

# Carbohydrate Changes during Maturation of Cucumber Fruit<sup>1</sup>

## IMPLICATIONS FOR SUGAR METABOLISM AND TRANSPORT

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### ABSTRACT

Changes in the carbohydrate profiles in the mesocarp, endocarp, and seeds of maturing cucumber (*Cucumis sativus*, L.) fruit were analyzed. Fruit maturity was measured by a decrease in endocarp pH, which was found to correlate with a loss in peel chlorophyll and an increase in citric acid content. Concentrations of glucose and fructose (8.6–10.3 milligrams per gram fresh weight, respectively) were found to be higher than the concentration of sucrose (0.3 milligrams per gram fresh weight) in both mesocarp and endocarp tissue. Neither raffinose nor stachyose were found in these tissues. The levels of glucose and fructose in seeds decreased during development, but sucrose, raffinose, and stachyose accumulated during the late stages of maturation. Both raffinose and stachyose were found in the seeds of six lines of *Cucumis sativus* L. This accumulation of raffinose saccharides coincided with an increase in galactinol synthase activity in the seeds. Funiculi from maturing fruit were found to be high in sucrose concentration (4.8 milligrams per gram fresh weight) but devoid of both raffinose and stachyose. The results indicated that sucrose is the transport sugar from the peduncle to seed, and that raffinose saccharide accumulation in the seed is the result of *in situ* biosynthesis and not from direct vascular transport of these oligosaccharides into the seeds.

Raffinose saccharides occur as storage carbohydrate in seeds of legumes (14, 15) and other species (4, 15, 24) and in vegetative tissues of a wide range of plants (6, 22, 30). In the *Cucurbitaceae*, stachyose appears to be the predominant transport sugar (12, 28, 29) rather than sucrose (31); however, raffinose and small amounts of verbascose also may be translocated (12). There is evidence that sucrose is also a transport sugar in some of these species (12, 28).

Although cucumber transports primarily stachyose (29), glucose and fructose are the major free sugars in the fruit (20). Lower levels of sucrose and no traces of either raffinose or stachyose are found in the maturing fruit (20). Sucrose is the predominant soluble sugar found in the fruit peduncle along with low amounts

of stachyose and raffinose (20). This distribution of sugars is evidently due to the metabolism of transported stachyose arriving from source leaves (9, 25). In the peduncle, stachyose is metabolized to sucrose which apparently enters the fruit and is further metabolized to glucose and fructose (9, 20).

The recent report of raffinose saccharides in the mature seeds of *C. sativus* 'Calypso' (11) suggests that *in situ* synthesis of these sugars occurs in the developing seed, particularly since these sugars were not detected by paper chromatography in the fleshy portions of the cucumber fruit (20). There is the possibility, however, that this method of analysis failed to detect these sugars if they were restricted to the vascular tissues of the fruit. They might have been diluted by the high levels of glucose and fructose in the nonvascular tissues.

This study was conducted to determine if *in situ* synthesis is responsible for the appearance of raffinose and stachyose in maturing cucumber seeds. To answer this question, changes in carbohydrate levels within the mesocarp, endocarp, and seed of maturing cucumber fruit were measured. In addition, galactinol synthase activity, the first unique enzyme in raffinose saccharide biosynthesis (7, 10, 11, 16, 19), and the sugars in funiculi were determined. In this study, decrease in endocarp pH was used as an index of fruit maturation (21). To determine if this was an adequate estimate of maturity, a separate study was conducted comparing this decrease in pH with a loss in peel Chl. Also, the organic acids malate and citrate were assayed from endocarp tissue during fruit maturation.

### MATERIALS AND METHODS

**Plant Material and Sampling Procedures.** Cucumber (*Cucumis sativus* L.) fruit utilized in these studies were harvested from plants of cv Calypso, grown in field plots in Raleigh (except as noted below). For sugar and starch analysis of fruit tissues, three separate harvests were made during the growing season. Fruit were hand harvested in early morning, washed, and separated into nine different categories based on the pH of the endocarp tissue (21). These pH categories ranged from 5.75 to 3.50 in 0.25 pH unit increments. Each fruit was cut crosswise at a point approximately one-third of the length of the fruit proximal to the blossom end. A pH electrode was inserted into the center of this one-third section, the pH recorded, and this section was discarded. The two-thirds section was immediately chilled in ice until processed. Three fruit were collected for each pH category. All further operations were performed at 0°C.

Endocarp tissue and seeds were scooped from the three fruit in each pH category. Endocarp tissue refers to the gelatinous tissue surrounding the seeds and includes the associated placental tissue. The seeds were separated from the endocarp tissue and wiped with cheesecloth to insure removal of the gelatinous covering

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surrounding the individual seeds. The fruit were then peeled and the mesocarp tissue was cut into small pieces. Mesocarp tissue was the fleshy tissue between the peel and gelatinous endocarp tissue. Each of the three tissue types; seed, endocarp, and mesocarp were pooled from the three fruit in each pH category and held at 0°C until further processed. The results reported are the mean values of the three harvests.

**Sugar and Organic Acid Analysis.** The endocarp and mesocarp tissues were blended on ice in a VirTis homogenizer. The seeds were washed with at least three exchanges of ice-cold distilled H<sub>2</sub>O, then blended in 1:2 w/v or 1:3 w/v ice-cold distilled H<sub>2</sub>O depending on their dryness. Ten ml of these homogenates were pipetted into 30-ml centrifuge tubes and placed in a boiling water bath for 5 or 10 min. After cooling, the tubes were centrifuged at 27,000g for 15 min. The supernatant was stored at -20°C until chromatographed.

Sucrose, glucose, fructose, raffinose, stachyose, malic acid, and citric acid were analyzed by HPLC. The HPLC system consisted of a Waters 6000A pump and model 401 refractive index detector, a Rheodyne 7125 loop injector, and a Spectra-Physics 4100 computing integrator. Sucrose, raffinose, stachyose, malate, and citrate were separated by reversed-phase chromatography as previously described (11). Samples were thawed, filtered through a 0.45- $\mu$ m filter and diluted with an equal volume of 2 $\times$  elution buffer, and a 10- $\mu$ l sample injected. Glucose and fructose were separated with a BioRad Aminex HPX-87 heavy metal column at 70°C with water as the elution solvent. Samples were thawed, filtered through a 0.45- $\mu$ m filter, and a 20- $\mu$ l sample injected.

**Starch Analysis.** For starch analysis, 2 ml of the homogenate prepared for the sugar analysis were pipetted into 16-ml centrifuge tubes. Eight ml of 100% ethanol were added to give a final ethanol concentration of 80%. The contents were mixed and stored at -20°C until further processed. The samples in the 16-ml centrifuge tubes were thawed, centrifuged at 27,000g for 15 min, decanted, and the supernatant discarded. The pellet was extracted with 5 ml hot 80% ethanol by stirring and incubating in a boiling water bath for 5 min. The tubes were then centrifuged at 27,000g and the supernatant discarded. This extraction procedure was repeated again and the resulting pellet saved. One or 2 ml of 50 mM Na-acetate buffer (pH 4.5) was added to the pellet and incubated in a boiling water bath for 30 min with occasional stirring. The tubes were removed and cooled to room temperature. Three ml of amyloglucosidase (*Aspergillus oryzae*, Sigma), dialyzed and diluted in 40 mM Na-acetate buffer (pH 4.5), were added and tubes incubated at 55°C with constant shaking for 3 h. Tubes were then placed for 10 min in boiling water bath, removed, centrifuged at 27,000g for 15 min, and the supernatant collected. The glucose liberated was measured using a glucose oxidase (Sigma) assay (2). Starch content is expressed as  $\mu$ g glucose eq/gfw.<sup>3</sup> This is similar to a method proposed by MacRae (17). The *Aspergillus amyloglucosidase* was free of  $\beta$ -glucanase.

**Peel Chl Determination.** In the second harvest, three fruit in each pH category were peeled and the peel tissue analyzed for Chl. The peelings from the whole fruit were weighed and blended with 1:5 w/v 80% ethanol in an Osterizer blender. Blender cups were incubated for 10 min in a boiling water bath to extract the Chl further. The homogenates were filtered through Whatman No. 1 filter paper and their  $A_{665}$  recorded as an estimate of total Chl content.

**Dry Seed Study.** Ten g of dry seeds of six *C. sativus* lines or cultivars from commercial and local sources were washed thoroughly in distilled H<sub>2</sub>O and ground in 20 ml ice-cold distilled H<sub>2</sub>O for 5 min in a VirTis homogenizer. Homogenates were analyzed for soluble sugars and starch using the procedures described above for seeds from developing fruit.

**Galactinol Synthase Assay.** Fruit were harvested from greenhouse-grown plants and the pH of their endocarp tissue determined using the procedures described above. Three fruit each in endocarp pH categories of 5.3, 4.7, or 3.5 were selected. Seeds were removed from each of these nine fruit, washed with cold distilled H<sub>2</sub>O, and kept at 0°C. Two g of seeds from each of the nine fruit were homogenized at 0°C in 10 ml 50 mM Na-phosphate buffer (pH 7.2), containing 10 mM DTT using a Polytron homogenizer. The homogenate was centrifuged at 27,000g for 15 min. The supernatant was filtered through one layer of Miracloth and each extract was desalted using G-25 Sephadex column chromatography (10 ml bed-volume). Galactinol synthase activity was determined using a procedure previously described (10, 11, 19). Enzyme activity was expressed as milliunits (munits), which is that amount of enzyme necessary to form a nmol of galactinol/min at 30°C.

**Funiculi and Seed Extraction.** Three greenhouse-grown fruit having an endocarp pH of 4.3, 4.5, or 4.8, were used to determine the carbohydrate content of funiculi and corresponding seeds. Immediately after collection, the fruit were chilled on ice and all subsequent excisions were performed at 0°C. From 250 to 500 mg of funiculi were removed from each of these fruit along with their attached seed. Funiculi and seeds were separated using a scalpel, placed in separate tubes containing 80% ethanol, and incubated for 5 to 10 min in a boiling water bath. The tissues were homogenized using a Polytron homogenizer and centrifuged at 27,000g for 15 min. The supernatants were evaporated and lyophilized to remove ethanol. Samples were redissolved in water and the concentrations of sucrose, glucose, fructose, raffinose, and stachyose determined by HPLC as described above.

## RESULTS

### Raffinose Saccharides in Seeds of Different *C. sativus* Cultivars.

Because the occurrence of raffinose and stachyose in cucumber seeds had only been documented in Calypso (11), six other lines or cultivars were assayed for the presence of these sugars. These sugars did occur in all six lines (Table I). Concentrations were fairly uniform except for 'Tablegreen 76' which contained lower concentrations of all three soluble sugars, but higher levels of starch.

**Chl Loss from Maturing Fruit.** It has been previously reported (21) that, as fruit of cucumber cv 'Chipper' mature, there is a drop in endocarp or seed cavity pH. Because this decrease in pH of the fruit endocarp was associated with a loss in peel Chl and an increase in fresh weight and ethylene and CO<sub>2</sub> production, it was used as a measure of relative fruit maturity. To substantiate further that this was a reliable method for determining maturity of Calypso fruit, a separate experiment was conducted comparing fruit endocarp pH with peel Chl during maturation. The results (Fig. 1) indicate that in Calypso the same relationship exists as reported for Chipper. As endocarp pH decreased, there was a

Table I. Concentration of Sucrose, Raffinose, Stachyose, and Starch in the Seeds of Six Different Lines or Cultivars of *Cucumis sativus* L.

Data are from a single analysis.

Line or Cultivar	Sucrose	Raffinose	Stachyose	Starch
				$\mu$ g glu eq/gfw
Addis	7.2	11.1	9.3	40.9
Aodai-nazare	7.2	8.4	9.9	177.9
Gy 3*	7.2	10.8	8.1	32.4
Poinsett 76	9.8	12.7	6.9	168.5
SMR 58*	11.0	12.2	6.0	67.0
Tablegreen 76	1.1	1.8	2.2	723.3

\* Breeding lines; other four are cultivars.

<sup>3</sup> Abbreviations: gfw, gram fresh weight.

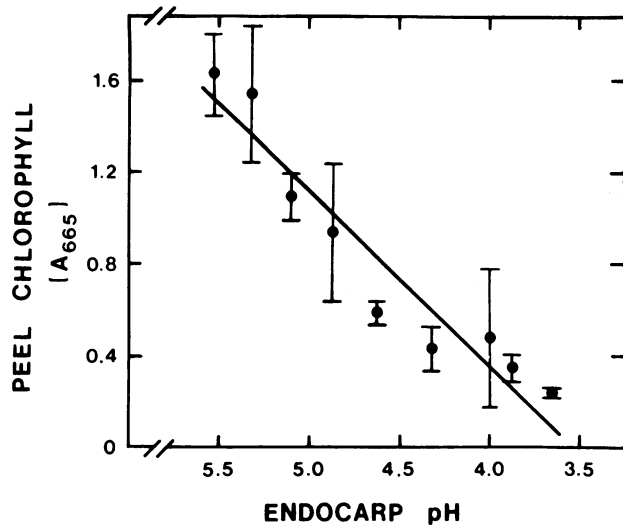


FIG. 1. Relationship between Chl concentration in the peel and endocarp pH in cucumber fruit of increasing maturity. Chl data are reported as the mean  $A_{665} \pm SE$ . The correlation coefficient between endocarp pH and peel Chl was 0.95.

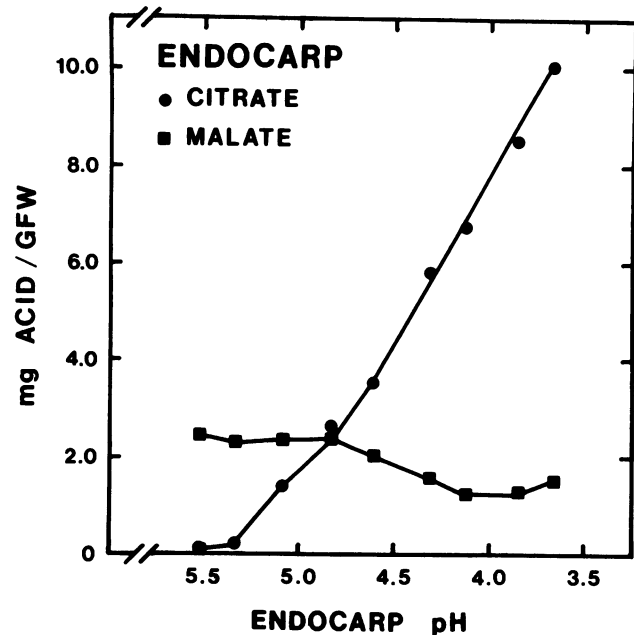


FIG. 2. Content of malic and citric acid in the endocarp tissue of cucumber fruit of increasing maturity. Analysis of variance indicated no significant ( $P = 0.05$ ) change in malate concentration over the decrease in endocarp pH.

corresponding loss in Chl from the peel. Thus, endocarp pH was used as an index of fruit maturation in these studies.

**Organic Acid Content of Fruit Endocarp Tissues.** Analysis for the organic acids malate and citrate by HPLC showed that citric acid accumulated during the decrease in endocarp pH (Fig. 2). The concentration of malic acid did not change over the pH ranges observed. Analysis of these two acids in mesocarp tissue indicated a slight rise in citric acid concentration, but again no change in malic (data not shown). These data are in agreement with previous work which demonstrated that the decrease in endocarp pH is primarily the result of citric acid accumulation (18).

**Sugar Content of Mesocarp, Endocarp, and Seeds of Maturing Fruit.** Analysis of the soluble sugars present in the mesocarp tissue

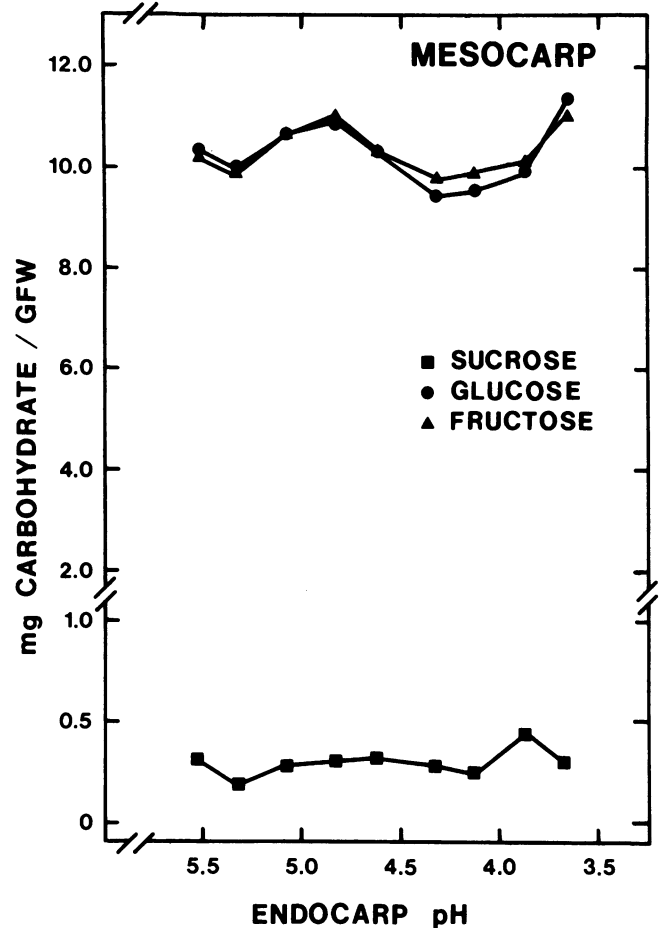


FIG. 3. Sugar content of mesocarp tissue from cucumber fruit of increasing maturity. Analysis of variance indicated no significant ( $P = 0.05$ ) change over maturity (endocarp pH) of the three sugars detected.

showed concentrations of glucose and fructose over 20 times that of sucrose (Fig. 3). The concentrations of these sugars did not change significantly as endocarp pH decreased ( $P = 0.05$ ). More importantly, neither raffinose nor stachyose were detected in the mesocarp tissue in fruit of any maturity. Starch levels in the mesocarp were somewhat variable during maturation (data not shown), but no significant change was found. The mean starch content was  $86 \mu\text{g}$  glucose eq/gfw.

Endocarp sugars showed almost the same pattern (Fig. 4). There were substantially higher concentrations of glucose and fructose than sucrose, and the concentrations of these sugars showed no significant ( $P = 0.05$ ) change during maturation. As in the mesocarp, raffinose or stachyose were not detected. Starch levels were also variable in this tissue (data not shown) with a mean concentration of  $236 \mu\text{g}$  glucose eq/gfw.

The profile of soluble sugar in seeds of maturing fruit showed that concentrations of sucrose and hexoses decreased during maturation (Fig. 5). Sucrose concentration increased during the late stages of maturity while glucose and fructose continued to decline. There was also an increase in the concentration of raffinose saccharides throughout fruit maturation. Low levels of raffinose were present in the youngest set of fruit sampled (mean pH 5.5); however, stachyose did not appear until fruit had a mean endocarp pH of 5.1. Starch content in the seeds tended to be variable as in other tissues. There was, however, a significant ( $P = 0.01$ ) decrease during development from  $6 \text{ mg}$  glucose eq/gfw at pH 5.1 to  $2 \text{ mg}$  glucose eq/gfw at pH 3.7. Starch may decrease further during the final stages of maturation as indicated by the low values found in fully mature seeds (Table I).

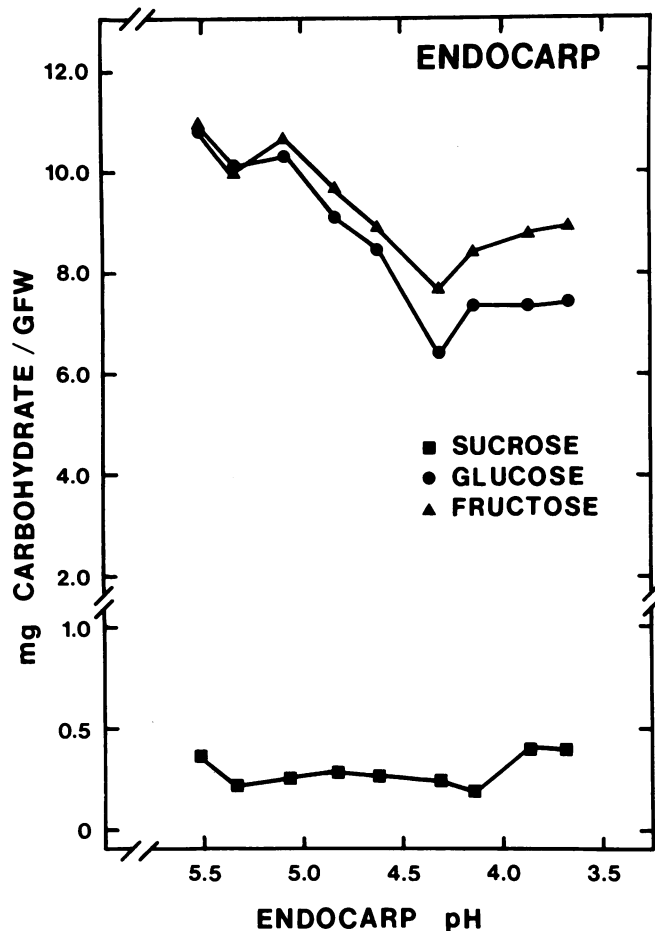


FIG. 4. Sugar content of endocarp tissue from cucumber fruit of increasing maturity. Analysis of variance indicated no significant ( $P = 0.05$ ) change over maturity (endocarp pH) of the three sugars detected.

**Galactinol Synthase Activity.** Seeds from greenhouse-grown fruit of three different pH categories showed increasing galactinol synthase activity as pH decreased (Table II). Mesocarp and endocarp tissues from fruit harvested on three separate occasions were assayed for galactinol synthase and no activity was detected.

**Funiculi and Seed Sugars.** Both raffinose and stachyose were found in the maturing seeds but were not present in funiculi (Table III). Glucose and fructose levels were higher in funiculi tissue than in the attached seed.

DISCUSSION

Data presented in Table I show that storage of the raffinose saccharides appears common to the seeds of *C. sativus*. The pattern of accumulation of these sugars in Calypso seeds (Fig. 5) is similar to that reported in other species which also accumulate raffinose saccharides in their seeds (14, 26, 27). In most of these species, stachyose is the predominant raffinose-type sugar stored (4, 15, 23). In *C. sativus*, as in cotton (24), raffinose appears to be the predominant form.

Analysis of the sugars present in the maturing cucumber fruit showed that at no time during development did the mesocarp and endocarp tissues contain detectable levels of raffinose or stachyose. These sugars appeared only in the developing seed during the later stages of maturation, well after the fruit was a marketable product.

The accumulation of raffinose and stachyose was also mirrored by the activity of the enzyme galactinol synthase. This enzyme is apparently unique to the pathway of raffinose saccharide biosyn-

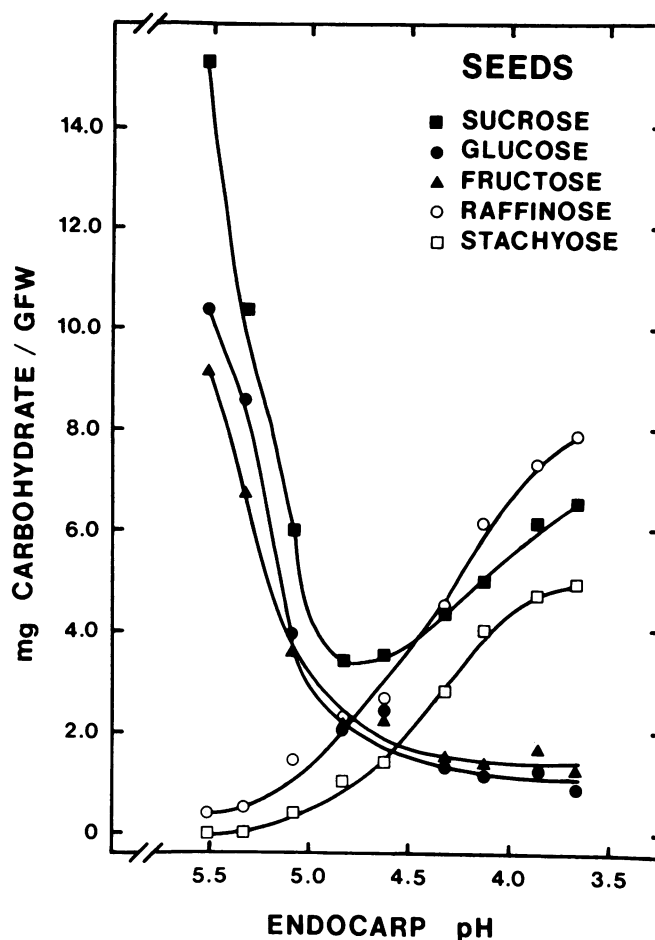


FIG. 5. Sugar content of seeds from cucumber fruit of increasing maturity. Analysis of variance indicated a significant ( $P = 0.01$ ) change over maturity (endocarp pH) of all sugars detected.

Table II. Galactinol Synthase Activity in Seeds of Cucumber Fruit of Increasing Maturity

Endocarp pH	Time from Pollination to Harvest of Fruit	Galactinol Synthase Activity
	<i>d</i>	<i>munits/gfw seeds</i>
5.3	11	1.5 ± 0.6 <sup>a</sup>
4.7	23	11.3 ± 3.2
3.5	45	16.8 ± 2.4

<sup>a</sup> Values are expressed as means ± SE.

Table III. Sugar Content of Funiculi and Seeds of Maturing Cucumber Fruit

Tissue	Sucrose	Raffinose	Stachyose	Glucose	Fructose
	<i>mg/gfw</i>				
Funiculi	4.8 ± 0.7 <sup>a</sup>	ND <sup>b</sup>	ND	7.3 ± 0.8	8.8 ± 1.0
Seeds	3.5 ± 0.3	2.1 ± 0.3	1.8 ± 0.5	0.9 ± 0.1	1.0 ± 0.2

<sup>a</sup> Values are expressed as means ± SE and are the average of samples from three individual fruit with endocarp pH values of 4.3, 4.5, and 4.8.

<sup>b</sup> Not detected.

thesis (11). Its activity has been shown to precede the appearance of raffinose and stachyose in developing soybean seed (11), and its product, galactinol, has been shown to accumulate before these sugars appear in the seeds of other legumes (26, 27). The only known function of this enzyme is the production of galactinol for

the biosynthesis of raffinose saccharides. Sugar accumulation data (Figs. 3-5) show that raffinose and stachyose were found only in seed tissue. Galactinol synthase activity was found to increase in seeds during development (Table II) and was not detected in other fruit tissues.

To confirm that raffinose saccharides were not being transported into the developing seeds, the sugar content of funiculi tissue was assayed. The funiculus is the stalk by which the developing ovule (seed) is attached to the placenta of the fruit (5, 13) and contains the vascular tissues connecting the two structures. If the raffinose saccharides were being transported through this vascular tissue, they should be detected by analysis of sugars in this structure. Raffinose saccharides were not present in the funiculi from fruit in which raffinose saccharides were present in seeds (Table III). Sucrose concentration in the funiculi (Table III) was approximately 10 times as high as that of the surrounding endocarp tissue (Fig. 4). Also, the sucrose concentration of the seed was lower than that of the subtending funiculi. Sucrose presumably, therefore, is the transport sugar present in the vascular tissues of the fruit.

These data, in addition to the enzyme and sugar profile data, indicate that biosynthesis *in situ*, rather than accumulation from the transport stream is responsible for the appearance of raffinose saccharides in cucumber seeds. Raffinose saccharides are synthesized *in situ* in the seeds of other species as well (1, 8, 24).

Because sucrose is the predominant sugar found in the fruit peduncle (20), it is evidently transported through the vascular tissues within the fruit, into the funiculus. Sucrose then enters the developing seed where it is utilized as an energy and carbon source for growth and development, part of which is the biosynthesis of the raffinose saccharides.

Synthesis of the translocated sugar in the source leaves, as well as utilization of the translocate at sinks in cucurbits, involves more extended biochemical sequences than those needed in plants which do not form raffinose saccharides. In the fruit peduncle of cucumbers, galactosyl-sucrose is converted to sucrose through a series of enzymic reactions (9, 25). Sucrose, transported within the vascular tissues of the fruit, may be accumulated as glucose and fructose in the flesh of the fruit or enter the seed where it is, in part, reconverted into raffinose and stachyose. In either case, the transport sugar is different from that accumulated in soluble form within the sink tissue. Thus, one role for these extended catabolic/anabolic sequences in stachyose transporting plants may be the maintenance of a chemical potential for movement of the transport carbohydrate into sink cells, and such a chemical potential could be an asset to accumulation of the relatively high concentrations of soluble sugars in the storage cells of fruits and seeds.

Considerable early research on phloem transport utilized cultivated cucurbits. The rapid rates of dry matter accumulation in developing fruits of these species led to the suggestion that translocation rates might represent the upper limits occurring in plants (3 and references therein). Rapid growth rates of fruit in this and closely related genera and the correlated fact that these plants transport raffinose saccharides rather than sucrose may or may not be fortuitous. Research is needed to contrast relative advantages and disadvantages to sucrose *versus* raffinose saccharide transport in plants.

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