Alteration of Cellular Compartmentation and Membrane Permeability to Sugars in Immature and Mature Apple Fruit

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Abstract. A study was conducted to determine the distribution of sugars in vacuoles, cytoplasm, and free space in apples (*Malus domestica* Bork) picked at the immature and mature stage of maturity. The volumes of free space and air space were 13.4% and 14.5%, respectively, in immature fruit, and 14.6% and 25.6%, respectively, in mature fruit. The inner cellular volume (vacuole + cytoplasm) was 72% and 60% for immature and mature fruit, respectively. About 90% of each sugar (glucose, fructose, sucrose, and sorbitol) was found in the vacuole. The concentration of total sugar in the inner cell or free space was 326 or 128 mM each in immature fruit and 937 or 406 mM each in mature fruit. Permeability to sugars across the plasma membrane and tonoplast also increased with fruit maturation, 7- to 30-fold for the tonoplast and 4- to 5-fold for the plasma membrane in mature compared to immature fruit. Cells in immature fruit apparently enlarge through higher turgor pressure from sequestering of sugars into vacuoles, and cease to enlarge in mature fruit as the amount of sugar unloading into the fruit is reduced due to the accumulation of sugar in the free space or cytoplasm.

Apple fruit enlarge and accumulate sugar after cell division has ceased. Turgor of storage cells and the extensibility of cell walls are important factors in cell enlargement. The osmotic pressure between sieve tubes and free space or cytoplasm is important in the unloading of sugar from sieve tube into storage cells. Many reports suggest active transport of sugars across the plasma membrane (Lin, 1985; Yamaki and Asakura, 1988) or tonoplast (Wyse et al., 1985; Yamaki, 1987). H^+ -ATPase is the major agent, and the sugar carrier is a minor agent in the transport of sugars.

If sieve tube unloading depends on the mass flow and if the turgor of the storage cell contributes to fruit enlargement, then the sugar concentration in free space and the cytoplasm has to be lower than in the vacuole or sieve tube. Furthermore, the concentration is expected to change greatly during fruit development. Consequently, it is of interest to clarify the concentration of sugars in the free space, cytoplasm, and vacuole.

However, direct determination of the solute content in each compartment is problematical, because collection of sap from each compartment is difficult. The compartmental analysis method (Macklon and Higinbotham, 1970; Pallaghy and Scott, 1969) is available for this purpose. Some researchers have applied this method for solute determining content in the apoplast or vacuole of storage tissue, for example, sucrose in sugar beet (*Beta vulgaris* L.) root (Kowallick et al., 1980; Saftner et al., 1983), sugar (Damon et al., 1988), or amino acids (Hsu et al., 1984), in tomato (*Lycopersicon esculentum* Mill.) fruit. The present experiment deals with the distribution of sugars in vacuoles, cytoplasm, and free space during fruit maturation and the relationship between sugar accumulation and fruit enlargement.

Materials and methods

Materials. 'Jonagold' apples were harvested on 18 July and 10 Oct. 1989 from an orchard of the Morioka branch of the

Fruit Tree Research Station. The fruit was kept at 4°C and used within 10 days.

Compartmental analysis method. Apple tissue was hollowed out radially toward the core with a cork borer (10 mm diameter) and sliced into disks 2-mm thick. About 10 g of disks from the middle of the tissue plug was preincubated for 10 sec in 50 ml of 2 mM $CaCl_2$ solution at 0°C to remove sugars from the surface. The disks were then put into the same volume of fresh solution (2 mM $CaCl_2$) and incubated under active aeration. At specified times, the disks were transferred into new solution and incubated for specified times (0 to 250 min). After each incubation, an aliquot of the incubation medium containing the released sugars was frozen and kept at -20°C until analysis by high-performance liquid chromatography (HPLC). Logarithmic values of each sugar remaining in the disks after each incubation period were plotted according to the compartmental analysis method (Macklon and Higinbotham, 1970; Pallaghy and Scott, 1969) to determine the content of each sugar in the vacuole, cytoplasm, and free space.

Determination of free space volume. About 10 g of disks was preincubated 1 h at 0°C in 0.25 M mannitol containing 2 mM $CaCl_2$ to achieve complete equilibration of solute in the free space. The disks then were transferred into 2 mM $CaCl_2$ solution to determine the distribution of mannitol in free space according to the compartmental analysis method noted. The volume of free space was calculated from the mannitol content in the free space divided by the free space volume equivalent to the mannitol concentration in the preincubation medium after equilibration.

Determination of air space. About 5 g of disks was preincubated for 1 h at 0°C in 50 ml of 0.3 M mannitol solution containing 2 mM $CaCl_2$. The disks were weighed, transferred into new solution, and infiltrated under vacuum at 10 mm Hg (Richter and Ehwald, 1983). The disks were weighed at 15 min intervals during infiltration. The volume of air space was calculated by $d = (W_t - W_0)/V_a$, where d = density of the incubation medium, W_0 and W_t = weight of tissue before and after infiltration, and V_a = volume of air space.

Determination of broken cells. The number of broken cells during disk preparation relative to total cells in the disk were obtained through determination of phenolic compounds that are

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localized only in vacuoles (Yamaki, 1984). The disks, carefully cut to minimize release of sap, were divided into two groups. One was directly soaked in 80% EtOH and the other was soaked in 80% EtOH after being washed 10 sec in 2 mM CaCl_2 . Then, both samples were homogenized and centrifuged at $3000 \times g$ for 10 min. The pellet was extracted three times with 80% EtOH and the supernatants were combined and assayed for phenolics by the Folin-Denis method (Folin and Denis, 1915). The proportion of broken cells was calculated from the ratio of phenolics in washed disks to that in nonwashed disks.

Sugar analyses by HPLC. Solution containing the released sugars was concentrated in an evaporator. Sugars were separated on a Shodex Sugar SC 1011 column (Showadenko Co., Tokyo) at 80°C with water ($1 \text{ ml} \cdot \text{min}^{-1}$) using a Hitachi 655A-11 liquid chromatograph with a refractive index detector (RI-3H).

Results

Determination of sugar content by the compartmental analysis method. According to general belief, sugar localized in tissue compartments is released first from the free space, next from the cytoplasm, and finally from the vacuole if sugar is postulated to leak only by diffusion across membranes. When log (sugar content) was plotted against incubation time given, the linear portion of the line (Fig. 1 a and d) indicates release of sugar from the vacuole. By extending the linear trend to time 0, one can obtain the sugar content in the vacuole. When the linear portion is removed from the original curve of Fig. 1 a and d and re-plotted against incubation time, the second linear portion appears (Fig. 1 b and e), which is used to calculate sugar in the cytoplasm. The same procedure can then be used to derive the third portion (Fig. 1 c and f) and the sugar content in the free space.

Volume of free space and air space. The volume of free space in immature fruit was 9% less than in mature fruit, but the volume of air space in immature and mature fruit differed by 76% (Table 1). Thus, air space increased significantly with maturation of the fruit. The inner-cellular volume was calculated by subtracting the free space and air space volumes from the total volume of tissue. The inner-cell volume was nearly equal to the protoplast volume and was predominantly occupied by vacuoles. It was 13% less in immature fruit than in mature fruit (Table 1).

Fig. 1. Distribution of individual sugars in the vacuole, cytoplasm, and free space of apple fruit tissue by compartmental analysis. Fruit were harvested 18 July (immature) and 10 Oct. (mature).

Table 1. Volume of free space, air space, and the inner cell ($\text{ml} \cdot \text{g}^{-1}$ fresh weight) in apple tissue.

Tissue ^z age	Free space	Air space	Inner cell ^y
Immature	0.134 ± 0.010	0.145 ± 0.019	0.721
Mature	0.146 ± 0.060	0.256 ± 0.028	0.598

^zPicked 18 July (immature) and 10 Oct. (mature).

^yObtained by subtracting the volume of free space and air space from the total volume of tissue.

Table 2. The number of intact and total cells and percentage of intact cells in apple disks as determined by testing for phenolic compounds.

Tissue ^z	Phenolic compounds ($\text{mg} \cdot \text{g}^{-1}$ fresh wt)		Intact cells (%)
	Nonwashed (total cells)	Washed (intact cells)	
Immature	30.1 ± 1.2	27.8 ± 1.2	92.4
Mature	12.8 ± 1.0	10.8 ± 1.6	84.4

^zPicked 18 July (immature) and 10 Oct. (mature).

Ratio of broken cells. There were about half as many in immature broken cells as in mature fruit (Table 2). The increased percentage of broken cells in mature fruit is a consequence of fewer cells per gram of tissue due to larger cell size.

Sugar content in free space, cytoplasm, and vacuole. Actual values at time 0 for individual sugar concentrations in the free space, cytoplasm, or vacuole, as determined by the compartmental analysis method, were corrected for broken cells by the factor $100/(100\% \text{ of broken cells})$. About 90% of the sugars were located in the vacuoles of immature and mature fruit (Table 3). This result suggests that the vacuole is the main storage compartment for sugars in apple tissue. The main sugars in immature fruit were fructose and glucose, and minor ones were sucrose and sorbitol. In mature fruit, fructose was the main sugar, followed by sucrose. However, there were no differences among the distribution patterns for fructose, glucose, and sucrose between the cytoplasm and the free space for both mature and immature fruit, and more sorbitol was in the cytoplasm maturities (Table 3). This result may indicate a preferential unloading of the polyol into the cytoplasm.

Sugar concentration in inner cell and free space. The vacuolar volume in the cell is difficult to distinguish from the cytoplasmic volume, although, in general, the cytoplasm occupies only a small portion of the cellular volume. Data in Tables 1, 2, and 3 were used to calculate sugar concentration in the free space and inner cell (vacuole + cytoplasm). The concentration of total sugar in the inner cell and free space was 326 and 128 mM in immature fruit and 937 and 406 mM each in mature fruit (Table 4). The difference in sugar concentration between inner cell and free space was numerically greater and the sugar concentration was higher in both entities in mature than in immature fruit.

Membrane permeability for each sugar. K values (velocity constant) were obtained from the slope of log (sugar $\text{mg} \cdot \text{g}^{-1}$ per fresh wt per min) in Fig. 1. Values were higher for the plasma membrane than for the tonoplast in both mature and immature fruit, but especially in immature fruit (Table 5). During fruit maturation, permeability of sugars increased 7- to 30-fold through the tonoplast and 4- to 5-fold through the plasma membrane. No prominent differences in permeability were found among the

Table 3. Sugar content in the vacuole, cytoplasm, and free space of apple tissue (mg·g⁻¹ fresh weight).

Tissue ^z	Sucrose		Glucose		Fructose		Sorbitol		Total	
	A ^y	C ^y	A	C	A	C	A	C	A	C
<i>Immature</i>										
Vacuole	3.04 ± 1.15*	3.29	11.9 ± 0.2	12.9	21.6 ± 0.3	23.4	1.51 ± 0.04	1.63	38.0 ± 0.7	41.2
Cytoplasm	0.34 ± 0.13	0.37	0.75 ± 0.05	0.81	1.32 ± 0.10	1.43	0.21 ± 0.03	0.23	2.61 ± 0.30	2.84
Free space	0.20 ± 0.02	0.22	0.88 ± 0.02	0.95	1.73 ± 0.10	1.87	0.15 ± 0.01	0.16	2.97 ± 0.18	3.20
Total	3.58 ± 1.04	3.88	13.5 ± 0.2	14.7	24.6 ± 0.3	26.7	1.86 ± 0.06	2.02	43.6 ± 0.6	47.2
<i>Mature</i>										
Vacuole	19.6 ± 0.8	23.3	12.7 ± 0.1	15.1	52.4 ± 3.7	62.2	4.01 ± 0.15	4.76	88.7 ± 3.1	105
Cytoplasm	1.37 ± 0.08	1.63	0.82 ± 0.08	0.97	3.20 ± 0.21	3.80	0.79 ± 0.03	0.94	6.18 ± 0.18	7.34
Free space	2.00 ± 0.19	2.37	1.49 ± 0.29	1.77	6.34 ± 0.03	7.52	0.41 ± 0.01	0.49	10.2 ± 0.5	12.2
Total	23.0 ± 1.1	27.3	15.0 ± 0.3	17.8	61.9 ± 4.0	73.5	5.20 ± 0.12	6.13	105 ± 3.0	125

^zPicked 18 July (immature) and 10 Oct. (mature).^yA = actual value calculated by the compartmental analysis method, C = corrected for broken cells.^xValue ± SD.

Table 4. Sugar concentration (mM) in the inner cell (IC) and free space (FS) of apple tissue.

Sugar	Immature ^z		Mature ^z	
	IC	FS	IC	FS
Sorbitol	14.3	6.6	53.0	4.6
Fructose	191	77.5	613	286
Glucose	106	39.4	149	67.4
Sucrose	14.8	4.8	122	47.5
Total	326	128	937	406

^zPicked 18 July (immature) and 10 Oct. (mature).

Table 5. Velocity constant (k) for individual sugars across the plasma membrane (PM) and tonoplast (TP) in immature and mature apples.

Sugar	Immature ^z		Mature ^z	
	TP (× 10 ⁻³ ·min ⁻¹)	PM (× 10 ⁻³ ·min ⁻¹)	TP (× 10 ⁻³ ·min ⁻¹)	PM (× 10 ⁻³ ·min ⁻¹)
Sorbitol	0.235 ± 0.025	29.7 ± 7.7	1.52 ± 0.08	56.9 ± 7.9
Fructose	0.066 ± 0.002	27.5 ± 3.5	1.39 ± 0.04	78.8 ± 7.3
Glucose	0.077 ± 0.002	32.3 ± 1.8	1.52 ± 0.02	80.8 ± 2.8
Sucrose	0.057 ± 0.013	28.6 ± 3.1	1.56 ± 0.09	88.7 ± 4.7

^zPicked 18 July (immature) and 10 Oct. (mature).

K values for each sugar, except for sorbitol through the tonoplast of immature fruit.

Discussion

Although compartmental analysis is a good method for determining the solute content in the free space, cytoplasm, and vacuole of apples, one must accept two premises: 1) solute released from each compartment conforms to the law of diffusion and is not energy dependent and 2) membranes (plasma and tonoplast) have functional and structural integrity during solute release. When sugar is unloaded from the sieve tubes in phloem tissue, it is sometimes considered to be effluxed into free space by co-transporting with H⁺ (Partrick, 1990). In some tissue, the unloading of sugar is reduced by inhibitors of H⁺-ATPase (Thorne and Rainbird, 1983; Wolswinkel and Ammerlaan, 1983). These results indicate that energy-dependent transport will play a role in the efflux of sugar across the membrane. However, the rate of sugar released into a medium is closely related to the osmolarity of the incubation medium (Porter et al., 1987). Thus, in a very hypotonic solution containing no sugar, such as in the present experiment, diffusion is expected

to occur. Also, plasmolysis may occur under such hypotonic conditions. When disks are incubated in water at room temperature, sugar release becomes rapid after 30 min, and no linear release from vacuole occurs (data not shown). However, if the disks are incubated in 2 mM CaCl₂ solution at 0°C, such an abrupt release does not take place. The linearity of release continues for >2 h until ≈50% of the total sugar is released. Consequently, the compartmental analysis approach was considered valid for our purposes.

The concentration of sugar of phloem exudate in woody plants was reported by Zimmermann (1957, 1958) to be between 0.3 and 0.5 M throughout the developing season. In the present results, sugar concentration in the free space (apoplast) was 128 mM in immature fruit and 406 mM in mature fruit. Thus, the apoplastic unloading in immature fruit functioned actively because of the high osmotic pressure between free space and sieve tube, whereas in mature tissue the unloading was weak due to the low osmotic pressure. The increase in free space with maturation is likely due to an absence of H⁺-ATPase coupled sugar-transport across the plasma membranes (Yamaki et al., 1989) and a higher leakage of sugar across the plasma membrane (Table 5). However, the symplastic unloading is considered weak in mature fruit due to the small difference in osmotic pressure between the cytoplasm and sieve tube. This small difference is explained by the following arguments: 1) equilibration of sugar concentration between cytoplasm and free space occurs because uptake of sugar across the plasma membrane depends on diffusion in mature fruit (Yamaki et al., 1989) and 2) the sugar concentration in the cytoplasm remains high because the tonoplast in mature fruit is very leaky for sugar permeation compared with that in immature fruit as shown in the velocity constants (Table 5). We suggest that sugar unloading into fruit flesh from sieve tubes must decrease due to the reduction in apoplastic and symplastic flows of sugar.

Sugar accumulation in vacuoles and in the free space proceeds with fruit maturation. The turgor formed between vacuoles and free space becomes higher in mature fruit. This relationship clearly indicates that vacuoles are the site of sugar deposits and that the H⁺-ATPase coupled sugar-transport system continues to function actively in mature fruit as it does in immature fruit (Yamaki, 1987).

Vacuoles can have a strong turgor pressure through immature and mature stages. Immature fruit can enlarge by increasing the turgor from sequestering unloaded sugar into the vacuole. However, the fruit ceases to enlarge when it becomes mature. This

cessation occurs because of a reduction of sugar unloading into fruit flesh and by the physical strength of the cell wall maintaining cellular structure. As Yamaki et al. (1979) pointed out in the development of Japanese pear (*Pyrus pyrifolia* Nakai) fruit, polysaccharides needed for cell walls in mature fruit have been already deposited during the immature stage, meaning that a thicker or folded cell wall is present in immature fruit. The cell wall can be unfolded easily through minor enzymic modification of polysaccharides and the fruit can enlarge by expansion of vacuoles much like a balloon expanding. However, when the fruit reaches the mature stage, cell walls are difficult to unfold because of a shortage of folded polysaccharides.

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