of species of higher plants is an active, carrier-mediated process (2, 3, 7, 17, 19). For a more extensive review, see reference 9.

The primary translocation compound in cotton (*Gossypium hirsutum* L.) is sucrose. Reported here is the uptake of sucrose and its inversion products, glucose and fructose, from free space by hypocotyl tissue from young cotton palnts.

MATERIALS AND METHODS

Uptake of glucose, fructose, and sucrose was observed in segments from fully expanded hypocotyls of cotton (*G. hirsutum* L.). Cotton plants ('Acala SJ 2') were grown at 5-cm spacing in a greenhouse (32 C day and 22 C night ± 5 C) in flats of peat, loamy soil and sand mixed in volumes of 1:1:1. The plants were watered daily with 0.5-strength modified Hoagland solution (18). Hypocotyl elongation was generally complete by the 12th day. Seeds were planted at weekly intervals, and uniform plants were selected between 14 and 21 days old. The top 1 cm of the hypocotyls was discarded, and the next 2 cm (approximately) was sliced into 1-mm segments. The segments were washed in distilled H_2O for 1 hr to remove free space solutes and cytoplasmic contamination due to cutting. When hexose uptake was to be measured, the prewashing period was extended to 2 or 3 hr.

Sugar solutions used in uptake studies were prepared fresh at the beginning of each experiment. Buffers, osmotica, hormones, and minerals were generally not included since they did not appear to be beneficial. One ml of the unlabeled sugar solutions was dispensed into 50-ml culture tubes, and 10 washed hypocotyl segments were added. After a 1-hr adjustment period, 50 μl of uniformly labeled sugar solution (0.1 μM) containing 250,000 cpm was added per tube for the uptake experiments. Labeled sugars were obtained from ICN Chemical and Radioisotope Division, Irvine, Calif. The constant amount of label and varied concentrations of unlabeled sugar gave a variable specific activity that was greater in the dilute solutions. Unless specified otherwise, all uptake studies were done on a rotary shaker at 33 C in the light (140 μE photosynthetic active radiation m^{-2} sec^{-1}). No special precautions were taken to keep the solutions sterile during uptake or preconditioning since no microbial growth was observed and treatment with 50 mg/l streptomycin had no effect.

After the uptake period (generally 2 hr), the segments were rinsed with several changes of distilled H_2O for 1 hr to remove free space label. They were then blotted dry and placed in scintillation vials, two segments/vial, with 2.5 ml of 95% ethanol, sealed, and heated at 70 C for 4 hr to extract alcohol-soluble compounds. After cooling, 5 ml of a toluene scintillation cocktail (6 g/l Omniscint, ICN) was added, and the samples were allowed to stand overnight. Adding the cocktail to the ethanol already in the vial resulted in a final combination of ethanol-toluene (1:2, v/v) plus Omniscint (4 g/l). Each sample was counted for 1 min on a scintillation counter. Over-all counting efficiency for ^{14}C was about 80%. Quench due to the tissue was less than 5%. After an initial counting, the hypocotyl segments, which now retained only alcohol-insoluble compounds, could be removed and counted separately in fresh scintillation fluid. The remaining cocktail, containing alcohol-soluble compounds, could also be counted separately. The results thus obtained are presented as total, soluble, and insoluble label in the tissue.

Counting the segments by twos allowed five replications/50-ml tube. The standard deviation among segments in one tube was generally less than 12%, yielding a standard error for their mean of about 6%. Unless stated otherwise, that may be taken as the standard error of the results. The standard deviation among tubes replicated on the same day was generally less than 8%. Experiments were usually done with one tube/treatment, and the entire experiment was replicated several times on different days. Representative experiments are shown here.

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RESULTS AND DISCUSSION

Time Course of Uptake. The characterization of transport kinetics generally requires steady-state conditions during observation. With sucrose, the time course of accumulation from a 60 mM solution was linear during the first 6 hr (Fig. 1A). Linear uptake was obtained with higher and lower concentrations of either sucrose or hexose and often continued for as long as 10 hr. It was assumed that experiments lasting 4 hr or less were done in steady-state conditions.

In contrast, hexose uptake in fresh tissue generally showed a lag period of about 2 to 3 hr before reaching a constant long term uptake rate. This lag period could be eliminated by prewashing in H_2O for several hr. Figure 1B shows the uptake by fresh (brief rinse only) and prewashed tissue of glucose from a labeled 10 mM solution, with distribution into soluble and insoluble fractions. Prewashing in 100 mM glucose or sucrose gave similar results to prewashing in H_2O. The acceleration of uptake may have been due to carrier induction, although in other experiments the K_m of glucose uptake did not differ significantly between fresh and aged tissue. If additional carriers were induced, they were not of a different "high affinity" form as observed in squash hypocotyl tissue (11, 12). (All other hexose-uptake experiments included prewashing for 3 hr so that the initial lag phase could be avoided.)

To learn whether label was redistributed between the alcohol-soluble and -insoluble pools during the rinse period following uptake, hypocotyl segments were incubated in labeled 10 mM sucrose for 4 hr. Immediately after uptake, the segments were placed in H_2O, and samples of tissue were removed periodically, blotted, and counted to determine the label distribution (Fig. 2). Insoluble compounds stopped forming when uptake ceased and, after the initial rapid efflux from the free space, no additional label was lost from the tissue. Results were identical when washing was in 60 mM sucrose. Over longer periods, soluble label was slowly depleted when the tissue was left in H_2O, but insoluble products apparently were not synthesized. It seems that synthesis was dependent on a continuous uptake of new substrate, and was unsupported by internal sugar. After 4 hr of uptake, over 90% of the alcohol-soluble label was in sugar (principally sucrose, regardless of whether sucrose or hexose was supplied to the tissue). About 25% of the insoluble label was in starch; the rest was mostly in cell wall material.

Temperature. Temperature strongly affected sucrose uptake from a 60 mM solution (Fig. 3). The optimal temperature for uptake, 35 C, was the same as the optimal temperature for growth of cotton hypocotyls. The temperature used in all other experiments was 33 C.

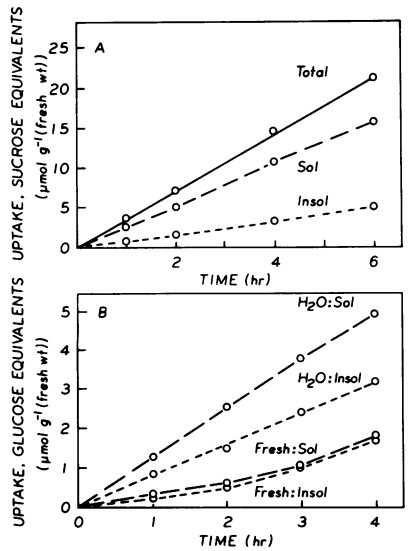

FIG. 1. A: Effect of incubation time on uptake and incorporation of 60 μM sucrose by hypocotyl segments. B: uptake and incorporation of 10 mM glucose by hypocotyl segments as affected by 4-hr pretreatment in H_2O. Curves labeled as (pretreatment:soluble or insoluble fractions).

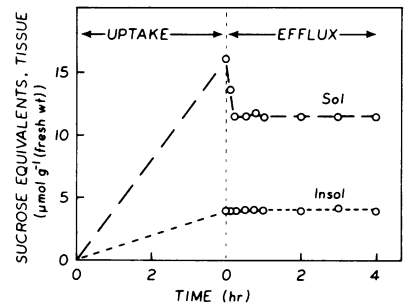

FIG. 2. Sucrose uptake and incorporation during incubation in a 10 mM sucrose solution and efflux into H_2O.

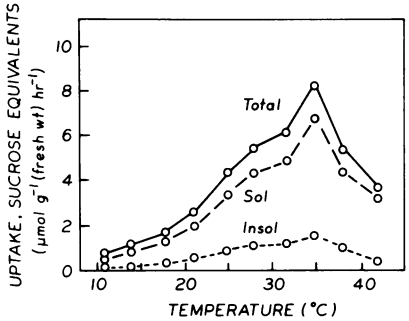

FIG. 3. Effect of temperature on uptake and incorporation of 60 mM sucrose.

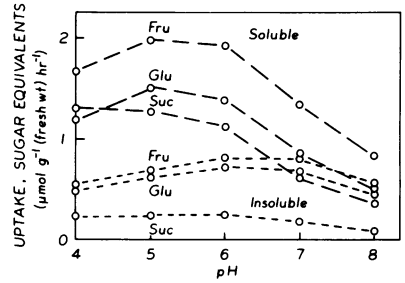

FIG. 4. Effect of pH on uptake and incorporation of 5 mM glucose, fructose, and sucrose into soluble and insoluble fractions.

pH. The pH of the incubation solution has been shown to have strong effects on sugar uptake and sucrose inversion (1, 5, 7, 14–16). The effect of pH on hexose and sucrose uptake was investigated over a pH range of 4 to 8 established with 50 mM Na-phosphate as buffer. That concentration was found not to be detrimental to uptake, and pH did not drift during incubation. Total uptake from 5 mM glucose, fructose, or sucrose, primarily reflecting uptake into the soluble pool, declined as pH increased above 6 to 7 (Fig. 4). In this experiment, hexose incorporation into insoluble compounds was not affected greatly. In other experiments, hexose incorporation was reduced at pH 7, but accumulation in the soluble pool was always more sensitive to pH. The inhibition of uptake above pH 8 could be reversed by adding acid, indicating that the effect was due to pH, not the amount of salt in solution. It is unlikely that the effect of pH on sucrose uptake was due to an effect on invertase activity, since sucrose was not inverted significantly during uptake (see "Carrier Specificity," below) and hexose uptake was also pH-dependent. Those results are consistent with the sugar-proton symport theory of sugar uptake (14–16). It is possible that internal (cytoplasmic and vacuolar) pH was also changed by this treatment, and processes such as sucrose synthesis and storage may also have been affected.

Through feedback, a reduction in utilization might have reduced uptake.

Buffers were not used in other experiments since the tissue generally stabilized the solution pH at about 6. It is unlikely that the normal apoplastic solution has significant buffering capacity, so it was intended that the unbuffered incubation solution would approximate that condition and allow the tissue segments to determine the pH of their environment, as *in vivo*.

Osmotic Potential. The effect of the osmotic strength of the incubation solution was investigated with mannitol additions. Concentrations of less than 0.5 M had no effect on sucrose uptake by tissue incubated in labeled 60 mM sucrose (Fig. 5A) or in 0.1 μM (label only). The tissue was slightly wilted at 0.5 M mannitol and turgor was lost at higher concentrations.

The effect of overnight preincubation in either H_2O or 100 mM sucrose on the mannitol response was observed with uptake of labeled 10 mM glucose (Fig. 5B) (Sucrose could not be used since overnight pretreatment of excised tissue induced large amounts of extracellular invertase.) Pretreatment with sucrose suppressed glucose uptake at low levels of mannitol (feedback) but contributed to adjustments (presumably osmotic) which reduced osmotic stress above 0.4 M mannitol. This effect was particularly pronounced in other experiments with 10-day-old plants.

Carrier Specificity. Experiments were conducted with mixtures of glucose, fructose, and sucrose to identify the specificity of the carriers involved in their uptake. A lack of interaction (competition) between sugars during uptake is evidence that they do not compete for a common carrier. A strong reduction of uptake of one sugar by another is evidence for a common carrier but does not rule out the possibility of cytoplasmic interactions that feed back on uptake.

A problem commonly encountered in measuring sucrose uptake is the occurrence of free space invertase. Sucrose and hexose seem to share a common carrier if a significant fraction of the sucrose is hydrolyzed before uptake. When hypocotyl tissue was incubated for 2 hr in labeled 40 mM sucrose with increasing concentrations of glucose, glucose competed with sucrose uptake only to a limited extent, and glucose beyond 10 mM had no additional effect (Fig. 6A). The specificity of sucrose uptake was investigated further by doubling the sucrose concentration through the addition of 40 mM

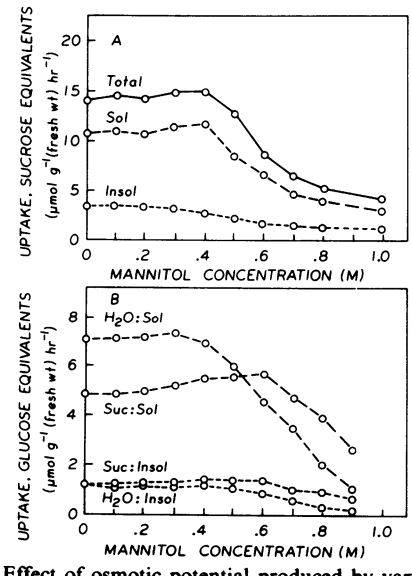

FIG. 5. A: Effect of osmotic potential produced by varying mannitol concentrations on uptake and incorporation of 60 mM sucrose by hypocotyl segments. B: Effect of osmotic potential produced by varying mannitol concentrations on uptake and incorporation of 10 mM glucose. Tissue pretreated overnight in either 100 mM sucrose or H_2O. Curves labeled as (pretreatment:soluble or insoluble fraction).

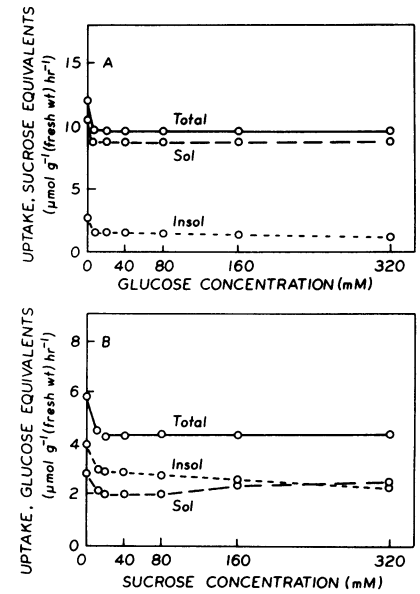

FIG. 6. A: Effect of glucose concentration on uptake and incorporation of 40 mM sucrose by hypocotyl segments. B: Effect of sucrose concentration on uptake and incorporation of 20 mM glucose.

unlabeled sucrose in the presence of 320 mM glucose. Sucrose uptake was essentially saturated at 40 mM sucrose. The number of counts found in the soluble fraction after uptake was 1,100 from 40 mM sucrose plus 320 mM glucose, and 650 from 80 mM sucrose (diluted label) plus 320 mM glucose. The insoluble fraction was reduced similarly (about 50%). This response to isotopic label dilution in the presence of saturating levels of glucose clearly demonstrates the specificity of sucrose uptake. Glucose competed to a greater extent with incorporation into the insoluble fraction than with storage into the soluble pool. That might be expected since sucrose must be inverted before being used in synthetic reactions. Fructose competed with sucrose uptake in a similar way, though to a lesser extent.

The reverse experiment of sucrose competition with uptake of labeled glucose also yielded results suggestive of limited free space sucrose inversion (Fig. 6B). Sucrose competed only slightly with uptake of 20 mM glucose. Glucose uptake was saturated at 20 mM, and, as in the previous experiment, the specificity of glucose uptake was demonstrated by a 50% isotopic dilution of the labeled glucose in the presence of 320 mM sucrose. As before, label uptake was reduced 50%, demonstrating that unlabeled glucose was competitive with labeled glucose but that sucrose was not.

Glucose and fructose appeared to share a common hexose carrier, since glucose competed with the entire flux of fructose (Fig. 7A). Fructose was a poor competitor with glucose, since even high concentrations of fructose did not completely suppress glucose uptake (Fig. 7B). Competition of fructose with glucose uptake did not follow a simple relation. In fact, in other experiments, the addition of small amounts of fructose sometimes stimulated glucose uptake; this may have been due to internal interactions between glucose and fructose that resulted in a rate of sucrose synthesis greater than when glucose was presented alone. Increased glucose utilization might then affect glucose uptake through reduction in feedback inhibition. Isotopic dilution of labeled glucose in the presence of 320 mM fructose reduced label uptake considerably, whereas dilution of labeled fructose in the presence of 320 mM glucose had no effect. When presented in equal concentrations, the superior competitive ability of glucose over fructose was maintained over a wide concentration range, with glucose uptake being about 2.5 times as great as fructose uptake (Fig. 7C). That experiment was done by preparing dupli-

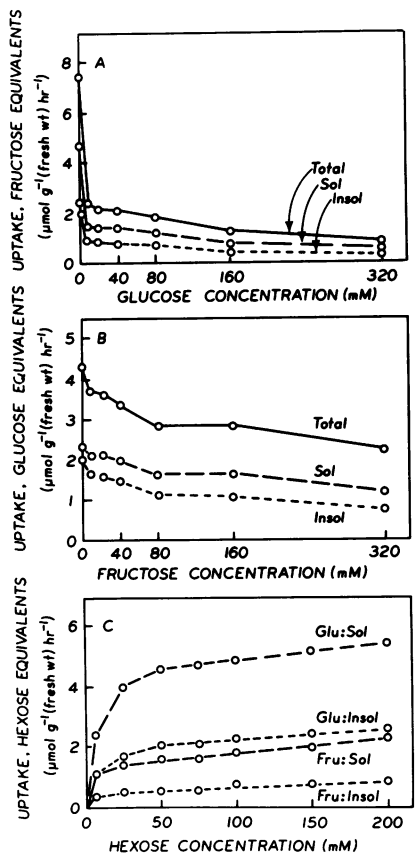

FIG. 7. A: Effect of glucose concentration on uptake and incorporation of 20 mM fructose by hypocotyl segments. B: Effect of fructose concentration on uptake and incorporation of 20 mM glucose. C: Effect of increasing equimolar glucose and fructose concentrations up to 100 mM (200 mM total) on uptake and incorporation of glucose and fructose. Curves labeled as (sugar taken up:soluble or insoluble fraction).

cate samples for each concentration and labeling one with glucose and the other with fructose.

The apparent competition in those experiments could have been due to internal interaction rather than competition for a carrier, although the absolute specificity of uptake in the presence of high levels of competitor is strong evidence for the existence of separate sucrose and hexose carriers. In keeping with that interpretation, asymmetrically labeled sucrose was not significantly randomized during uptake (data not shown).

Kinetics of Sugar Uptake. The concentration responses of glucose, fructose, and sucrose uptake are graphed in a Lineweaver-Burk plot (Fig. 8) to emphasize the Michaelis-Menten constants, V_{max} and K_m. The linear plots indicate that the uptake of all three sugars can be fitted quite precisely with rectangular hyperbolae, as would be expected in carrier-mediated uptake. Values of V_{max} were 4.5, 20 and 14, μmol g^{-1} (fresh weight) hr^{-1} for glucose, fructose, and sucrose, respectively; corresponding values of K_m were 1.0, 8 and 8 mM. Although the constants varied among experiments, those relative values were representative of a large number of experiments. In general, glucose had the smallest K_m and V_{max}, while the K_m values for fructose and sucrose uptake were larger and quite similar to each other except that the sucrose constants are doubled if expressed in hexose equivalents. The V_{max} was generally greater for fructose uptake than for sucrose, although comparison at high concentrations was complicated by the fact that uptake sometimes displayed an apparently diffusive component in addition to the carrier-like phase. The observed K_m was always smaller for glucose uptake than for fructose uptake, so the competition between the hexoses may be due to the carrier's greater "affinity" for glucose than for fructose.

The differences between winter and summer material was noteworthy. K_m varied considerably and in some experiments done in the winter the results were as much as three times as great as the values given above for summer material. The basis for the variation is not known, but it demonstrates the potential range over which sugar transport might be regulated.

Another aspect of uptake that deserves attention is the partitioning of label between the soluble and insoluble pools. Growth, as measured by dry weight accumulation, can be strongly determined by the kinetics of synthetic reactions. Hunt and Loomis (13) demonstrated the relationship of tobacco callus growth to external sucrose concentration. Their results described a rectangular hyperbola with a K_m of 3.7 mM. In cotton hypocotyl tissue, when sugar uptake was observed over a wide concentration range (Fig. 9), the insoluble pool became saturated at lower external concentrations than did the soluble pool. Being a multistep process, incorporation kinetics might be expected to be complex, and for fructose and sucrose they generally were. Below 4 mM, however, glucose incorporation described a rectangular hyperbola quite precisely, with a K_m of 0.8 mM and a V_{max} of 1.33 μmol g^{-1} (fresh weight) hr^{-1}. That V_{max} is equivalent to 5.7 mg g^{-1} (fresh weight) day^{-1}, and incorporation of sucrose was often double that. The growth rate observed in the greenhouse for tissue of that age was about 4 mg (dry weight) g^{-1} (fresh weight) day^{-1}. Clearly, uptake and incorporation of free space sugars by excised tissue could be rapid enough to support the growth rates observed *in vivo*. Uptake kinetics of fructose and sucrose generally reflected the rate of soluble sugar accumulation since that was the major fate of the sugars, particularly at the higher concentrations.

Free Space Sugars. Free space sugar concentration *in vivo* was estimated by slicing hypocotyls into segments of 0.5, 1.0, and 2.0 mm and then washing out and analyzing the free space sugars by GLC. The amount of cytoplasmic sugar released through slicing doubled when the size of the tissue segments was halved, whereas the contribution of actual free space sugars presumably did not change. Experiments using that method yielded free space sucrose concentrations between 1 and 8 mM. Hexoses were not detected in the free space. *In vitro*, a 2 mM sucrose solution was sufficient to support the synthesis of insoluble compounds at a rate equal to the growth rate of cotton seedlings observed in the greenhouse.

Other Aspects. The soluble sugar found in cotton tissues was

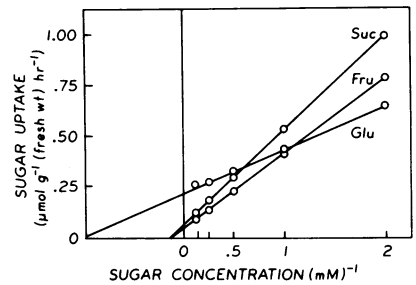

FIG. 8. Lineweaver-Burk plot of glucose, fructose, and sucrose uptake by hypocotyl segments.

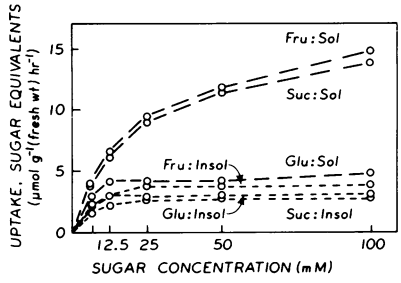

FIG. 9. Effect of substrate concentration on uptake and incorporation of glucose, fructose, and sucrose by hypocotyl segments.

largely sucrose, no matter which sugar was supplied, and the synthesis and storage of sucrose may have been controlling factors in uptake. Incubation in fructose generally increased glucose, fructose, and sucrose levels in the tissue, whereas uptake of glucose often resulted in a decrease in fructose levels and increases in glucose and sucrose. This suggests that the large V_{max} of fructose uptake may have resulted from rapid sucrose synthesis, while sucrose synthesis from glucose was limited by slow fructose formation. Why glucose might be formed from fructose more rapidly than the reverse was not apparent.

The low K_m for incorporation into insoluble compounds suggests that synthesis had a higher priority for sugar use than did storage. With sugar concentrations that were saturating to incorporation but less than saturating to the soluble pool, manipulations that reduced uptake generally reduced the soluble pool more than the insoluble pool.

Sucrose uptake kinetics often suggested some free space inversion and uptake as hexose since an acid invertase (K_m around 2 mM) was present in various amounts (generally less than a V_{max} of 2 μmol g^{-1} (fresh weight) hr^{-1} in the free space of the tissue. Washing 1-mm slices for as little as 4 hr significantly increased free space invertase activity, and washing overnight greatly increased activity to a V_{max} of around 20 μmol g^{-1} (fresh weight) hr^{-1}. Those results were obtained by incubating hypocotyl slices in a sucrose solution, and analyzing the incubation solution by gas chromatography for the disappearance of sucrose and the appearance of glucose and fructose. Presumably because of inversion, sucrose uptake may appear biphasic, with the lower phase being characteristic of glucose uptake. It was possible to eliminate that effect in fresh tissue by observing sucrose uptake in the presence of 100 mM glucose. Inverted sucrose would then compete with such a large amount of unlabeled glucose for the hexose carrier that the only label taken up would be uninverted sucrose. The invertase activity present in aged tissue was too great for that technique to be used.

It is likely that applied glucose also competed with cytoplasmically hydrolyzed sucrose in utilization. If internal inversion and utilization of sucrose were to regulate sucrose uptake, internal glucose competition might achieve the same results as external competition. In either event, the uptake and storage of sucrose can be observed independently from its inversion products when uptake is measured in the presence of glucose.

There was some evidence of a second phase of hexose uptake between 50 and 200 mM, although, because of its highly variable nature and the unphysiological concentrations involved, that range was not investigated extensively. Also, the monophasic responses observed below 50 mM did not necessarily rule out the possibility of a greater number of closely spaced phases. If uptake is regulated by the utilization rate, each process utilizing sugar might potentially affect the kinetics of uptake. Although uptake did not always conform to perfect rectangular hyperbolae, there was no consistent evidence for a multiphasic response.

Sugar uptake by hypocotyl tissue was active, inasmuch as overnight incubation in 2 mM sucrose of fructose increased the tissue sucrose content from 5 to 10 mM to over 20 mM. Treatment with metabolic inhibitors such as 2,4-dinitrophenol, rotenone, and cyanide or exposure to anaerobic conditions or low temperature often reduced uptake by over 90% (10).

In summary, it seems that sugar uptake from the free space in cotton hypocotyl tissue is an active process mediated by separate carriers which are specific between sucrose and hexose. The hexose carrier seems to have a higher affinity for glucose than for fructose. Significant invertase activity was not detected in the free space of fresh hypocotyl tissue, although considerable activity was induced beginning several hr after excision. When sucrose was presented at a concentration equal to that found in the free space *in vivo*, it was incorporated into alcohol-insoluble compounds at a rate equal to growth rates *in vivo*. That is taken as evidence for an apoplastic route in short distance transport, and for the significance of sugar uptake from free space by excised tissue as a model of sink activity. Regulation of this phenomenon is considered by Hampson *et al.* (10).

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