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Sucrose-Metabolizing Enzymes in Transport Tissues and Adjacent Sink Structures in Developing Citrus Fruit[1]

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ABSTRACT

Juice tissues of citrus lack phloem; therefore, photosynthates enroute to juice sacs exit the vascular system on the surface of each segment. Areas of extensive phloem unloading and transport (vascular bundles + segment epidermis) can thus be separated from those of assimilate storage (juice sacs) and adjacent tissues where both processes occur (peel). Sugar composition, dry weight accumulation, and activities of four sucrose-metabolizing enzymes (soluble and cell-wall-bound acid invertase, alkaline invertase, sucrose synthase, and sucrose phosphate synthase) were measured in these transport and sink tissues of grapefruit (*Citrus paradisi* Macf.) to determine more clearly whether a given enzyme appeared to be more directly associated with assimilate transport *versus* deposition or utilization. Results were compared at three developmental stages. Activity of sucrose (per gram fresh weight and per milligram protein) extracted from zones of extensive phloem unloading and transport was significantly greater than from adjacent sink tissues during the stages (II and III) when juice sacs grow most rapidly. In stage II fruit, activity of sucrose synthase also significantly surpassed that of all other sucrose-metabolizing enzymes in extracts from the transport tissues (vascular bundles + segment epidermis). In contrast, sucrose phosphate synthase and alkaline invertase at this stage of growth were the most active enzymes from adjacent, rapidly growing, phloem-free sink tissues (juice sacs). Activity of these two enzymes in extracts from juice sacs was significantly greater than that form the transport tissues (vascular bundles + segment epidermis). Soluble acid invertase was the most active enzyme in extracts from all tissues of very young fruit (stage I), including nonvascular regions, but nearly disappeared prior to the onset of juice sac sugar accumulation. The physiological function of high sucrose synthase activity in the transport tissues during rapid sucrose import remains to be determined.

Two alternative functions have been attributed to sucrose-metabolizing enzymes in sink tissues. Several studies have emphasized a role in the capacity for storage/utilization in sink cells (16–18, 25, 35), while others have suggested a more direct association with transport and/or phloem functioning (13–16, 30, 34). In some instances a given enzyme may be involved in both (16). The two functions are often difficult to distinguish, however.

Initial work with maize kernels (30) focused on this point and showed with serial tissue sections that acid invertase was most active where sucrose exited the transport path, rather than at sites of eventual storage. This finding was consistent with an earlier proposal that sucrose entering sugarcane stems moved from phloem into extracellular space where it was hydrolyzed by an extracellular invertase prior to uptake and resynthesis (possibly by SPS[4]) (13–15). Similarities between results obtained in maize and sugarcane led to the general view that the described sequence of events proceeded the same way in many tissues. More recent histochemical studies of invertase in maize tissues have confirmed earlier localizations (8), but sucrose hydrolysis no longer appears essential for all sucrose import into kernels (29) or many other sink structures (15). Likewise, the extent of sucrose breakdown and activity of associated enzymes during sucrose import is now believed to vary considerably. Activity of both alkaline invertase and the reversible enzyme, SS appear to be related to the extent of assimilate uptake by numerous sink tissues (6, 16, 25), while the involvement of SPS in sucrose-importing organs remains equivocal (12, 22, 36).

Particularly intriguing among studies in this area have been attempts to separate the involvement of sucrose-metabolizing enzymes in transport processes from those of storage or metabolic functions. SS, for example, is believed to function primarily in sucrose degradation. The prevailing viewpoint and majority of data favor a role for this enzyme in metabolism of sink cells, either for production of respiratory substrates (17, 25, 35) or precursors for synthesis of complex carbohydrates (1, 16, 35). However, Hawker and Hatch (15) and Slack (34) initially reported that the vast majority of SS in sugarcane stems appeared to be localized in vascular bundles. Dick and ap Rees (7) later found elevated SS activity in the stele (vascular tissues) *versus* the cortex (nonvascular) of pea roots. Claussen *et al.* (5) also observed greater SS activity in midribs compared to adjacent leaf blades.

The structure of citrus fruit (Fig. 1) allows still further resolution of enzyme involvement in transport tissues *versus* sink cells because the two tissues can be physically separated: phloem-free sink cells (multicellular juice sacs) and transport tissues (vascular bundles + segment epidermis [both along the path for assimilates entering juice sacs (20)]. Assimilate entry into juice sacs occurs via three vascular bundles (one dorsal

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[4] Abbreviations: SPS, sucrose phosphate synthase; SS, sucrose synthase; MCW, methanol:chloroform:water; PVPP, polyvinylpolypyrrolidone; UDPG, uridine diphospho-glucose; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; RuBP, ribulose bisphosphate.

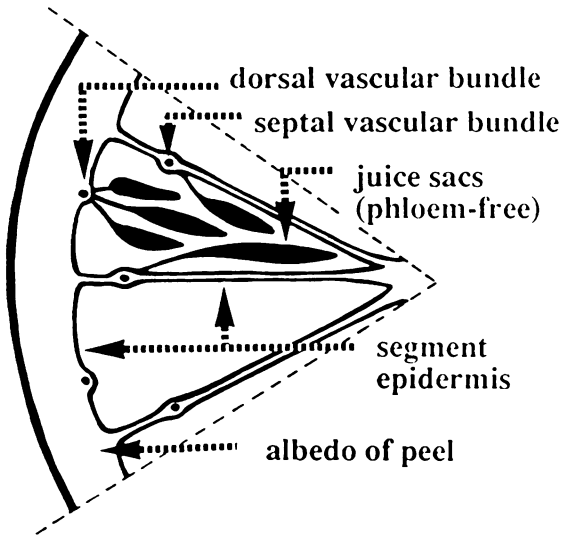

Figure 1. Diagram of vascular and nonvascular areas of developing citrus fruit.

and two septal) exterior to the segment epidermis of each juice section (20). All photosynthates enroute to juice sacs must exit the phloem at these sites because the vascular system does not extend into the juice tissues (23). Sucrose cleavage at these points is generally minimal (20; and our unpublished data). The majority of assimilates then enter the segment epidermis through which they are transported to the bases of hair-like stalks subtending juice sacs (KE Koch, Avigne, unpublished data). Areas of extensive phloem unloading and transport (vascular bundles and segment epidermis, respectively) can thus be separated from those of phloem-free storage areas (juice sacs), and adjacent tissues where both processes occur (peel). Invertase (EC 3.2.1.26), SS (EC 2.4.1.13), and SPS (EC 2.4.1.14) were assayed in extracts of these tissues to more clearly determine the association between activity of a given enzyme and processes more directly related to assimilate transport *versus* deposition or utilization.

MATERIALS AND METHODS

Plant Material

'Marsh' grapefruit (*Citrus paradisi* Macf.) were collected from the outer, southern canopy of 6 mature trees in Lake Wales, FL, during the 1985 growing season. Samples for enzyme assays from this season were harvested on June 1, August 6, and September 30. Each of three replications at a given stage of development consisted of four fruit from three trees. Fruit also were collected in a similar manner form nine mature trees in Lake Alfred, FL, biweekly during the previous season. In this instance, six fruit from three trees were used for each replication. Extreme winter cold destroyed all trees used for the first set of data, so the comparable, but more southerly Lake Wales site was used for subsequent sampling.

Equatorial and longitudinal diameters were measured and individual fruit weighed. These data, together with anatomical examinations of developing grapefruit (23), were used to determine timing of the three classic stages of citrus fruit growth initially described for oranges by Bain (2). Grapefruit in the present study were undergoing active cell division (stage I), during the first 10 weeks of development, when their equatorial diameters were less than 6.0 ± 1.0 cm. The expansion phase (stage II) took place in fruit with diameters 6.0 ± 2.0 cm to 9.3 ± 2.0 cm, and from 10 to 25 weeks after fruit set. The maturation phase (stage III) occurred in grapefruit larger than this, after approximately 25 or more weeks' development. Sugar levels and dry weight generally increase slowly during this final period of growth, which varies widely in duration depending on climatic conditions (32, 33).

Tissues were separated into albedo (inner, nonpigmented peel), juice sacs, and a fraction containing major vascular bundles + segment epidermis (with the central axis in very young fruit). Fresh and dry weights were determined for samples of each. The tissues were frozen and stored at −80°C until use. Enzyme activities and sugar compositions varied little between extracts from fresh *versus* frozen tissues, and remained constant under stated conditions for at least 1 year.

Sugar Analysis

Samples were homogenized for 5 to 10 min with a Polytron (Brinkman Instruments, Westburg, NY) in 5 volumes (v/w) of MCW (12:5:3, v/v). Extract was filtered through Whatman No. 2 paper, rinsing insolubles with the above solution. Water and chloroform were then added to bring the final M:C:W ratio to 10:6:5. Subsequent separation of a chloroform layer allowed removal of lipids and pigments. The remaining aqueous-alcohol phase was evaporated to dryness and resuspended in a known volume of water. A 2 mL aliquot was passed through a C-18, Sep-pak column (Water's Associates, Inc., Millford, MA) and a 0.45 μm filter before injection into a Bio-Rad and HPLC system (Richmond, CA). Water flowing at 0.6 mL min^{-1} was used for the mobile phase. A calcium-form cation exchange column (HPX-87C, Bio-Rad) was used at 85°C and eluted sugars were detected with a refractometer (Bio-Rad). Peaks were quantified using known standards.

Enzyme Extraction

All enzymes were extracted and desalted at 0 to 5°C. For soluble invertases, tissues were homogenized for 5 min with a Polytron (Brinkman, Westbury, NY) using a ratio of 1 g tissue:5 mL 200 mM potassium phosphate buffer (pH 7.5), with 5 mM $MgCl_2$, 5 mM DTT, and 5% PVPP. The relatively high molarity of buffer (200 mM) was used to inhibit binding of soluble enzymes to the insoluble fraction during homogenization. Homogenate was filtered through 4 layers of cheesecloth, rinsed with 5 mL extraction buffer minus PVPP, and centrifuged at 20,000*g* for 10 min. Proteins in the supernatant were precipitated by gradual addition of $(NH_4)_2SO_4$ up to 80% saturation and centrifuged as above for 20 min. Solution pH was adjusted to 7.5 when necessary with 50 mM NH_4OH. Proteins were desalted on a Sephadex G-25 column (1.0 × 6.0) with 10 mM potassium phosphate buffer (pH 7.5), 0.25 mM $MgCl_2$, and 0.25 mM DTT. Eluent between 1 and $1.5V^e$:V^o was collected and protein content assayed as described by Bradford (4) using a BSA standard. Soluble acid invertase also was extracted the previous season (1984) as per

Purvis and Rice (27), using 100 mM Hepes (pH 8.0), with 5 mM EDTA and 5% PVPP. Crude supernatant was dialyzed for 20 to 24 h against 10 mM potassium phosphate buffer (pH 7.5). Cell wall material for assays of insoluble invertase was washed in 150 to 200 mL dilute (1:40 v/v) extraction buffer minus PVPP.

SS and SPS were extracted similarly except Hepes buffer was substituted for potassium phosphate and extraction buffer also contained 1 mM EGTA (SPS only), 20 mM sodium ascorbate, 10 mM cysteine-HCl, and 10% PVPP. After the first centrifugation, proteins in the supernatant were either dialyzed 8 h (for SPS) against 5 mM Hepes buffer (pH 7.5) containing 25 μm DTT, 25 μm EGTA, and 125 μm $MgCl_2$, or precipitated with 80% $(NH_4)_2SO_4$ (for SS) and centrifuged as above for 20 min. Dialysis resulted in little or no loss of SPS activity when compared to samples desalted by gel filtration. Salt-precipitated proteins were washed by suspending in diluted extraction buffer (1:5 v/v) containing 80% $(NH_4)_2SO_4$, and repelleted by centrifugation. The washed pellet was resuspended in 10 mM Hepes (pH 7.5), 0.25 mM $MgCl_2$, 0.25 mM DTT, and 5 mM EDTA, then centrifuged as above for 10 min. The supernatant was desalted as for invertase except the mobile phase was the resuspension buffer described above.

Extraction procedures for both invertase and SS/SPS were optimized for reductant, divalent cations, and chelators. Addition of a protease inhibitor, PMSF, did not affect results of extraction. Also, activity of the above enzymes from sweet potatoe were unchanged after incubation with citrus extracts.

Enzyme Assays

Soluble invertases were assayed for 15 to 30 min in a total volume of 0.5 mL containing extract, 80 mM acetate-K_3PO_4 (pH 4.5 or 7.5 for acid or alkaline forms, respectively) and 100 mM sucrose. Acid invertase was assayed during the previous season at 37°C using 0.67 M acetate buffer (pH 4.7). A relatively narrow pH optimum of 4.5 was found for acid invertase while that of alkaline invertase was broader and maximal at 7.5. Both assays were linear over time (to 60 min) and with amount of enzyme added. Insoluble invertases were assayed in a similar manner except that 0.2 to 0.5 g cell wall material was used, the final reaction volume was 1.0 mL, and reactions were incubated for 15 to 30 min at 45°C. Addition of Nelson's reagent A (26) terminated each invertase assay, and production of glucose and fructose was quantified by completing the Nelson assay.

SS was assayed in both the synthetic and degradative directions. Reaction medium for SS contained in a 0.5 mL volume: extract, 80 mM Hepes (pH 8.5), 5 mM KCN, 5 mM NaF, 100 mM fructose, and 15 mM UDPG. The medium for sucrose cleavage in a similar volume consisted of extract, 80 mM Mes (pH 5.5 [5.0–6.0 found optimal]), 5 mM NaF, 100 mM sucrose, and 5 mM UDP. Reactions proceeded for 15 min at 30°C and were terminated by boiling for 1 min. Assays for SS activity were optimized in both directions for pH and linearity for 1 min. Assays for SS activity were optimized in both directions for pH and linearity with time and protein concentration (data not shown). Complete recovery also was obtained for nucleotide sugars produced when the reaction proceeded in the degradative direction. Possible action of a UDPase was detected only in nonvascular tissues of very young fruit, where assays of SS in the synthetic direction resulted in lesser values than did the degradative reactions. UDP production was quantified by measurement of pyruvate kinase-specific loss of NADH in the presence of lactic dehydrogenase (3), and UDPG production was quantified by measuring UDPG dehydrogenase-specific synthesis of NADH (3).

SPS was assayed at 30°C as modified from Echeverria and Humphreys (11) in a 0.7 mL volume containing 50 mM Hepes buffer (pH 8.0), 20 mM phenyl-β-glucoside, 5 mM EDTA, 5 mM NaF, 15 mM UDPG, 15 mM F-6-P, 35 mM G-6-P, and extract. NaF and EDTA were added to inhibit phosphatases, and phenyl-β-glucoside to inhibit sucrose synthase. Production of UDP was quantified as described above.

RESULTS

Fresh and Dry Weight Accumulation

Dry weight increases by tissues of developing grapefruit indicated the peel was by far the major sink tissue in young, stage I fruit, whereas weight increases by juice sacs predominated during the last two-thirds of fruit development (Fig. 2A). Both observations were consistent with previously reported growth data (33). Little dry matter was associated with the segment epidermis surrounding juice tissues at any stage of development (Fig. 2A), and minimal weight increases were observed in this tissue during the second half of fruit growth.

Accumulation of fresh weight (Fig. 2B) after the start of stage II development was due almost entirely to expansion of juice sacs, which do not begin their rapid increase in fluid content until after the first 8 to 10 weeks of fruit growth (33).

Sugar Composition

Juice sacs initially accumulated higher levels of sucrose than did other tissues sampled (Fig. 3A) and the concentration continued to increase through much of the subsequent tissue expansion (stage II). During the final portion of grapefruit development (stage III and immediately preceding it), sucrose levels in juice sacs were less than those in albedo (the nonpigmented portion of the peel) and vascular bundle/segment epidermis fraction. The albedo began to accumulate sucrose several weeks later than did juice tissues, after over half the final expansion of peel had been completed (Figs. 2B and 3A). Other data on sugar levels in peel of developing grapefruit are consistent with this observation (32). Overall, sucrose levels in the vascular bundle + segment epidermis fraction approximately paralleled those of the albedo.

In contrast to sucrose, levels of hexoses in young fruit were considerably greater in albedo than in juice tissues; however, albedo hexoses dropped midway in fruit growth (Fig. 3B). Hexose concentration in juice sacs rose only slightly, and did so primarily during the last portion of development examined (Fig. 3B). The hexose/sucrose ratio is known to increase in this tissue late in development and also after harvest of grapefruit (32).

For the entire albedo (Fig. 3C, and D), sucrose showed a relatively constant increase, but not hexoses. Sucrose accumulation by the combined mass of juice tissues was nearly

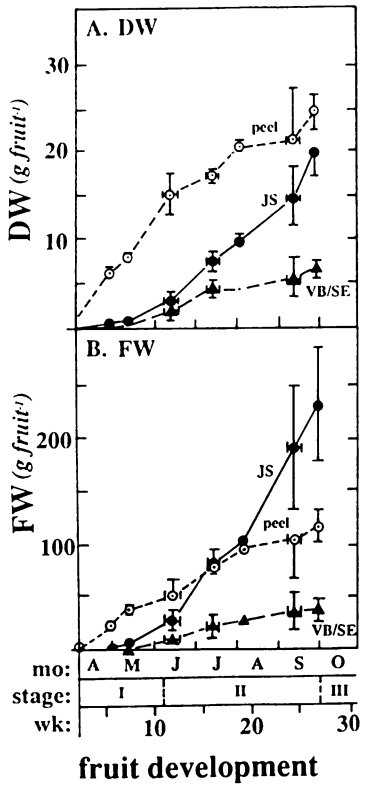

Figure 2. Accumulation of (A) dry weight and (B) fresh weight by tissues of developing 'Marsh' grapefruit; (⊙ - - ⊙), peel (pigmented flavedo + white albedo); (▲ - - - ▲), segment epidermis; (●——●), juice sacs. Vertical bars denote standard errors of five or more field-grown fruit during two growing seasons and are smaller than symbols where not shown. Horizontal bars adjacent to each point show the time period during which samples were harvested for a designated mean. Stages I, II, and III are described in the text. Note minimal growth of juice sacs prior to the onset of stage II.

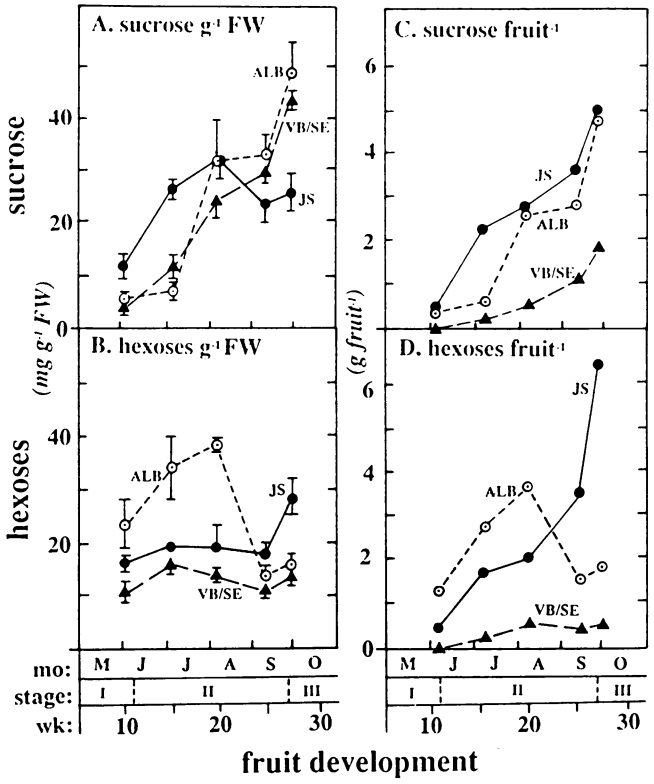

Figure 3. Accumulation of (A) sucrose g^{-1} FW^{-1}, (B) Hexoses g^{-1} FW^{-1}, (C) sucrose $fruit^{-1}$, and (D) hexoses $fruit^{-1}$ in tissues of developing 'Marsh' grapefruit; (⊙ - - ⊙), albedo (inner, nonpigmented portion of peel); (▲ - - - ▲), transport tissues (vascular bundles + segment epidermis); (●——●), phloem-free juice sacs. Vertical bars represent variability among field-grown fruit, and denote standard errors of at least three replications each consisting of four or more fruit. Values are smaller than symbols where not shown.

linear (r = 0,996) from June through September. The rate of hexose increase in juice tissues rose somewhat during later development resulting in total accumulations equal to or greater than those of sucrose. Sucrose and hexoses in the vascular bundle + segment epidermis fraction of the whole fruit were considerably less than those of adjacent sink tissues, but this was primarily due to differences in total mass (refer back to Fig. 3A). Combined degradative activity of enzymes reported in the present study are ample enough to account for the relatively long-term increase in hexose level. Echeverria and Burns (9) have also shown nonenzymic cleavage of sucrose at low pH can contribute to hexose levels in some citrus fruit. However, the period of lowest pH for grapefruit juice sac extracts occurred early rather than late in development.

Acid Invertase

Soluble acid invertase was active in extracts of both the transport and sink tissues during initial fruit growth (stage I), but dropped to near or below the limits of detection after 12 or more weeks of fruit development (Table I). Results from the following year (1985) were comparable, regardless of whether data were expressed per unit protein (Fig. 4A) or g fresh weight (Fig. 4B). Similar changes and activity levels have been reported for juice sacs of developing tangerines (18). Present values for young fruit tissues are also comparable to those of other sucrose-importing tissues during their earliest phases of growth (13).

Activity of soluble acid invertase g^{-1} fresh weight (Fig. 4B) and its insoluble (cell-wall-bound [13, 15, 16, 21, 24]) counterpart (Fig. 4C) dropped to barely detectable levels by the stage II sampling point. In young, stage I fruit, substantial activity of soluble and insoluble acid invertases were evident in extracts of phloem-free juice sacs as well as the adjacent zone of phloem unloading and transport (vascular bundles + segment epidermis)

The solubility of acid invertase in tissue homogenates is highly variable and often only partly reflects the extent of cell-wall-binding or localization *in vivo* (1, 13, 16, 24). Cell-wall-binding can occur during extraction, depending on ionic strength and media composition (1, 24). However, several authors have found biochemical differences between what they believe to be a soluble, vacuolar enzyme, and a cell-wall-bound form (13, 15, 16, 21, 24). Changes in the ratio of soluble to insoluble enzyme have also occurred during development (13, 15, 24, 31). In the present work and in other

Table I. *Activity of Soluble Acid Invertase from Transport Tissues and Adjacent Sink Structures with or without Vascular Bundles (Albedo and Juice Sacs, Respectively)*

Development		Tissue		
Date	Age	Inner peel (albedo)	Transport tissues (vascular bundles + segment epidermis)	Phloem-free juice sacs
	weeks[a]	*μmol glucose equiv. g^{-1} FW*		
5/18	8	263.6 (±18.9)[b]	236.8 (±13.1)	944.9 (±407.0)
6/5	10	105.4 (±2.3)	91.0 (±5.3)	117.9 (±10.5)
6/18	12	0[c]	0	0
7/18	18	0	0	0.6 (±0.5)
Aug–Dec (4 other samplings): no detectable activity in any tissue				

[a] Approximate number of weeks past flowering. Fruit did not set simultaneously, and effects of age variation would have been proportionally greater in earliest samples. [b] Values in parentheses represent variability among field-grown fruit and denote standard errors for means of two to three replications, each from extracts of six different fruit sampled from different trees. [c] Below detectable levels.

instances, the insoluble acid invertase activity was generally a small component of the total (18). Higher percentages have been observed in sugarbeet roots (31), and cell cultures (24). Changes in proportions of soluble to insoluble invertase during development were not considered to be related to photosynthate partitioning in intact sugar beets (31), but Masuda *et al.* (24) report differing affinities for sucrose among soluble and insoluble invertases of cell cultures.

Alkaline Invertase

Specific activity of alkaline invertase during the juice sac expansion of stage II fruit was greater in extracts of these phloem-free sacs and albedo than those from adjacent transport tissues (vascular bundles + segment epidermis) (Fig. 4E). The difference was not as pronounced when data were expressed per unit fresh weight (due to expansion of storage tissues [Fig. 4F]). However, when data for the insoluble fraction were also considered (Fig. 4G), the total soluble alkaline invertase activity remained markedly greater in extracts of phloem-free juice sacs than from those of transport tissues.

Alkaline invertase from juice sacs of young, stage I fruit was less active per unit protein than that from the same tissues during their more rapid growth in stage II fruit (Fig. 4E). Activity from all tissue samples of stage I fruit was also considerably less than that of acid invertase (compare activity units for Figs 4, A–D to those of 4, E–F), but this had reversed in stage II fruit. It is typical for an alkaline invertase, where present, to remain active for a longer portion of sink tissue development than does acid invertase (13, 18, 22, 31).

Total invertase activity (soluble + insoluble) measured at pH 7.5 is likely to be that of the same 'neutral' or 'alkaline' enzyme, because activity typically recovered in the insoluble fraction (18, 31) is not believed to be a separate, cell-wall-bound form (1). Alkaline invertase activities in the present report are comparable to those found for sugar beet roots (31), muskmelon (22), sugarcane (14), and immature juice tissues of tangerine (18). Some reports of neutral or alkaline

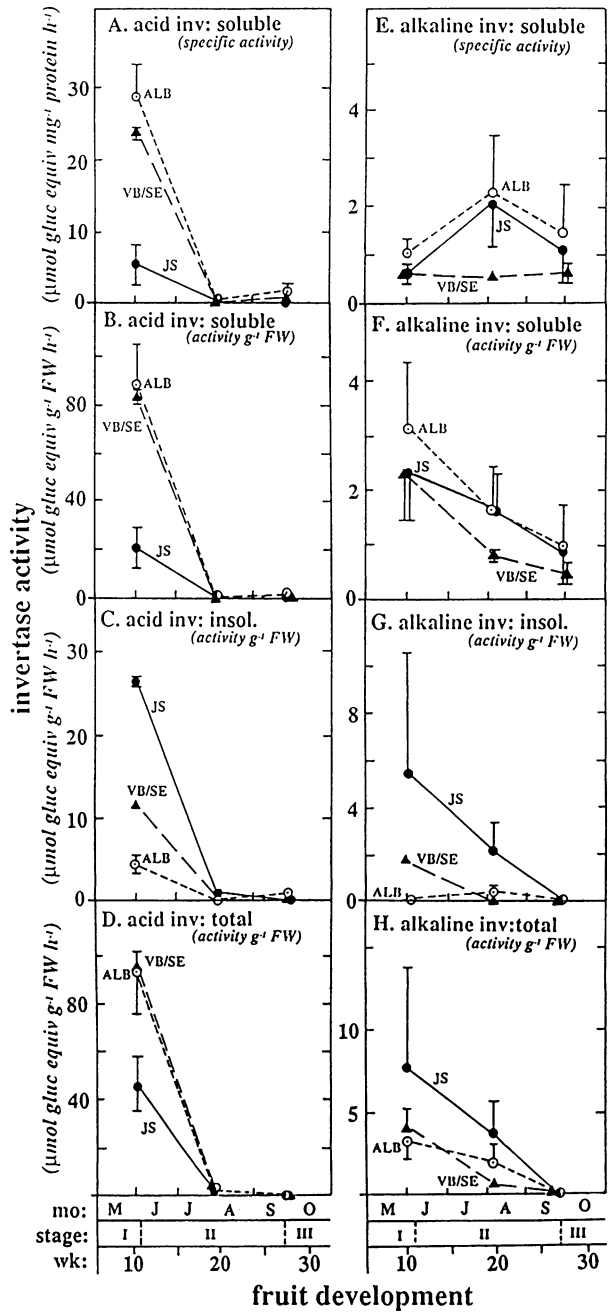

Figure 4. Respective activities of acid and alkaline invertases (A, E) in the soluble fraction mg^{-1} protein (B, F) in the soluble fraction g^{-1} FW, (C, G) in the insoluble fraction g^{-1} FW, and (D, H) total extracted enzyme g^{-1} FW of transport and adjacent sink tissues at three stages of grapefruit development; (⊙ – – ⊙), albedo (inner, nonpigmented portion of peel); (▲ – – – ▲), transport tissues (vascular bundles + segment epidermis); (●——●), phloem-free juice sacs. Vertical bars represent variability among field-grown fruit, and denote standard errors of at least three replications each consisting of four or more fruit. Values are smaller than symbols where not shown. Statistically significant differences in results are not specifically designated, but samples from stage II fruit in E, J, and H showed significant differences in activity of alkaline invertase from transport tissues (vascular bundles + segment epidermis) *versus* phloem-free juice sacs. Data are expressed both per unit fresh weight and protein so that effects of tissue expansion can be assessed. Stages I, II, and III are described in the text.

invertases may result from a relatively broad pH optimum for acid invertase which can allow substantial activity at pH 7 or above. In the present study, distinct, separate pH optima for acid and alkaline invertase were apparent in extracts from fruit at all developmental stages (pH data not shown).

SS

In vitro SS activity from the transport tissues (vascular bundle + segment epidermis) was significantly greater than that from sink tissues of fruit at stages II and III, when juice sacs accumulate the majority of their dry weight and volume ($P < 0.05$). SS predominated in and/or near the zone of phloem unloading regardless of whether data were expressed per unit protein or fresh weight (Fig. 5). Its hydrolytic action probably occurs primarily in the cytoplasm of citrus cells (10), as suggested for mature sugarcane stems (14, 15) and sugar beet roots (31). Activities from optimized assays were within the range of those obtained for other sink tissues (11), but varied in grapefruit depending on the direction of the assay (Fig. 5). Many aspects of assay conditions can affect the SS reaction differently (1).

In the present study, SS activities in extracts of stage I fruit differed markedly depending on whether the degradative or synthetic assay was used. Results form citrus leaves also varied during development depending on assay direction (28). Differences in magnitude of the *in vitro* SS reaction associated with assay direction in the present study indicate little about its *in vivo* role, but the common assumption of a solely degradative function should be avoided. The majority of data from other species favor a degradative direction for SS in sink tissues due to locally high levels of sucrose, typically rapid utilization of fructose and/or UDPG, and the presence or absence of other enzyme effectors (1, 10, 16). Extensive circumstantial evidence also favors this view (2, 16, 25), but it does not necessarily hold true in all instances.

SPS

Extracts of the rapidly growing, phloem-free juice sacs of stage II fruit showed greater mean activity of SPS than did adjacent transport tissues (vascular nodules + segment epidermis) (Fig. 6, A and B). This difference was evident regardless of whether data were expressed per unit fresh weight or protein (Fig. 6, A and B). SPS generally is more active in chlorophyllous tissues (22), especially those which export sucrose (1, 19), but photosynthetic capacity is minimal in all but the outermost tissues of citrus fruit (PT Tomlinson, and KE Koch, unpublished data).

Comparative Activities of Sucrose-Metabolizing Enzymes

Of prime importance in Table II are the data on the most rapidly growing juice sacs and adjacent transport tissues of stage II fruit. At this stage, SS activity in extracts from the zone of most extensive phloem unloading and transport (vascular bundles + segment epidermis) significantly surpassed that of all other sucrose-metabolizing enzymes ($P < 0.001$). It was also markedly greater than that of SS in either of the adjacent sink tissues. Data were expressed per unit fresh

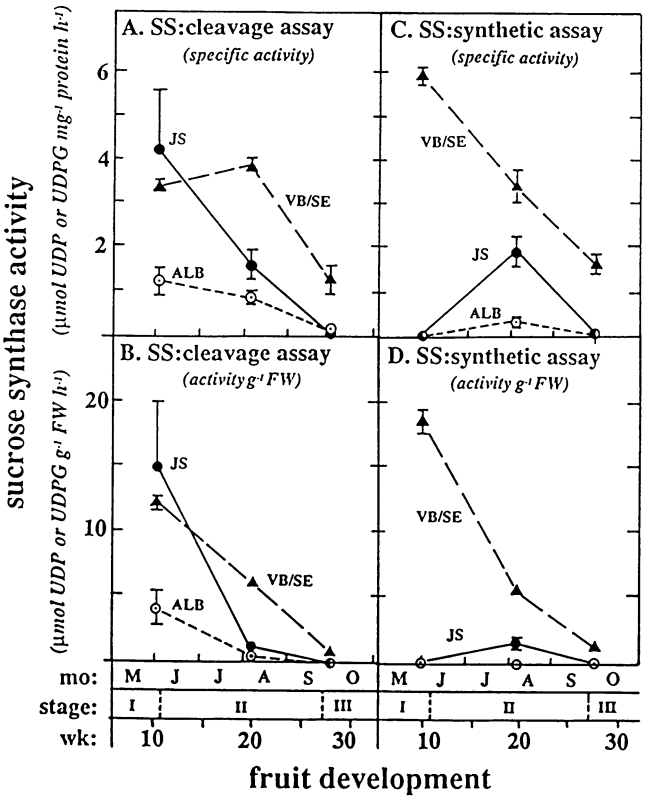

Figure 5. Sucrose synthase activity assayed as (A) sucrose cleaved mg^{-1} protein, (B) sucrose cleaved g^{-1} FW, (C) sucrose synthesized mg^{-1} protein, and (D) sucrose synthesized g^{-1} FW of transport tissues at three stages of grapefruit development; (⊙ - - ⊙), albedo (inner, nonpigmented portion of peel); (▲ - - - ▲), transport tissues (vascular bundles + segment epidermis); (●——●), phloem-free juice sacs. Rates of sucrose cleavage and synthesis were assayed by quantifying UDPG and UDP production, respectively (see text). Vertical bars represent variability among field-grown fruit, and denote standard errors of at least three or more replications each consisting of four or more fruit. Values are smaller than symbols where not shown. Statistically significant differences in results are not specifically designated, but sucrose synthase activity in extracts of transport tissues (vascular bundle + segment epidermis) was significantly greater than from phloem-free juice sacs of stage II and III fruit in A to D. Data are expressed both per unit fresh weight and protein so that effects of tissue expansion can be assessed. Stages I, II, and II are described in the text.

weight to allow comparisons of total activities (including insoluble fraction), but as noted earlier, the high activity of SS in transport *versus* sink tissues was also observed per unit protein. Alkaline invertase and SPS provided a striking contrast to SS in that their activities were greater in sink than transport tissues of stage II fruit. Both of the former enzymes were more active in extracts of stage II fruit than was acid invertase.

A comparison between stage II and younger, stage I fruit (Table II) showed higher activities of most enzymes in the less expanded tissues. Juice sacs in particular undergo little fresh or dry weight increases during stage I development. Activity of acid invertase in all samples from stage I fruit was disproportionately greater than at subsequent stages of development.

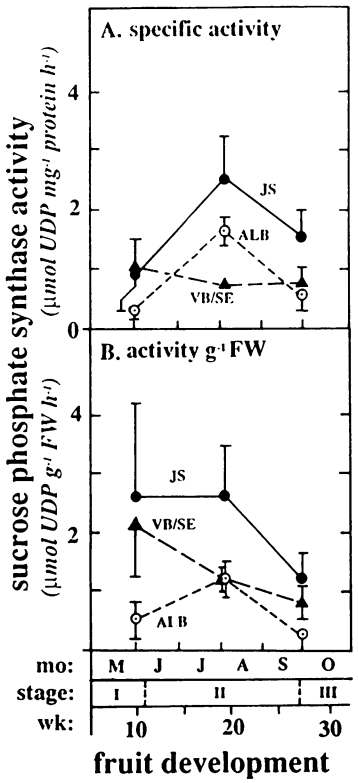

Figure 6. Sucrose phosphate synthase (A) activity mg^{-1} protein and (B) activity g^{-1} FW in extracts from transport and sink tissues at three stages of grapefruit development; (⊙ – – ⊙), albedo (inner, nonpigmented portion of peel); (▲ – – – ▲), vascular bundles + segment epidermis; (●——●), phloem-free juice sacs. Vertical bars represent variability among field-grown fruit, and denote standard errors of at least three replications each consisting of four or more fruit. Values are smaller than symbols where not shown. Statistically significant differences in results are not specifically designated, but sucrose phosphate synthase activities in the vascular bundle + segment epidermis fractions were significantly less than those of phloem-free juice sacs of stage II fruit in A and B. Data are expressed both per unit fresh weight and protein so that effects of tissue expansion can be assessed. Stages I, II, and III are described in the text.

Acid invertase activity from stage III fruit tissues also rose relative to that of other sucrose-metabolizing enzymes. However, the onset of cool night temperatures has been shown to induce this enzyme in citrus fruit tissues (27). Differences among other enzymes of stage III fruit, where significant, were not as pronounced as at stage II where the majority of juice sac growth occurs.

DISCUSSION

Claussen *et al.* (5) proposed that "sucrose synthase might have an important role in the regulation of sucrose content in the conducting tissue." This intriguing hypothesis has been difficult to test, however, and extensive data support the alternative view, that involvement of SS in sink activity is predominantly through sucrose metabolism in sink cells (resulting in production of substrates for respiration [17, 25, 35] or UDPG for synthesis of complex carbohydrates [16, 35]). Data in the present report are not inconsistent with the latter,

Table II. *Activities of Total Acid and Alkaline Invertase, SS, and SPS from a Phloem-Unloading Zone and Adjacent Sink Tissues of Fruit Tissues Sampled at Three Stages of Development*

Values from tissues of stage II fruit represent a period during which the majority of sugars are accumulated by phloen-free juice sacs, and the largest amount of sucrose is imported through the segment epidermis and associated vascular bundles. Data are also expressed per unit protein (see Figs. 3–5) to allow an appraisal of dilution effects by tissue expansion.

Developmental Stage and Enzyme	Tissue		
	Inner peel (albedo)	Transport tissues (vascular bundles + segment epidermis)	Phloem-free juice sacs
	μmol sucrose synthesized or degraded g FW^{-1} h^{-1}		
Stage I			
Acid invertase[a]	91.5 (±18.0)	48.6 (±2.2)	24.7 (±5.6)
Alkaline invertase[a]	1.6 (±0.6)	2.0 (±0.6)	3.9 (±3.0)
SS[b]	4.0 (±1.3)	12.1 (±0.5)	14.9 (±5.1)
SPS	0.5 (±0.3)	2.1 (±0.9)	2.6 (±1.6)
Stage II			
Acid invertase	0.1 (±0.1)	0.3 (±0.2)	0.5 (±0.4)
Alkaline invertase	1.0 (±0.5)	0.4 (±0.1)	1.9 (±1.0)
SS	0.6 (±0.6)	6.1 (±0.1)	1.0 (±0.3)
SPS	1.2 (±0.3)	1.2 (±0.2)	2.6 (±0.9)
Stage III			
Acid invertase	1.9 (±0.8)	1.3 (±0.5)	0.4 (±0.4)
Alkaline invertase	0.6 (±0.4)	0.3 (±0.2)	0.5 (±0.3)
SS	0.1 (±0)	1.0 (±0.3)	0.1 (±0)
SPS	0.3 (±0.3)	0.8 (±0.3)	1.2 (±0.4)

[a] Acid and alkaline invertase values are totals from assays of both soluble and insoluble fractions. Data were converted to μmol sucrose hydrolyzed by halving values for μmol glucose equivalents produced. [b] Values from cleavage assays for sucrose synthase. These were generally equal to or greater than those from the synthetic reaction.

because SS was found active in extracts of sink tissues completely lacking vascular tissues (juice sacs; Fig. 5; Table II). This was particularly evident during stage I of fruit development when cell division, cell wall synthesis, and respiration rates were maximal.

Nevertheless, evidence presented here additionally demonstrates an association between SS and transport tissues (vascular bundles + segment epidermis). The specific function of this enzyme in the transport process clearly remains speculative at present, but the localized activity is most apparent during a period of extensive phloem unloading and assimilate transfer at the same site. A role for SS in wholly or partially symplastic phloem unloading could be analogous to that proposed for acid invertase in apoplastic phloem unloading (16, 30). Sucrose cleavage in the symplast at or near the site of unloading could thus increase the sucrose gradient at the sink end of the translocation path and indirectly increase the hydrostatic pressure gradient within phloem between source and sink.

Hawker and Hatch (15) and Slack (34) reported that the majority of SS in sugarcane stems appeared to be present in or near vascular bundles. More recently, Dick and ap Rees (7) found elevated SS activity in the stele (vascular tissues) *versus* the cortex (nonvascular area) of growing pea roots.

Claussen *et al.* (5) also reported that SS activity of vascular midribs was substantially greater than that of the attached blade from the same leaf. In citrus it has been possible to separately assay SS extracted from phloem-free sink tissues (juice sacs) and from the adjacent transport tissues (vascular bundles + segment epidermis) [both part of the path for assimilates enroute to juice sacs (20]). The present data have thus been obtained from clearly defined components of a sucrose-importing system. In addition, quantification of this comparison has shown that elevated SS activity in transport tissues is not simply due to greater cell density in and near vascular tissues.

In direct contrast to data on SS from stage II fruit (primary period of juice sac growth) activities of alkaline invertase and SPS are greater in extracts from phloem-free structures (juice sacs) than from transport tissues (vascular bundles + segment epidermis). These results are consistent with earlier work of Hawker and Hatch (16) who reported that neutral/alkaline invertase was active in the parenchyma, but not vascular bundles of sugarcane stems. Results of Krishnan in wheat differ, but activities of acid and alkaline invertase were not separated in that study (21).

The presence of alkaline invertase in juice sacs could conceivably result in simultaneous degradative action by both this enzyme and SS. Such dual activities have been noted in numerous sink tissues (13, 15, 24, 25, 31) and could be significant to metabolic partitioning of imported sucrose (17, 25, 35). Morell and Copeland (25) proposed that in soybean nodules, the balance between *in vivo* degradative activity of the two enzymes might be controlled by the different K_ms for sucrose and by varying sensitivities to other attributes of the cellular environment. This in turn could affect the partitioning of imported sucrose between metabolic events which most effectively utilize the degradative products of the two enzymic reactions, glucose and UDPG, respectively (25). Production of UDPG could be important in a sucrose-storing tissue such as citrus, if UDPG were utilized for rapid resynthesis of sucrose for storage. Huber and Akazawa (17) also suggested K_m differences between alkaline invertase and SS from cultured cells (although different than those from soybean nodules noted above) could be important to controlling the fate of sucrose.

Considerable interest has centered on the role of SPS in regulation of sucrose synthesis in source leaves (1, 19, and references cited therein). However, SPS also may be important to sucrose synthesis in many sucrose-storing sink tissues. For example, SPS in sugarcane was initially proposed to operate as part of a 'pull' system contributing to sink strength (13, and references cited therein), and later, as part of a multienzyme group translocator in the tonoplast (36). Soluble SPS has been assayed during muskmelon development (22), where its activity was reported to increase during most rapid sucrose accumulation. Also, Fieuw and Willenbrink (12) found active SPS in the portion of sugar beet roots accumulating greatest sugars, while Pavlinova and Prasolova (as cited in 12) obtained minimal activity from vascular tissue.

Timing and localization of soluble and insoluble acid invertase activities confirmed their involvement in processes other than phloem unloading. Earlier studies have clearly demonstrated the importance of acid invertase during assimilate transfer into immature sugarcane stems (13, 14) and developing corn kernels (30). However, sucrose hydrolysis later was found not to occur during photosynthate entry into a variety of other sink tissues (16 and references cited therein, 29). In grapefruit, the capacity of both soluble and insoluble acid invertases drops to very low levels in tissues still rapidly accumulating dry matter. In fact, high rates of juice sac expansion do not begin until after this decrease in total acid invertase activity, and several studies have reported that increases in sucrose levels occur only after decreases in acid invertase (22, 24, 31). Periodic late season increases in acid invertase of citrus have been found linked to low temperature exposure and chilling resistance rather than transport processes (27).

The presence of insoluble acid invertase in completely nonvascular tissues of young grapefruit also suggests that this enzyme, at least in some instances, may be more important to sink strength through association with specific metabolic processes than by direct involvement in phloem unloading. A close association, for example, has been found between acid invertase activity and the rate of cell division and expansion in meristematic zones of sugar beet tap roots (31), expanding citrus leaves (28), and other immature tissues (1, 24). In these instances, hexoses produced during the invertase reaction could be utilized rapidly in respiratory and biosynthetic processes (16, 31). Large portions of imported photosynthate also must be allocated to the synthesis of cell wall constituents in very young citrus fruit, where cellulose, hemicellulose and particularly pectin comprise over 60% of the tissue dry weight (32). In addition, the invertase activity may be important to the maintenance of, or increase in osmotic pressure of newly-formed, expanding cells.

CONCLUDING REMARKS

SS activity was greater in extracts of transport tissues, where extensive phloem unloading and subsequent transfer are known to occur (vascular bundles + segment epidermis during stages II and II of juice sac growth), than in adjacent sink structures completely lacking phloem (juice sacs). Moreover, SS was significantly more active than any other sucrose-metabolizing enzyme in extracts of transport tissues from stage II fruit (when the majority of juice sac growth occurs). Results confirm and extend initial reports of high SS activity from vascular bundles by Hawker and Hatch (15) and Slack (34). SS may thus in some instances be closely allied with functioning of transport tissues (in a manner as yet undefined) in addition to its established role in deposition and/or utilization of imported assimilates (16, 17, 25, 35). Results also indicate the opposite for alkaline invertase and SPS, which are most active in completely nonvascular sink structures. Acid invertase was nearly absent during the majority of fruit development, but had been active in extracts of both vascular and nonvascular portions of very young fruit.

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LITERATURE CITED

1. **Avigad G** 1982) Sucrose and other disaccharides. *In* FA Loewus, W Tanner, eds, Encyclopedia of Plant Physiology (New Series). Springer-Verlag, Berlin, pp 216–347
2. **Bain JM** (1958) Morphology, anatomical, and physiological changes in the developing fruit of the Valencia orange. *Citrus sinensis* (L.) Osbeck. Aust J Bot **6:** 1–28
3. **Bergmeyer H** (1974) Nucleotides, coenzymes and related compounds. *In* H Bergmeyer, ed, Methods of Enzymatic Analysis, Vol 4. Academic Press, New York, pp 1967–2203
4. **Bradford MM** (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem **72:** 248–254
5. **Claussen W, Loveys BR, Hawker JS** (1985) Comparative investigations on the distribution of sucrose synthase activity and invertase activity within growing mature and old leaves of some 3-carbon photosynthetic pathway and 4-carbon photosynthetic pathway species. Physiol Plant **65:** 275–280
6. **Claussen W** (1983) Investigations on the relationship between the distribution of assimilates and sucrose synthetase activity. Z Pflanzenphysiol Bd **110:** 175–182
7. **Dick PS, ap Rees T** (1976) Sucrose metabolism by roots of *Pisum sativum.* Phytochemistry **15:** 225–259
8. **Doehlert DC, Felker FC** (1987) Characterization and distribution of invertase activity in developing (*Zea mays*) kernels. Physiol Plant **70:** 51–57
9. **Echeverria E, Burns JK** (1989) Vacuolar acid hydrolysis as a physiological mechanism for sucrose breakdown. Plant Physiol **90:** 530–533
10. **Echeverria E, Valich J** (1988) Carbohydrate and enzyme distribution in protoplasts from Valencia orange juice sacs. Phytochemistry **27:** 73–76
11. **Echeverria E, Humphreys T** (1984) *In vitro* and *in vivo* inhibition of sucrose synthesis by sucrose. Phytochemistry **23:** 2727–2731
12. **Fieuw S, Willenbrink J** (1987) Sucrose synthase and sucrose phosphate synthase in sugar beet plans (*Beta vulgaris* L. ssp. altissima). J Plant Physiol **131:** 153–162
13. **Glasziou KT, Gayler KR** (1972) Storage of sugars in stalks of sugar cane. Bot Rev **38:** 471–490
14. **Hatch MD, Sacher JA, Glasziou KT** (1963) Sugar accumulation cycle in sugar cane II. Studies on enzymes of the cycle. Plant Physiol **38:** 338–343
15. **Hawker JS, Hatch MD** (1965) Mechanism of sugar storage by mature stem tissue of sugarcane. Physiol Plant **18:** 444–453
16. **Hawker JS** (1985) Sucrose. *In* PM Dey, RA Dixon, eds, Biochemistry of Storage Carbohydrates in Green Plants. Academic Press, London, pp 1–51
17. **Huber SC, Akazawa T** (1986) A novel sucrose synthase pathway for sucrose degradation in cultured sycamore cells. Plant Physiol **81:** 1008–1013
18. **Kato T, Kabota S** (1978) Properties of invertases in sugar storage of citrus fruit and changes in their activities during maturation. Physiol Plant **43:** 67–72
19. **Kerr PS, Huber SC** (1987) Coordinate control of sucrose formation in soybean leaves by sucrose-phosphate synthase and fructose-2,6-bisphosphate. Planta **170:** 197–204
20. **Koch KE** (1984) The path of photosynthate translocation into citrus fruit. Plant Cell Environ **7:** 647–653
21. **Krishnan HB, Blanchette JT, Okita TW** (1984) Wheat invertases: characterization of cell wall-bound and soluble forms. Plant Physiol **78:** 241–245
22. **Lingle SE, Dunlap JR** (1987) Sucrose metabolism in netted muskmelon fruit during development. Plant Physiol **84:** 386–389
23. **Lowell CA** (1987) Structure and sucrose metabolizing enzymes of the transport path: Implications for assimilate translocation in grapefruit. PhD thesis, University of Florida
24. **Masuda H, Takahashi T, Sugawara S** (1988) Acid and alkaline invertases in suspension cultures of sugar beet cells. Plant Physiol **86:** 312–317
25. **Morell M, Copeland L** (1985) Sucrose synthase of soybean nodules. Plant Physiol **78:** 149–154
26. **Nelson N** (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem **153:** 357–380
27. **Purvis AC, Rice JD** (1983) Low temperature induction of invertase activity in grapefruit flavedo tissue. Phytochemistry **22:** 831–834
28. **Schaffer AA, Sagee O, Goldschmidt EE, Goren R** (1987) Invertase and sucrose synthase activity, carbohydrate status and endogenous IAA levels during *citrus* leaf development. Physiol Plant **69:** 151–155
29. **Schmalstig JG, Hitz WD** (1987) Transport and metabolism of a sucrose analog (l′-fluorosucrose) into *Zea mays* L. endosperm without invertase hydrolysis. Plant Physiol **85:** 902–905
30. **Shannon JC, Dougherty TC** (1972) Movement of ^{14}C-labeled assimilates into kernels of *Zea mays*. L. II. Invertase activity in the pedicle and placentochalazal tissues. Plant Physiol **49:** 203–206
31. **Silvius JE, Snyder FW** (1979) Comparative enzymatic studies of sucrose metabolism in the taproots and fibrous roots of *Beta vulgaris* L. Plant Physiol **54:** 1070–1073
32. **Sinclair W** (1972) The grapefruit: its composition, physiology, and products. University of California, Division of Agricultural Sciences, Riverside, CA.
33. **Sinclair W** (1984) The biochemistry and physiology of the lemon and other citrus fruits. University of California, Division of Agricultural Sciences, Riverside, California
34. **Slack CR** (1966) Inhibition of UDP glucose: D-fructose 2-glucosyl transferase from sugarcane stem tissue by phenol oxidation products Phytochemistry **5:** 397–403
35. **Sung S-J, Xu D-P, Galloway CM, Black CC** 1988) A reassessment of glycolysis and gluconeogenesis in higher plants. Physiol Plant **72:** 650–654
36. **Thom M, Maretzki A** (1985) Group translocation as a mechanism for sucrose transfer into vacuoles from sugarcane cells. Proc Natl Acad Sci USA **88:** 4697–4701