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Functional Characterization of a Putative Disaccharide Membrane Transporter in Crustacean Intestine

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Functional Characterization of a Putative Disaccharide Membrane Transporter in Crustacean Intestine

by

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Abstract

The mechanisms of transepithelial absorption of dietary sucrose in the American lobster, *Homarus americanus,* were investigated in this study to determine whether sugars can be transported across an animal gut intact or as monosaccharides following hydrolysis. Lobster intestine was isolated and mounted in a perfusion chamber to characterize the mechanisms of mucosal to serosal (MS) ^{14}C -sucrose transport across the intestine MS fluxes were measured by adding varying concentrations of ^{14}C-sucrose to the perfusate which resulted in a hyperbolic curve following Michaelis-Menten kinetics. The kinetic constants of the proposed sucrose transporter were K_M = 15.84 ± 1.81 μM and J_{max} = 2.32 ± 0.07 ρmol cm^{-2}min^{-1}. The accumulation of ^{14}C-sucrose in the bath in the presence of inhibitors, phloretin, phloridzin, and trehalose was observed. Inhibitory analysis showed that phloridzin, an inhibitor of Na^+-dependent mucosal glucose transport, decreased MS ^{14}C-sucrose transport suggesting that MS ^{14}C-sucrose radioactive flux may partially involve an SGLT-1-like transporter. Phloretin, a known inhibitor of Na^+-independent basolateral glucose transport, decreased MS ^{14}C-sucrose transport, suggesting that some ^{14}C-sucrose radioactivity may be transported to the blood by a GLUT 2-like carrier. Decreased MS ^{14}C-sucrose transport was also observed in the presence of trehalose, a disaccharide containing D-glucose moieties. Thin-layer chromatography (TLC) was used to identify the chemical nature of radioactively labeled sugars in the bath following transport. TLC revealed ^{14}C-sucrose was transported across the intestine largely as an intact molecule with no ^{14}C-glucose or ^{14}C-fructose appearing in the serosal bath or luminal perfusate. Bath samples evaporated to dryness and resuspended disclosed only 15% volatile metabolites. Results of this study strongly suggest that disaccharide sugars can be transported intact across

animal intestine and provide support for the occurrence of a disaccharide membrane transporter that has not previously been functionally characterized.

Introduction

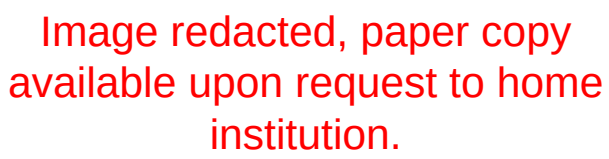
The transport of sugars, both complex and simple, from within the intestine to the bloodstream of vertebrates and invertebrates has been studied for many years. Complex carbohydrates are converted into smaller molecules to be transported across the phospholipid bilayer of intestinal plasma membranes following digestion. Because of their permeability coefficients and size, complex sugars require integral proteins for facilitated diffusion or active transport to enter cells (Walter, 1986 and Finkelstein, 1976). As primary carbon sources, most heterotrophs utilize carbohydrates, specifically D-glucose, D-fructose, and D-galactose for energy (Walmsley, 1998). Glucose and fructose, both simple sugars, have transporters that have a history of evolutionary development to facilitate uptake from the lumen. Transport occurs via passive and active mechanisms. Facilitated diffusion transport mechanisms involve mobile carriers to allow substances to move down a concentration gradient across a liquid membrane without requiring the cell to expend energy (Cussler, 1989). Active transport is a process where a protein requires energy to move molecules against their concentration gradients. The sodium-potassium ATPase is an example of an energy-dependent primary active transport process in which there is an unequal exchange of three sodium ions exported out of the cell for two potassium ions that are imported into the cell. Secondary active transport uses a transport protein that indirectly utilizes the sodium-potassium ATPase to transport ions and molecules across the plasma membrane.

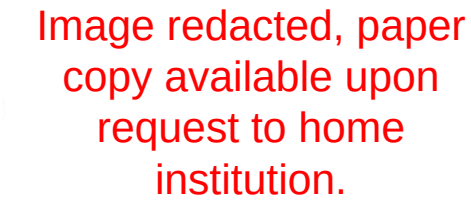
Specifically, glucose transporter families are facilitated glucose transporters, GLUT, and the sodium-coupled glucose cotransporters, SGLT. The GLUTs are responsible for the downhill, passive transport of glucose across cell membranes and SGLT1 is responsible for the secondary active transport of glucose across the brush border membrane of the small intestine (Wright, 2007).

The solute carrier 5 (SLC5) co-transporter gene family is a large group of glucose transporter proteins that utilize protons or sodium as co-transported substrates. The sodium-glucose transpoter1 (SGLT1) is a co-transporter of sodium and glucose or galactose found on the apical side of the intestinal cells and plays a dominant role in glucose absorption in the small intestine (Gould and Holman, 1993; Ma et al., 2010). Sodium first binds to the negatively charged co-transporter to make the binding site for glucose available. Once glucose binds to its binding site, the co-transporter undergoes a conformational change which causes the release of glucose into the cytosol followed by Na^+ release into the cytosol. The co-transporter regains its negative charge and undergoes a conformational change returning to original state (Sala-Rabanal et al, 2012).

The direction and rate of glucose transport by SGLT1 are functions of the direction and magnitude of the Na^+-gradients across the plasma membrane. In normal cells, it is the Na/K-ATPase that sets the direction and magnitude of the sodium gradient (Wright, 2007). Therefore, Na^+ and sugar co-transport by SGLT1 is referred to as secondary active transport because the driving forces and concentration gradients are maintained by the primary active sodium-potassium pump (Figure 1).

Another group of sugar transporter proteins are those within the glucose transporter (GLUT) family which are sodium independent. Classes I and II consist of glucose transporters 1-4 (GLUT1-4), and Class III consists of GLUT 6, 8, 10, and 12 (Wilson-O'Brien et al, 2010). The overall structure of the GLUT proteins is conserved across classes (Figure 1). GLUT5 mediates the uptake of fructose on the apical side of the intestine (Burant et al., 1992). The GLUT2 transporter on the basolateral side of the cell allows glucose transport from within the epithelial cell to the bloodstream (Goodman, 2010).





A)

B

Figure 1 A model of Na^+-glucose cotransporters (SGLTs) binding Na^+ first (step 2) then binding glucose (step 3), undergoing a conformational change (step 4), releasing the glucose and Na^+(steps 5 and 6) then returning to the original state (step 1)(A)(Sala-Rabanal et al , 2012). GLUT transport proteins are sodium independent and act as a facilitated diffusion system. Glucose binds to its binding site on the transporter, the transporter undergoes a conformational change releasing glucose in the cytosol. Overall structure of GLUT transporters is conserved (Wilson-O'Brien et al, 2010) (B).

For many higher plants, sucrose is the dominant form of sugar translocation (Lalonde and Frommer, 2012). In plants, sucrose is also the major transport form for photoassimilated carbon and is both a source of carbon skeletons and energy for plant organs unable to perform photosynthesis (Lemoine, 2000). For sucrose to move from its source to various organs of the plant, the disaccharide has to be transported across several membranes involving specific sucrose carriers called SUTs.

Like monosaccharide transporters, sucrose transporters also belong to a large gene family. The first sucrose transporter gene (SUT1) was identified by expression cloning from spinach and potato leaf cDNA libraries (Kuhn et al., 1999). Interestingly, the sucrose transporter also mediates transport of the disaccharide maltose and a variety of glucosides. A second sucrose

transporter, SUT2, is characterized by an extended central loop. This central loop contains several conserved domains across species of plants with sucrose transporters present (Lalonde et al., 2004).

Humans also have sucrose transporter homologs known as the solute transporter family (SLC45) composed of 4 genes: SLC45A1, SLC45A2, SLC45A3, and SLC45A4. The SLC45 family is composed of a co-transporter along with 3 orphan transporters, transporters without identified substrates. Although only Slc45A1 has been demonstrated to translocate sugars across membranes thus far, there is some evidence that the other members of the human SLC45 gene family encode sugar transporters. Compared with plant families, the novel SLC45 family is a small group which was named a "putative" sugar transporter family because all members exhibit an apparent amino acid sequence that is more than 20% similar to plant sucrose transporters (Lemoine, 2000). On average, sucrose transporters share approximately 30% similar and 16% identical amino acids (Lalonde and Frommer, 2012).

A phylogenetic analysis of a sucrose transporter (SCRT) identified in *Drosophila melanogaster* found the transporter to be a sister to a clade comprising SLC45A1, SLC45A2, and SLC45A4 (Fig 2). The H^+/sucrose symporter was termed by FlyBase as Slc45-1 and shows a significant similarity to members of the human SLC45 family (Vitavska and Wieczorek, 2013). Figure 2 indicates the apparent phylogenetic relationship of the animal proteins not only with their plant counterparts, but also with those found in bacteria and fungi.

Figure 2 Phylogenetic tree of SLC45 family of animal proteins and similar proteins from plants, fungi and bacteria. SLC45A1–4: *Homo sapiens*; Slc45-1:*Drosophila melanogaster*; DP: *Daphnia pulex*; ZP: *Zunongwangia profunda*; Gn: *Glaciecola nitratireducens*; Am: *Alteromonas macleodii*; Pi: *Piriformospora indica*; Ao: *Aspergillus oryzae*; Ao: *Ajellomyces capsulatus*; AtSUC2-4: *Arabidopsis thaliana*. Substitutions per position are indicated by the scale bar. (Vitavska and Wieczorek, 2013).

The evolutionary history of SCRT reveals that it, like SLC45, has a highly conserved sucrose transporter signature (R-X-G-R-R). This transmembrane protein has 12 domains, an elongated N-terminus, and an extended central loop (Figure 3). However, since the expression of this gene was not tested in the organism from which it was isolated, its potential nutritional role still remains to be shown.

Figure 3 Hypothetical topology membrane spanning model of the *Drosophila* Slc45-1 sucrose transporter. Each circle represents one amino acid. Black circles are sites of serines or threonines which may be targets for phosphorylation by the protein kinases. Gray circles with asterisks: R-W-G-R-R (in circle), correspond to the signature sequence for sucrose transporters (Vitavska and Wieczorek, 2013).

The first animal membrane disaccharide transporter gene for a sucrose transporter, SCRT, from the genome of the fruit fly, *Drosophila melanogaster*, facilitating the absorption of the disaccharide sugar in *Saccharomyces cerevisiae* as a heterologous expression system, was recently reported (Meyer et al., 2011). The gene was identified in *Schizosaccharomyces pombe* to test the uptake of disaccharide sugars then expressed in in *Saccharomyces cerevisiae* (Meyer et al., 2011). Immunostaining of the late embryonic fly hindgut indicated the localization of SCRT and suggested the nutritional involvement of the sucrose transporter.

The disaccharide sugar, sucrose, is a complex sugar containing 1 glucose and 1 fructose joined with a glycosidic bond. It was historically accepted that disaccharides were hydrolyzed

before passing through absorptive gut epithelial cells since a disaccharide transporter was not known to be present in mammals (Alvarado, 1984). Furthermore, in mammals, extracellular hydrolases split complex sugars into their monomers, although there remained a question concerning the amount of sucrase present versus the disappearance rate of disaccharides (Miller and Crane, 1963)(Figure 4). In the hamster intestine, sucrose is hydrolized along the wall of the intestine and the remaining glucose and fructose move into the cell using SGLT1 and GLUT 5, respectively. This is the accepted model in other mammals as well (Miller and Crane, 1963). In humans, sucrose hydrolysis was studied and compared to monosaccharide absorption to reveal sucrose hydrolysis rates that exceeded the monosaccharide product absorption rates (Gray, 1966). Therefore, in animal cells sucrose was accepted to be hydrolyzed into glucose and fructose which subsequently passed across the plasma membrane.

Figure 4 Sucrose hydrolyzed into glucose and fructose by sucrase to pass across mammalian intestinal membranes as monosaccharides (Gray,1975).

Disaccharide transport has never been observed in animal cells, meaning sucrose has never been shown to be transported as an intact, whole molecule. Sucrase is the specific hydrolase present to cleave the glycosidic bond between glucose and fructose (Miller and Crane, 1963).

Sucrase does not exist free within the lumen but bound to the brush border membrane via a hydrophobic polypeptide segment of the intestinal cell to hydrolyze sucrose efficiently then release glucose and fructose for absorption (Reiser et al., 1974 and Brunner, 1979).

Beginning around 1990, studies of membrane transport proteins facilitating cellular uptake of di- and tripeptides from protein digestion by gastrointestinal epithelial cells showed that a considerable fraction of dietary proteins are absorbed as larger molecular units than as simple amino acids (Thamotharan et al., 1996). These studies argued that, under certain conditions, a variety of nutrients might be transported across intestinal epithelia as polymers. With this in mind, a hypothesis was formulated that proposed polysaccharide transport, specifically sucrose transport, might also occur in some animal species (Meyer, 2011).

The experimental organism used in the present study was the American lobster, *Homarus americanus.* Although sugar transport has been studied extensively in mammals, very little is known regarding sugar transport in crustaceans. In crustaceans, the digestive tract consists of three major divisions: the foregut, the midgut, and the hindgut (Wright and Ahearn, 1997). The foregut and hindgut are lined with a chitinous cuticle and are understood to play a minimal role in nutrient absorption compared to the midgut (Wright and Ahearn, 1997). Although the primary source of energy for crustaceans is not carbohydrates, the amount of carbohydrates available to crustaceans have a direct effect on growth and survival (Verri et al. 2001).

Crustaceans are a very diverse group of organisms, living in a wide variety of habitats, including freshwater, marine, and terrestrial. The large numbers of species found in these very different environments are largely a function of the physiological plasticity of the group as a whole (Ahearn et al., 1999). The American lobster, *Homarus americanus*, is an economically important crustacean harvested from the wild catch fishery. Functional challenges to organisms

inhabiting these markedly dissimilar environments often involve specialized adaptations of epithelial cell layers found in the gills, integument, gut, and antennal glands, which allow the animals to regulate the passage of molecules. Although the lobster diet is mainly protein, carbohydrates are encountered through marine plants (Conklin, 1995) and complex carbohydrate storage molecules such as glycogen in prey orangisms. Obi et al., (2011) reported transport of glucose and fructose across lobster intestine is qualitatively similar to sugar uptake in mammalian intestine, suggesting evolutionarily conserved absorption processes.

The crustacean midgut consists of the hepatopancreas and intestine, with the primary roles of dietary digestion and absorption. The hepatopancreas is a major site of sugar absorption (Ahearn and Maginniss, 1977) and is located bilaterally in the thoracic cavity consisting of E, F, R, B, and M cells (Verri, et al. 2001). The variety of cells in the hepatopancreas reflects the variety of hepatopancreatic functions in digestion and absorption. In the crustacean hepatopancreas, sucrase is one of several digestive enzymes (Saxena, 1982). The intestine is comprised of a single epithelial cell type with various transport proteins in the membranes of these cells and is considered to be a scavenger organ since it is secondary in nutrient absorption to the hepatopancreas (Wright and Ahearn, 1997). The intestine plays a significant role in the absorption of D-glucose and D-fructose (Verri et al., 2001 and Obi et al, 2011).

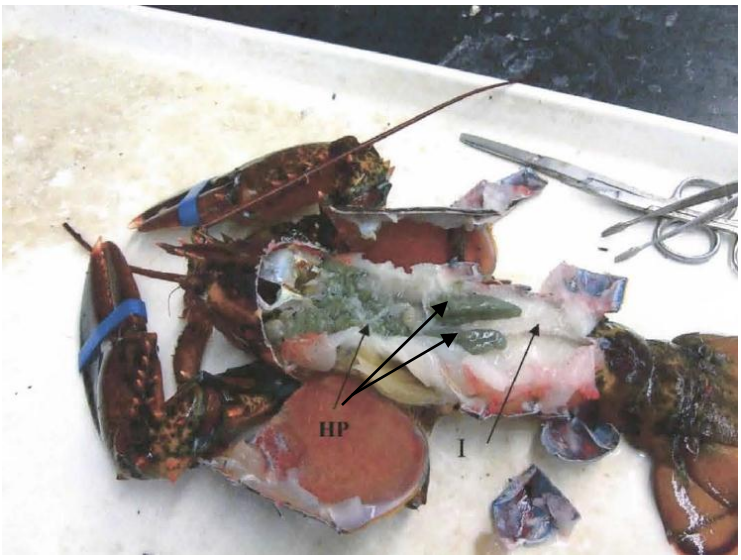


Figure 5 A dissected lobster showing the bilobed hepatopancreas (HP) and the intestine (I). Part of the intestine lies underneath the hepatopancreas and runs through the tail of the lobster. This picture was taken at the University of North Florida Physiology Research Laboratory, Jacksonville, Fl.

Carbohydrate digestion for these animals begins in the gastric chamber by secretion of a digestive enzyme mixture including α-amylase, glycosidases, maltase, and α-glucosidase (Johnson, 2003). Food is pulverized and recirculated between chitinous foregut chambers and tubules of the hepatopancreas for digestion and initial absorption, followed by final nutrient uptake by the intestine. Crustaceans have the strongest ability to degrade carbohydrates compared to other classes of invertebrates (Glass and Stark, 1995). The α-amylase in decapod crustaceans has been shown to be similar to amylolytic activity associated with mammals. Food passed through the foregut and midgut for digestion and some absorption continues to the intestine for final handling. The epithelial cells of the heptopancreas and intestine are lined with transporters on the apical and basolateral sides of the cells to ensure digested carbohydrates and other nutrients are transferred from the lumen of the organs to the bloodstream.

Sucrose, as an intact molecule, has been shown to pass across single cell membranes *in vitro* in cell culture, but trans-membrane or trans-intestinal transport has not previously been reported *in vivo* for animal intestinal epithelial cells. The low concentration of disaccharides, and high concentration of monosaccharides during intestinal adsorption in humans, suggested that either disaccharides entered cells and were hydrolyzed internally or were hydrolyzed by a membrane-bound enzymes before entering the cells (Gray, 1966).

Because the initial description and expression of the SCRT transporter in *Drosophila* was not followed by the functional characterization of this carrier system, the present study was undertaken that utilizes another arthropod species to assess the physiological properties of a putative intestinal sucrose transporter. Key methods used in this study included transport inhibition to slow or eliminate transport. Drugs and other sugars were presented as inhibitors of the proposed transporter. By using the transport-blocking drugs, phloridzin and phloretin, which respectively block glucose uptake across the apical membrane, and glucose and fructose efflux across the basolateral membrane of the intestinal epithelium, the resulting experiments helped establish whether ^{14}C-sucrose, or its hydrolyzed products (^{14}C-glucose and ^{14}C-fructose), passed through the lobster intestine. Phloridzin is a competitive inhibitor of SGLT1 that acts in a two-step process. Initially, instead of glucose binding, phloridzin binds with SGLT1, followed by a slow isomerization that results in phloridzin bound to the receptor-site of the SGLT (Raja et al., 2003). With phloridzin blocking the receptor, glucose cannot bind to the symporter and uptake is blocked on the apical side of the intestinal cell (Zheng et al., 2012). Phloretin has been observed to strongly inhibit monsaccharide transport by orienting itself in the opposite direction as the monosaccharide on GLUT2 that releases glucose or fructose to the serosa (Verkman and Solomon, 1982 and Zheng et al., 2012).

A similarly structured sugar was also used as a potential inhibitor of sucrose transport (Figure 6). Trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) is a sugar containing two glucose monomers attached by a glycosidic bond. Due to its similarity in nature to sucrose and increased potential to be part of the natural lobster diet, it was used as an alternative disaccharide to act as a possible competitive inhibitor. Trehalose is a non-reducing disaccharide that is used as an osmolyte, transport sugar, carbon reserve, and stress protectant in a wide range of organisms including bacteria, fungi and invertebrates and also in algae, mosses and liverworts in very low levels (Carillo, 2013). Because trehalose is found in a lobster's natural diet, trehalose is more apt to be digested by a lobster than sucrose.

Figure 6 Similar structures of trehalose (A) and sucrose (B) (Vilen 2013)

Thin layer chromatography (TLC) is an accepted chemical analysis method that was used for separating and identifying small quantities of compounds in a mixture and was used in the present study to assess whether the disaccharide, sucrose, was hydrolyzed into the constituent monosaccharides, glucose and fructose, during trans-intestinal transit. TLC is simple yet widely used for the analysis of synthetic organic molecules and natural products including carbohydrates because of their polarity (Zhang , 2009). Chromatography uses a stationary phase and a mobile liquid phase. The mobile phase flows through the stationary phase and carries the components of

the mixture with it based on polarity and molecular weight. TLC uses a thin, uniform layer of silica gel coated on a piece of glass or plastic as the stationary phase and the mobile phase being the developing solvent which is chloroform and methanol for carbohydrates.

The overarching purpose of this research was to investigate the presence of a potential SCRT-like transporter located in the intestine of the American lobster. The existence of a SCRT-like transporter would identify a possible nutritional purpose for the transporter. These results would be unique because sugar transport across animal membranes is widely believed to be restricted to monosaccharides

Materials and Methods

Animals

Male *H. americanus* lobsters were purchased from a local seafood dealer (Fisherman's Dock, Jacksonville, Florida) and were maintained unfed at 15 °C for no more than 1 week in an aquarium containing filtered seawater. The portion of the intestine that was used was cut from 1 cm posterior to the stomach to about two-thirds of the length of the tail. *In vitro* transmural mucosal to serosal (MS) transport of ^{14}C-sucrose (American Radioactive Chemicals, St. Louis, Missouri) was investigated using a perfusion apparatus as previously described (Ahearn and Maginniss, 1977) (Figure 7). The midgut tissue from the intestine was flushed with physiological saline (410 mM NaCl, 15 mM KCl, 5 mM $CaSO_4$, 10 mM $MgSO_4$, 5 mM Hepes/ KOH at pH 7.1) and mounted on a 18 gauge needle at both ends of the perfusion apparatus using surgical thread. The length and diameter of the experimental intestine were measured and the intestinal surface area was calculated using the equation $A = \pi ld$, where l and d represent the length and diameter of the intestine, respectively. The perfusion bath (serosal medium) was filled with 35 mL of physiological saline. The experimental perfusate (the experimental saline plus appropriate experimental treatments) was pumped through the intestine using a peristaltic pump (Instech Laboratories Inc., Plymouth Meeting, PA,) at a rate of 0.38 mL min^{-1} (Figure 7). This rate was previously shown to provide constant transmural transport in lobster intestine for more than 3 hr of incubation without added oxygen at 23°C (Conrad and Ahearn, 2005).

Figure 7 Example perfusion set up with perfusate containing the [^{14}C] sucrose and saline serosal bath (Ahearn and Maginniss, 1977).

Mucosal to Serosal (MS) Transport

Prior to the start of experimentation, ^{14}C-sucrose (400-700µCi/mmol) was purchased from Perkin-Elmer Biotechnology Company (Waltham, MA). Triplicate aliquots of each experimental perfusate (200 µL) were collected in separate Falcon tubes to determine the total counts of radioactively labeled sugar in each tube, and from the bath to determine the amount of background radioactivity at the beginning of an experiment. Experimental solutions were then perfused through the intestine for varying times, but not more than 3 hr. All experimental procedures were carried out at 23°C and timed with a laboratory timer. Triplicate aliquot radioactive samples (200 µL) were collected from the serosal medium after passage across the intestine every 10 min for the duration of each experimental treatment. An equal amount of physiological saline was added back to the serosal medium in order to maintain a constant volume in the bath.

Data Analysis

The radioactive experimental samples collected were placed in a 7 mL tube containing 3 mL scintillation cocktail and counted for radioactivity in the Beckman LS6500 scintillation counter. The mean background count was subtracted from each triplicate sample at each time

point. The specific activity of the perfusate was used to convert sample cpm to ρmol of sugar transported. Radioactive counts obtained from bath samples (200 µL) were corrected for radioactivity in the full volume of the bath (35 mL). Transmural mucosal to serosal transport rates were expressed in ρmol cm^{-2} min^{-1}. Slopes of the data were determined by linear regression analysis and data curve fitting procedures using Sigma Plot 10.0 software (Systat Software Inc. Point Richmond, CA, USA). Experiments were repeated a minimum of three times using three separate animals providing similar results between animals. Significance of slopes apart from one another were obtained and determined using paired t-tests with SPSS Statistics 19 software (IBM. Armonk, NY, USA).

Thin Layer Chromatography

Prior to the start of experimentation, triplicate aliquots of each experimental perfusate (200 µL) were collected in separate Falcon tubes to determine the total counts of radioactively labeled sugar in each tube, and from the bath to determine the amount of background radioactivity at the beginning of an experiment. Experimental solutions were then perfused through the intestine for 3 hr. All experimental procedures were carried out at 23°C and timed with a laboratory timer. At the end of each hour, three 200 µL samples were collected from the serosal medium after passage across the intestine and placed in a 7 mL tube containing 3 mL scintillation cocktail and counted directly for radioactivity. At each hour, two 200 µl samples were taken from the bath, dried then resuspended in water to be counted in the Beckman LS6500 scintillation counter. One 200 µl sample was removed and placed in a 10mL test tube to be used to spot on a silica gel sheet. An equal amount of physiological saline was added back to the serosal medium in order to maintain a constant volume in the bath.

The TLC procedure was adapted from Farag (1978) and Young (1970). The conventional silica gel sheets (Analtech Inc., Newark, DE) were 20 x 20 cm. which were subdivided into lanes. The sheets remained grease-free and thoroughly clean before use. The glass developing chamber used was approximately 25 x 25 x 10 cm. with a lid that was sealed with petroleum jelly. To prepare the sheet for development, an origin line was marked 1.75cm from the bottom of the sheet and the solvent front was marked 14cm from the origin. The composition of individual samples on each separate lane was 15µl of ^{3}H-glucose, ^{3}H-fructose, ^{14}C-sucrose standards, bath samples at 1hr., 2hr, and 3hr (respectively), 3hrs dry and resuspended, and perfusate effluent. Standards were created by combining 2 µL of ^{3}H-glucose, ^{3}H-fructose, or ^{14}C- sucrose with 1mL of physiological saline. Lanes were labeled and marked, then 15 µL of each sample was applied to the origin 5 µL at a time. The spots were dried with a Conair (18755 watt) hair dryer between spotting. The solvent consisted of choloroform and methanol (80:20) by volume respectively. The solvent was poured into the developing chamber, then the silica gel sheet was allowed to develop until the solvent reached the solvent front after approximately 1hr. The TLC sheet was removed and the solvent allowed to evaporate for 15min. The sheet was then placed back into the chamber to be developed a second time. Once the sheet had developed twice, it was allowed to air dry for 15min. The sheets were visualized by cutting each lane into 2.5cm strips vertically then 0.5cm horizontally and placed separately in 7 mL tubes containing 3 mL scintillation cocktail and counted for radioactivity in the Beckman LS6500 scintillation counter. The mean background count was subtracted from each sample. The experiment was repeated a minimum of 3 times using different lobsters (Figure 8).

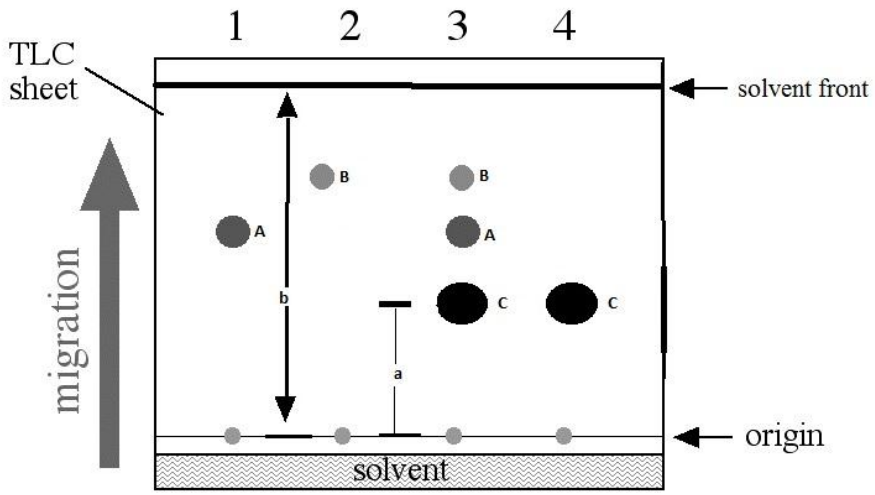


Figure 8 TLC sheet with origin and solvent front marked. Distance of each dot was measure from the starting point to the middle of the spot (a). The solvent front (b) was consistent for each silica gel sheet.

Volatilization

Prior to the start of experimentation, triplicate aliquots of each experimental perfusate (200 µL) were collected from separate Falcon tubes to determine the total counts of radioactively labeled sugar in each tube, and from the bath to determine the amount of background radioactivity at the beginning of an experiment. Experimental solutions were then perfused through the intestine for 1 hr. At the end of 1 hr, at least two 200 µL samples were removed from the serosal bath and counted directly. Two additional 200 µL samples were placed in test tubes to be evaporated in a drying oven for 5hrs at 85°C. The dried samples were then resuspended in 200 µL de-ionized water and counted.

Results

Time course of ^{14}C-sucrose transport across perfused intestine

Figure 9 illustrates a 2 hr time course of 0.1 mM ^{14}C-sucrose transport across perfused lobster intestine. Values displayed on this figure were collected at 10 min intervals and are displayed as means ± 1 SEM of 3 animals. Each time point sample for each experiment was collected in triplicate. All intestines were perfused with radiolabelled saline for 8 min without taking bath samples prior to initiating the 2 hr transport interval in order to equilibrate intestinal tissues with perfusate radioactivity. The calculated transmural rate of transport, determined from linear regression, was 6.96 +/- 0.58 pmol/cm^{-2} min^{-1}, assuming all serosal radioactivity was present as the original ^{14}C-sucrose molecule. These results suggest that over the time period selected, ^{14}C-sucrose transport was a linear function of time and did not show a tendency toward equilibration or reduced flux due to depletion of intracellular energy reserves.

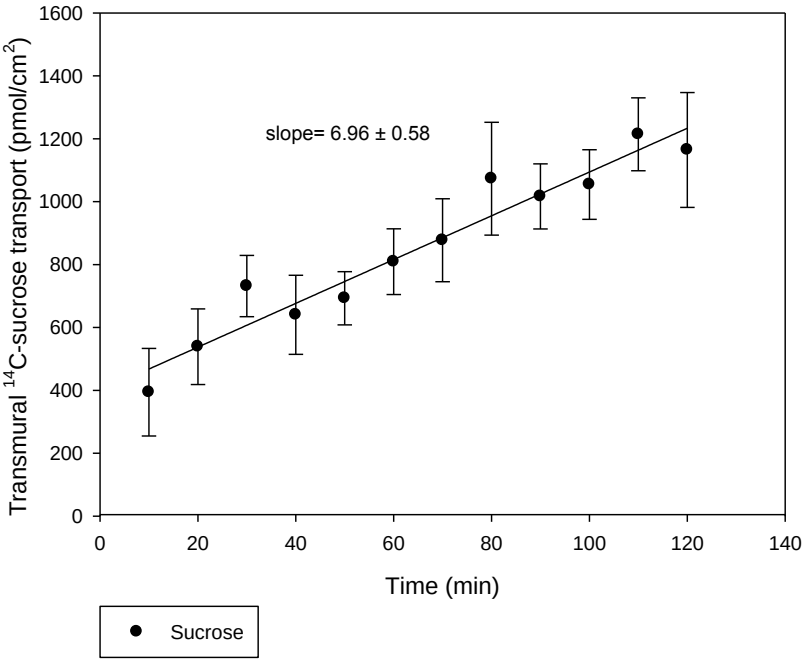


Figure 9 Transmural MS transport of radioactivity from ^{14}C-sucrose as a function of incubation time. Estimated sucrose transport was 6.96 ± 0.58 in ρmol cm^{-2} min^{-1} assuming all radioactivity in bath was in the form of ^{14}C-sucrose and not as its metabolites. Each experiment was conducted a total of three times. The slope was obtained using linear regression analysis (Sigma plot 10.0 software).

Effect of Inhibitors of ^{14}C-sucrose Transport

Transport blocking drugs, phloridzin and phloretin, were introduced separately into the experimental system as transport inhibitors. For the phloridzin experiments, 25 mL of 0.1 mM ^{14}C-sucrose was perfused through the intestine for 1hr (bath samples taken every 10 min). After 1hr, the perfusate was changed to 25 mL of 0.1mM ^{14}C-sucrose with 0.5 mM phloridzin. The intestine was first perfused for 8 min, the bath being stirred consistently for 30 seconds every

min, with no samples being taken. After 8 min, the intestine was perfused for 1hr under the phloridzin conditions and bath samples were collected every 10 min during this perfusion. This experiment was performed in quadruplicate using four separate animals. In Figure 10, transmural transport of 0.1 mM ^{14}C-sucrose from mucosa to serosa under control conditions (no drug) was 29.95 ± 2.83 ρmol cm^{-2} min^{-1}. Transport of ^{14}C-sucrose from mucosa to serosa in the presence of 0.5mM phloridzin was 20.82 ± 1.77 ρmol cm^{-2} min^{-1}. Using a paired samples t-test, these slopes were statistically different ($p = 0.04$, $t(3) = 3.23$). Phloridzin inhibited approximately 36% of the perfused ^{14}C-sucrose from being transported across the intestinal wall.

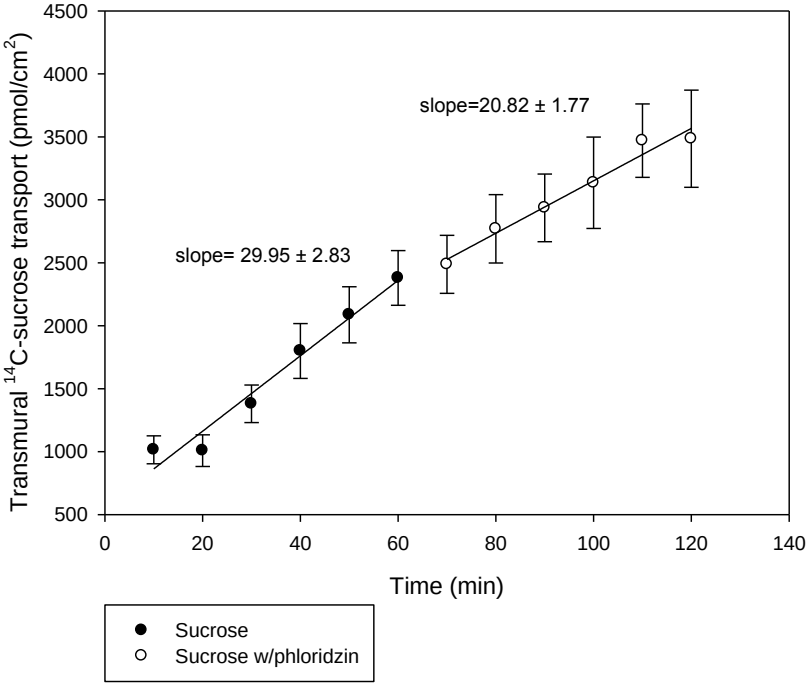


Figure 10 Effect of luminal 0.5 mM phlordizin on transmural transport of 0.1 mM ^{14}C-sucrose. During the first 60 min control interval, no phloridzin was added to the perfusate (29.95 ± 2.83 ρmol cm^{-2} min^{-1}). During the second 60 min interval, 0.5 mM phloridzin was added to the luminal perfusate along with 0.1 mM 14C-sucrose (20.82 ± 1.77 ρmol cm^{-2} min^{-1}). These slopes are significantly different using a paired samples t-test ($p < 0.05$). Each experiment was

conducted a total of four times. Slopes were obtained using linear regression analysis (Sigma plot 10.0 software).

The next experiment was designed to determine if the efflux process of ^{14}C-radioactivity from intestinal epithelium to serosal medium was affected by phloretin, a known inhibitor of basolateral glucose-transporting GLUT2 transporters. For 1 hr, 25 mL of 0.1mM ^{14}C-sucrose was perfused through the intestine. After 1hr, 0.5mM phloretin was added to the serosal bath. The peristaltic pump remained off for 8 min as the bath was stirred occasionally. After 8 min, the intestine was perfused 1hr with 25 mL of 0.1mM ^{14}C-sucrose. In Figure 11, the results from the phloretin experiment showed that the slopes were significantly different ($p = 0.006$, $t(2) = 13.20$) with the control slope being 20.99 ± 2.10 ρmol cm^{-2} min^{-1}, and under the phloretin conditions the slope was 14.26 ± 3.04 ρmol cm^{-2} min^{-1}. Transport in the presence of phloretin was 38% less than it was under control conditions. This experiment was performed in triplicate (three separate animals).

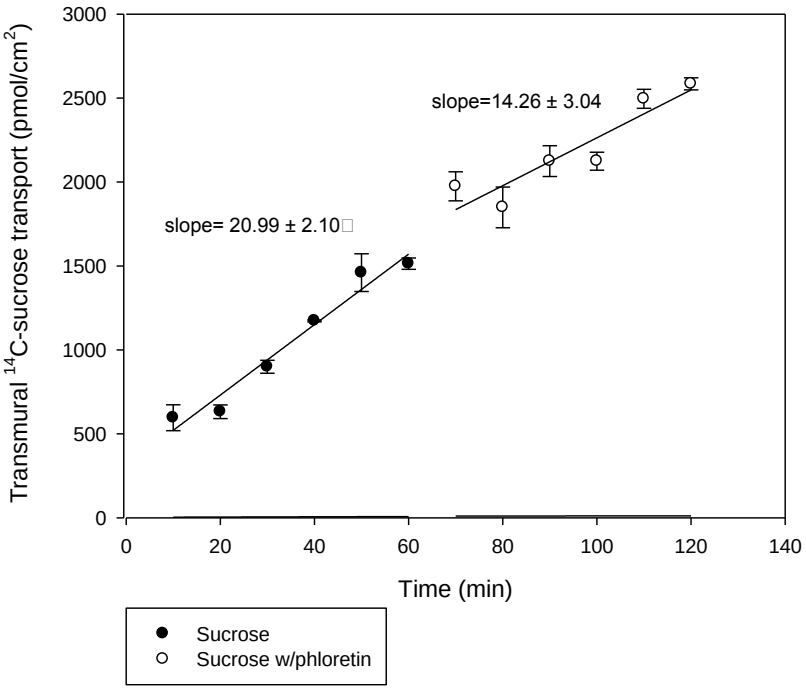


Figure 11 Effect of serosal 0.5 mM phloretin on transmural transport of 0.1 mM ^{14}C-sucrose. During the first 60 min control interval, no phloretin was added to the bath (20.99 ± 2.10 ρmol cm^{-2} min^{-1}). During the second 60 min interval, 0.5 mM phloretin was added to the bath and 0.1 mM ^{14}C-sucrose was perfused through the lumen (14.26 ± 3.04 ρmol cm^{-2} min^{-1}).These slopes are significantly different using a paired samples t-test ($p < 0.05$). Each experiment was conducted a total of three times. Slopes were obtained using linear regression analysis (Sigma plot 10.0 software).

To test the ability of the putative lobster SCRT-like transporter to transport disaccharides in addition to sucrose, trehalose was used as an alternative disaccharide. The experiment was perfused using 25 mL of 0.1mM ^{14}C-sucrose perfused through the intestine for 1hr as a control condition. After 1hr, 5 mM trehalose was added to the perfusate, and the intestine was perfused for a second 1hr interval. Bath samples were collected and counted continuously over the 2 hr

perfusion period. In Figure 12, the results from the experiment with trehalose showed that the control slope was 24.11 ± 3.29 ρmol cm^{-2} min^{-1} and trehalose treatment slope was 14.14 ± 1.84 ρmol cm^{-2} min^{-1}. Based on a paired samples t-test, the control and trehalose slopes were significantly different ($p = 0.001$, $t(3) = 6.46$). The average of 4 experiments indicated trehalose inhibited sucrose transport by approximately 52%.

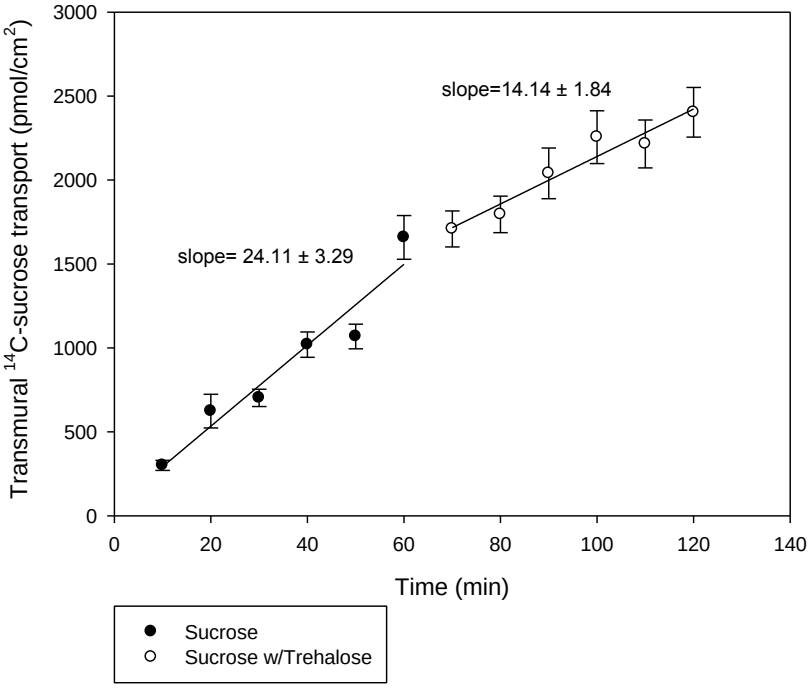


Figure 12 Effect of 5 mM luminal trehalose on transmural 0.1 mM ^{14}C-sucrose transport. During the first 60 min control interval, no trehalose was added to the perfusate (24.11 ± 3.29 ρmol cm^{-2} min^{-1}). During the second 60 min interval, 5 mM trehalose was added to the perfusate and 0.1 mM ^{14}C-sucrose was perfused through the lumen (14.14 ± 1.84 ρmol cm^{-2} min^{-1}). These slopes are significantly different using a paired samples t-test ($p < 0.05$, $t(3) = 6.46$). Each experiment was conducted a total of four times. Slopes were obtained using linear regression analysis (Sigma plot 10.0 software).

^{14}C-Sucrose Transport Kinetics

To determine the presence and characteristics of a putative sucrose transporter, transmural ^{14}C-sucrose was studied in the presence of increasing mucosal concentrations of sucrose. Perfusate saline containing 2.5µM, 5.0µM, 10µM, 25µM, 50µM, and 250µM sucrose was labeled with tracer amounts of ^{14}C-sucrose. After the standard 8 min perfusion with the selected ^{14}C-sucrose concentration prior to beginning each test period, respective ^{14}C-sucrose concentrations were subsequently perfused through the intestine for 1 hour stirring the serosal bath every 15 min then taking three 200µL samples at the end of each hour. Because the intestinal transporters were only usable for approximately 4hrs in *in vitro* experimental conditions, only 3 concentrations were sequentially tested at a time. The 2.5µM, 5.0µM, 10µM sucrose concentrations were sequentially tested for 1 hr each in 1 experiment in triplicate, and the 25µM, 50µM, and 100µM sucrose concentrations were sequentially tested for 1 hr each in a second experiment in triplicate. The individual transport rates at each concentration were obtained by linear regression analysis of the uptake over 60 min. at each concentration (Figure 13). Resulting transport rates of ^{14}C-sucrose at each concentration were plotted as a function of varying mucosal sucrose concentrations, resulting in a hyperbolic curve (Figure 14).

The hyperbolic curve followed the Michaelis-Menten equation for carrier-mediated transport given below:

$$J = \frac{Jmax\ [S]}{(K_M + [S])} \qquad \text{Equation 1}$$

Where J is transmural ^{14}C-sucrose transport in pmol/cm^{-2} min^{-1}, J_{max} is the maximum transport velocity in pmol/cm^{-2} min^{-1}, K_M is the concentration at half maximal transport velocity in µM ^{14}C-sucrose, and [S] is substrate concentration in µM ^{14}C-sucrose. The kinetic constants of

the proposed sucrose transporter, computed from curve-fitting analysis of the hyperbolic curve in Fig. 14, are: K_M = 15.84 ± 1.81 µM and J_{max} = 2.32 ± 0.07 ρmol $cm^{-2}min^{-1}$. These findings of hyperbolic ^{14}C-sucrose transport suggest that the movement of this disaccharide across the lobster intestine is mediated by a saturable, carrier-mediated process.

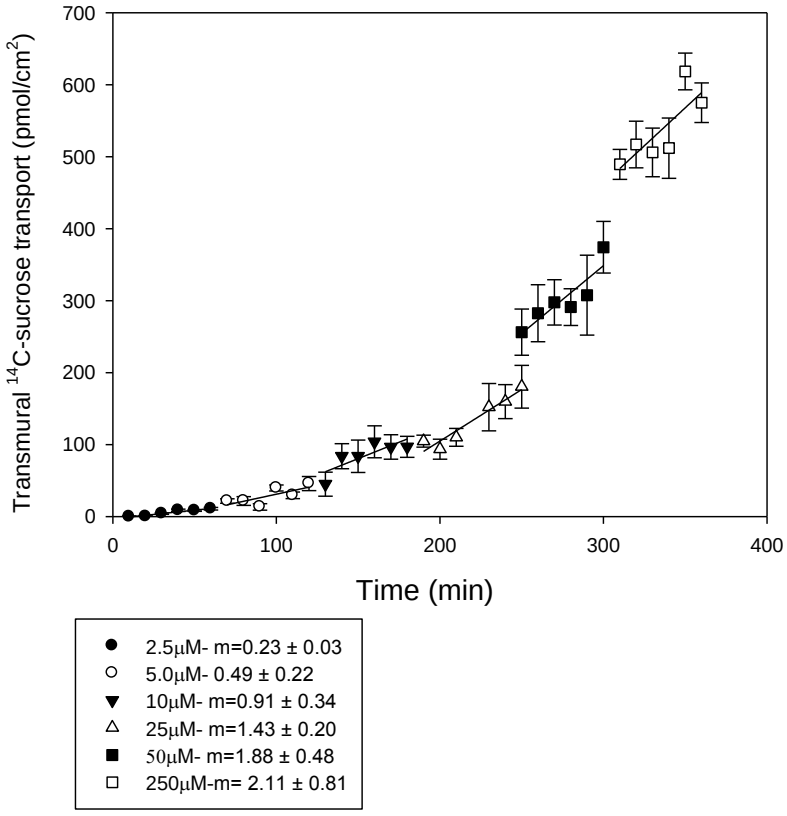


Figure 13 Transmural MS transport of radioactivity from ^{14}C-sucrose as a function of incubation time was obtained by linear regression analysis of uptake over 60 min and was computed using Sigma plot 10.0 software. ^{14}C-sucrose that was transferred from M to S during the 8 min tissue equilibration period was subtracted from each time point before plotting the sequential 10 min MS transport rate for each concentration. The experiments were repeated in triplicate (3 separate animals) and values represent means ± 1SEM.

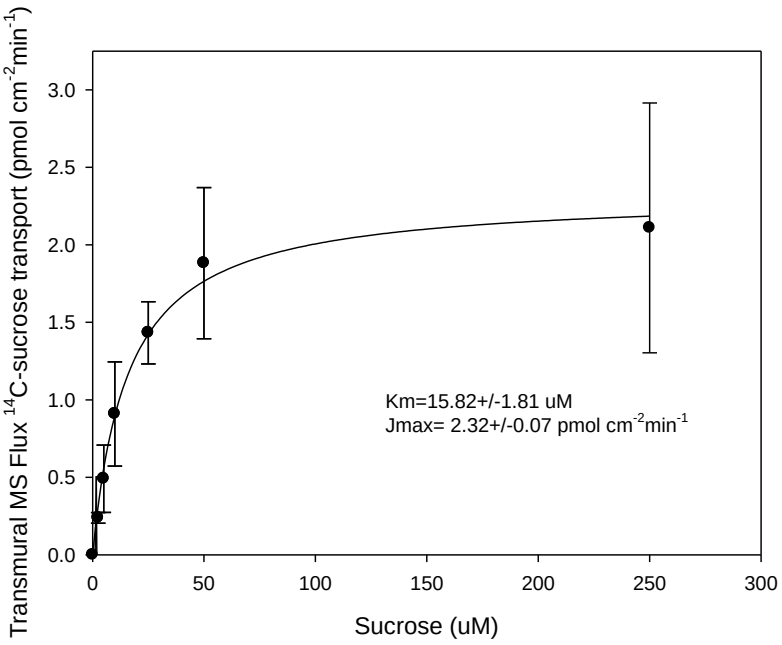


Figure 14 An experiment showing the effect of increasing mucosal ^{14}C-sucrose concentration on transmural MS flux of ^{14}C-sucrose transport. Each experiment was conducted a total of three times with three replicates each. The kinetic constants of the proposed sucrose transporter are: K_M = 15.84 ± 1.81 µM and J_{max} = 2.32 ± 0.07 ρmol cm^{-2}min^{-1}. The hyperbolic curve was obtained using Sigma plot 10.0 software.

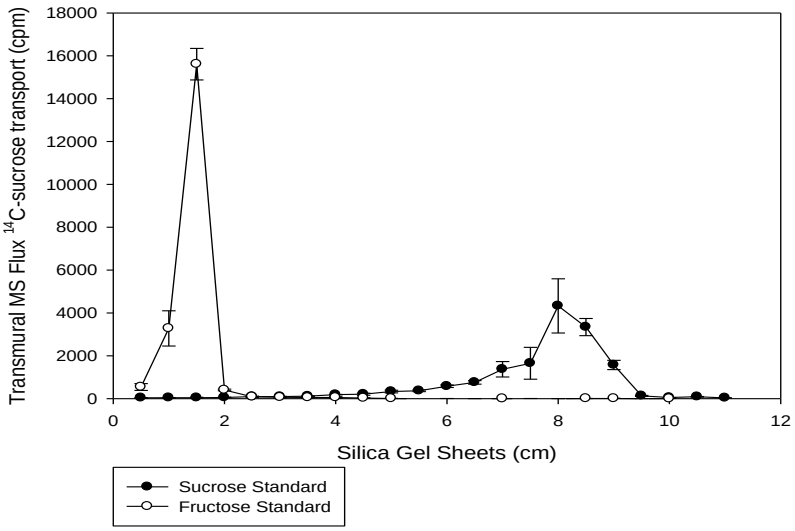
Thin Layer Chromatography

Thin-layer chromatography (TLC) was used to characterize the chemical form of ^{14}C-radioactivity present in the serosal bath after transit of ^{14}C-sucrose across intestinal tissues. In a preliminary experiment, 0.1 mM ^{14}C-sucrose was perfused through an intestine for 1 hr and 200 µL samples were taken of the bath, applied to a chromatogram sheet, and following development, the separation path was cut into 1 cm pieces which were counted for radioactivity alongside ^{14}C-sucrose and ^{3}H-D-fructose controls. At this ^{14}C-sucrose concentration,

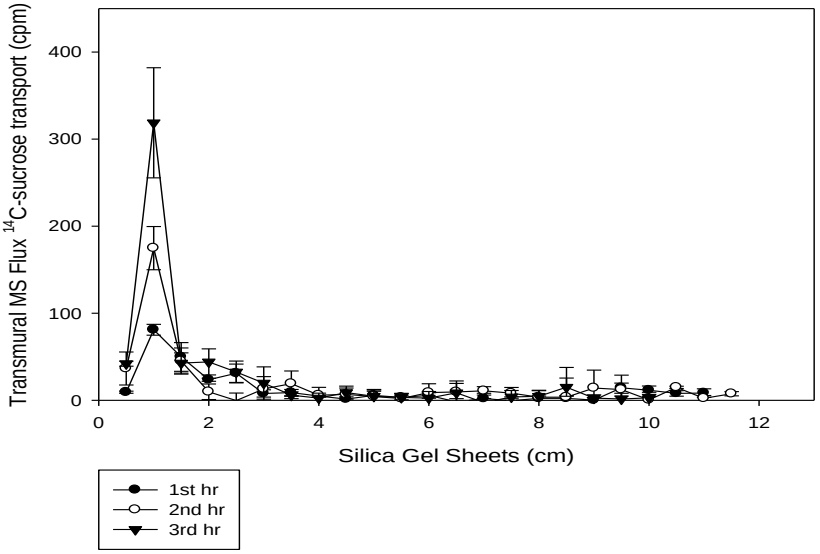
insufficient radioactivity from any sugar was observed along the disaccharide separation path on the silica gel sheet. Therefore, transport of ^{14}C-sucrose at a higher concentration was tested in quadruplicate experiments. ^{14}C-sucrose concentration was increased from 0.1 mM to 5 mM and the perfusion time was increased from 1 to 3 hr.

Following perfusion of 5 mM ^{14}C-sucrose for 3 hrs, triplicate 200 μL samples of the serosal bath and 200 μL of the collected perfusate effluent were spotted on a silica gel TLC sheet along with ^{14}C-sucrose and ^{3}H-D-fructose standards to assess the chemical nature of the ^{14}C-radioactivity on both sides of the transporting intestine. As displayed in Figure 15A, the ^{14}C-sucrose standard localized near the chromatogram origin, while the ^{3}H-D-fructose localized around 8 cm from the origin. Figure 15B indicates that after 1, 2, or 3 hrs of isotope perfusion, essentially all of the serosal isotope localized at the ^{14}C-sucrose standard position near the origin, with only a faint trace of isotope at the ^{3}H-D-fructose location. Figure 15C shows that during the perfusion period, only ^{14}C-sucrose could be identified in the perfusion effluent collected over the entire 3 hr experiment. These results suggest that during transport across the intestine ^{14}C-sucrose was not significantly converted into ^{14}C-glucose and ^{14}C-fructose metabolites which would have appeared either in the serosal bath and/or the perfusate effluent.

A


Transmural MS Flux ^{14}C-sucrose transport (cpm)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
12
Silica Gel Sheets (cm)
Sucrose Standard
Fructose Standard

B


Transmural MS Flux ^{14}C-sucrose transport (cpm)
400
300
200
100
0
0
2
4
6
8
10
12
Silica Gel Sheets (cm)
1st hr
2nd hr
3rd hr

C

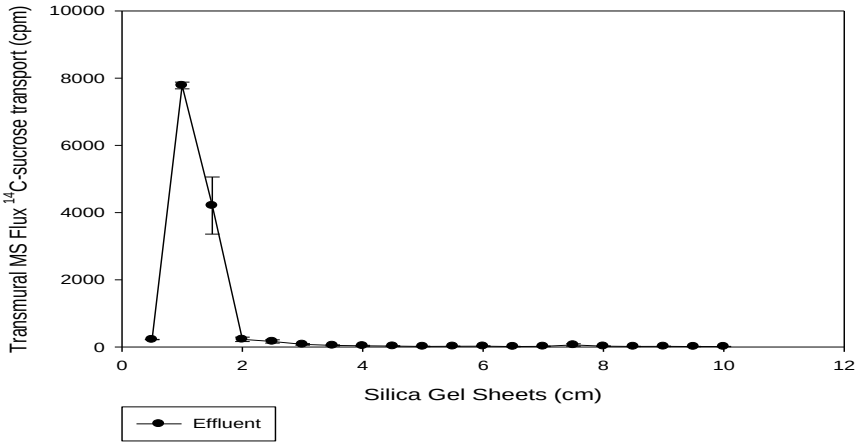

Transmural MS Flux ^{14}C-sucrose transport (cpm)
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
12
Silica Gel Sheets (cm)
Effluent

Figure 15 TLC of ^{14}C-sucrose and ^{3}H-D-fructose standards (A), sample of the serosal bath after a 3 hr perfusion (B), and sample of the perfusate effluent after a 3hr perfusion (C). Each experiment using 5mM ^{14}C-sucrose at a bilateral pH of 8.5 was conducted a total of four times (4 separate animals). Identification of radioactive counts per centimeter of silica gel sheet were obtained by cutting individual pieces of the separation path, placing them is scintillation cocktail, and counting in a scintillation counter.

Volatilization

An analysis of ^{14}C-sucrose metabolism into serosal bath volatile cpm and non-volatile cpm was conducted following a 3 hr transport experiment using 0.1mM and 5mM ^{14}C-sucrose (experiment was conducted in triplicate). Following the transport period, a 200 μL bath sample was counted for radioactivity directly and another 200 μL sample was evaporated to dryness, resuspended in distilled water, and subsequently counted for total cpm. Table 1 presents total cpm values for samples counted directly without evaporation, non-volatile cpm remaining after evaporation, and computed volatile cpm as the difference between the first two. In addition, Table 1 also presents the percent of total cpm due only to volatile counts. Results suggest that only a minor fraction of original ^{14}C-sucrose radioactivity passing through perfused intestines was metabolized to a volatile molecule, most likely $^{14}CO_2$. As shown in Table 1 and Figure 15, the great majority of radioactivity in the serosal bath following trans-intestinal transit was unchanged ^{14}C-sucrose.

Table 1 Comparison of volatile fraction to non-volatile fraction when intestines were perfused with 0.1mM ^{14}C-sucrose and 5mM ^{14}-sucrose

0.1 mM ^{14}C-Sucrose

Animal	Total Bath Cpm	Non-Volatile Cpm	Volatile Cpm	% Volatile Fraction
1	10,267	5,833	4,433	55.07
2	24,675	24,442	233	0.95
3	10,967	9,100	1,867	18.60

5 mM ^{14}C-Sucrose

Animal	Total Bath Cpm	Non-Volatile Cpm	Volatile Cpm	% Volatile Fraction
1	76,417	74,492	1,925	2.55
2	180,425	157,150	23,275	13.79
3	174,592	152,483	22,108	13.52
4	58,100	46,200	11,900	22.82
5	44,683.33	38,908.33	5,775.00	13.82
6	112,642	98,583	14,058	13.31

A bath sample analysis of 0.1 mM and 5 mM ^{14}C-sucrose metabolism into volatile and non-volatile fractions following 3 hr perfusions. The 0.1mM sucrose experiment was repeated 3 times and the 5mM sucrose experiment was repeated 6 times and individual values for the replicate experiments are displayed in respective rows. Using a paired samples t-test ($p < 0.05$, $t(3) = 0.21$), the 0.1mM ^{14}C-sucrose non-volatile fraction cpm was not significantly different from the total bath cpm. However, using a paired samples t-test ($p < 0.05$, $t(6) = 0.048$), the 5mM sucrose non-volatile fraction cpm was significantly different from the total bath cpm.

Discussion

Carbohydrates are major sources of energy for numerous organisms across the planet. Because of their importance for several biological processes, sugar transport has been studied across a wide variety of life forms. The finding of an SCRT-like gene in *Drosophila melanogaster* and expressed in *Saccharomyces cerevisiae* that transports sucrose is what led to this study. This study was conducted by investigating the transport of ^{14}C-sucrose across the *Homerus americanus* intestine in physiological saline that mimics the ionic composition of the lobster hemolymph. Until now, sucrose transport has not been shown to occur by a sucrose transporter in animals. The proposed work strongly suggests that sucrose is able to be transported as an intact molecule across crustacean intestine epithelium.

Comparative Sugar Transport

Sugar absorption in the lobster digestive tract occurs sequentially as food travels from stomach to rectum with transport characteristics being determined by substrate and ion concentrations in each organ to fully remove glucose and fructose from the lumen. The apparent binding affinities for sugars in lobster and prawn intestines are reported to be in the low μM range (Ahearn and Maginniss, 1977; Obi et al., 2011). It was noted that the crustacean intestine (posterior) is considered a scavenger organ that is resonsible for absorbing excess dietary sugars that were not otherwise absorbed by the hepatopancreas (anterior). As a result, the transport proteins located in the intestine should have a higher affinity for the uptake of sugars than in the hepatopancreas. This point is supported by comparing the kinetic constants of sugars in the hepatopancreas and in the intestine. The K_M for both D-glucose and D-fructose in the hepatopancreas is in the high μM or low mM range in comparison to the low μM range in the intestine supporting a higher affinity binding protein (Ahearn et al., 1985; Verri et al., 2001).

Crustaceans do the posterior organs have higher affinities for sugars, and also in a teleost fish. In the rockfish (*Sebastes caurinus*) pyloric caeca (anterior organ) D-glucose transport had a glucose binding affinity four times lower than the D-glucose transporters in the upper intestine (posterior organ) (Ahearn et al., 1992). In the hepatopancreas (anterior organ) luminal substrate concentrations are high, and the cells employs low affinity sugars transporters; while in the intestine (posterior organ) where luminal substrate concentrations are depleted, intestinal epithelial cells employ high-affinity sugar transporters to mediate maximum sugar removal from the lumen.

In Table 2, kinetic constants are compared across various species and absorptive organs to reinforce the higher affinity for sugars in posterior organs than anterior organs across various species. Also, similar substrates have similar kinetic constants across organisms. The current findings on intestinal ^{14}C-sucrose transport are comparable to previous research of apparent binding affinity (K_M) and maximal transport rate constants. A comparison of MS K_M values for glucose and fructose in the lobster intestine also reveals a similar high affinity transport protein for sucrose as well. The K_M values represent the concentration of substrate available to the transporter and the efficiency of the transporter. The monosaccharide transporters in lobster intestine had a K_M = 15.2 ± 3.5 µM for D-glucose and K_M = 10.1 ± 1.9 µM for D-fructose (Obi, et. al, 2011). The SCRT-like transporter observed in this study had a K_M = 15.82 ± 1.81 µM, indicating a high affinity system for sucrose uptake across the intestine similar to that of D-glucose and D-fructose. The apparent maximal transport velocity for D-fructose, J_{max} = 5.75 ± 0.4 ρmol cm^{-2} min^{-1} compared to sucrose, J_{max} = 2.32 ± 0.13 ρmol cm^{-2} min^{-1}, are very similar and also support the hypothesis of a high binding affinity of intestinal transport system working at low substrate concentrations for sucrose.

By comparison, plant sucrose transporters, as in *Beta vulgaris*, type I SUT, AtSUC2, which are responsible for loading high concentrations of sucrose into phloem, has a ten-fold higher K_M (affinity) for sucrose of 2,000 µM with a wide substrate specificity for α and β glucosides (Reinders, 2012). Data from Table 2, including the present study, confirm efficient sugar transporters based on the kinetic constants indicating increased transport across absorptive organs that is comparable to the present SCRT-like kinetic constants.

Table 2 Comparable kinetic constants of sugars in absorptive organs across various species.

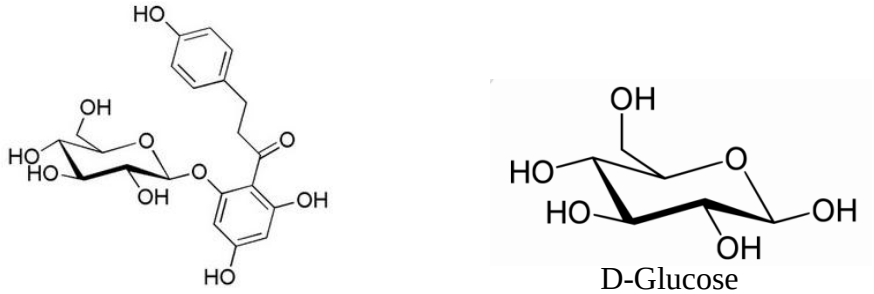
Species	Tissue	Substrate	K_M	Reference
Invertebrates				
Homarus americanus (lobster)	Hepatopancreas	D-glucose	620 ±50 μM	Wright and Ahearn, 1997
		D-fructose	325 ±81 μM	Sterling et. al, 2009
	intestine	D-glucose	15.2 ± 3.5 μM	Obi et. al, 2011
		D-fructose	10.1 ± 1.9 μM	Obi et. al, 2011
		sucrose	15.8 ± 1.81 μM	
Callinectes sapidus (Blue crab)	intestine	D-glucose	87 ± 19μM	Chu, 1986
Macrobrachium rosenbergii (Freshwater prawn)	intestine	D-glucose	170 μM	Ahearn and Maginniss, 1977
Fishes				
Sebastes caurinus (Copper Rock Fish)	pyloric caeca	D-glucose	580±120 μM	Ahearn et al., 1992
	intestine	D-glucose	140 ± 20 μM	Ahearn et al., 1992
Oreochromis mossambicus (Tilapia)	Upper intestine	D-glucose	670 ± 150 μM	Reshkin and Ahearn, 1987
	Lower intestine	D-glucose	390 ± 50 μM	
Mammals				
Rattus norvegicus (Rat)	intestine	D-glucose	31,400 ± 11,800 μM	Kellett and Helliwell, 2000
Homo sapiens (Human)	small intestine	D-fructose	1,120 ± 110 μM	Gould and Holman, 1993,
		D-glucose	3,600 ± 600 μM	Read et. al., 1977
Plants				
Beta vulgaris (sugar beet)	root	sucrose	2,200 ± 150 μM	Willenbrink and Doll, 1979
Saccharum officinarum (sugar cane)	phloem	sucrose	826 ± 203 μM	Reinders, et al., 2006

The SCRT-like transporter in crustacean cells has a lower K_M than those of plants understandably for two reasons. First, the intestine is a scavenger organ and would require the uptake of very specific for carbohydrates necessary for energy storage and creation. Second, plants have disaccharides readily available in their lumen that would require transport; whereas, the crustacean has the use of hydrolases to break down any remaining complex sugars into monomers for transport. The use of a disaccharide transporter would be greater in plants than crustaceans; however, the transport system can still be used to remove any remaining carbon sources to aid in the animal's energy and growth. The concentrations of monosaccharide to disaccharide in the lumen can also affect sucrose transport across the epithelium. After sucrose hydrolysis by sucrase, the glucose product by a feedback inhibition system saturates the glucose transporter's active transport mechanism (Gray, 1966). Therefore, as glucose was being produced, the intestinal sucrase enzyme reacted at a reduced rate increasing the amount of intact sucrose molecules available for MS transport.

Effects of Inhibitors on MS Transport of Sucrose

The results of TLC suggest no hydrolysis of sucrose into glucose and fructose monomers (Figure 15). Phloridzin is a competitive inhibitor of SGLT1 by using the glucose moiety in its structure to bind on the SGLT1 to prevent glucose from binding. Phloridzin is hypothesized to bind similarly to the glucose binding position on the SCRT-like transporter on the brush border membrane of the lobster intestine. Phloridzin prevented 34% of luminal sucrose from being transported across the intestinal epithelium (Figure 10). A mechanism to account for the sucrose that was not transported to the bath is the structural similarity of phloridzin to glucose (Figure 16). The glucose portion of phloridzin may bind to the SCRT-like transporter and inhibit the binding of the glucose portion of the sucrose molecule. This competitive binding of phloridzin to

the SCRT-like transporter likely accounts for 34% inhibition of the radioactivity being transferred to the serosal bath.



Phloridzin D-Glucose

Figure 16 Comparison of structure of Phlorizin and D-glucose noting the presence of a glucose in the phloridzin molecule.

Phloretin data (Figure 12) suggest that at least some of the perfused ^{14}C-sucrose radioactivity was prevented from being transported across the epithelial cell. The efflux of this radioactivity from epithelial cell to serosal medium was being blocked by serosal phloretin as a result of GLUT2-like inhibition. Again, phloretin has been observed to strongly inhibit monosaccharide transport by orienting itself in the opposite direction of the monosaccharide on GLUT2 during release of glucose or fructose to the serosa. Phloretin does not have a glucose moiety like phloridzin to bind to the glucose active site on the transporter. Instead, phloretin orients itself on the active site of GLUT2 to prevent glucose or fructose release from the cell (Krupka, 1985). It is being suggested that phloretin acts the same way with the presumptive SCRT-like transporter. Phloretin is able to bind to the transporter on the basolateral membrane to prevent sucrose from exiting the cell. In the presence of phloretin, 40% of the sucrose was not able to exit the cell. Approximately 60% of the ^{14}C-radioactivity originally appearing in the serosal bath during control perfusions still took place in the presence of these two drugs,

suggesting that at least one other mechanism of trans-intestinal transport of ^{14}C-radioactivity occurred when these drugs were used.

The ability of the putative lobster SCRT-like transporter was tested for specificity using trehalose. Trehalose is a disaccharide, very similar to sucrose, in its molecular weight and structure (Figure 6). If the SCRT-like transporter has a proposed glucose and fructose binding site, trehalose would be able to easily bind. Trehalose was able to inhibit 52% of sucrose transport. These results suggest that trehalose, as a disaccharide, was a more effective inhibitor of the SCRT-like transporter than phloretin and phloridzin. Trehalose is also a substrate of SCRT in plants and could be also in the SCRT-like transporter in lobster (Meyer, 2011). These results provide evidence toward a disaccharide transporter with glucose as one or both monomers since the 2 glucose molecules of trehalose can effectively inhibit the sucrose binding site on the SCRT-like transporter.

Phloridzin and trehalose have glucose in their structures, and phloretin is able to orient itself in the glucose binding site of a transporter. This information leads to the assumption that the SCRT-like transporter has sugar binding sites. Since sucrose has fructose and glucose both as monomers, the binding sites on the SCRT-like transporter are proposed to be able to bind glucose and fructose alike. The inhibitors were able to bind to the SCRT-like transporter competitively to decrease sucrose transport across the cell.

The TLC showed that there was only sucrose present in the serosal bath and effluent over a 3 hr perfusion. The molecular weight of fructose and glucose is the same; therefore, in TLC, glucose and fructose would separate at the same place on the chromatography sheet. There were no observed sugar metabolites in the serosal bath or effluent. A reason why metabolites were not observed in the bath or effluent could be because of their rate of conversion. The glucose and

fructose may have been produced by sucrase on the brush border membrane then transported into the cell, but then used for the cell's own cellular respiration instead of being transported. The amount that was not used for the cell's energy could have been transported, but at a rate that was undetectable by TLC.

The volatilization results indicate steady directionality of ^{14}C-sucrose transport across each intestine with non-volatile activity consistently greater than volatile activity (Table 1). The non-volatile fraction indicates sucrose; while the volatile fraction would likely indicate the metabolism of sucrose to volatile metabolites. On average, transmural 0.1mM sucrose transport produced an approximate 25% volatile fraction; while the 5mM sucrose perfusate had an approximate 13% volatile fraction.

Animal 1 (Table 1) that was tested with a perfused 0.1mM sucrose concentration had a 55% a volatile fraction which was higher than the other animals (Table 1). This phenomenon may be explained by a starved condition as the history of each experimental animal prior to its purchase is unknown. If the lobster was in a starved state, the sucrose present in the lumen may have been metabolized at a higher rate than would occur in a normally fed animal. Under this condition, intestinal hydrolases may have rapidly broken down the disaccharide into monomers to create energy for its own cell instead of transporting the monomers to the blood.

Proposed sucrose transport model

Michaelis-Menten kinetics, inhibition of ^{14}C-sucrose transport by phloridzin, phloretin, trehalose, analysis of serosal bath and effluent by TLC, and metabolite volatilization strongly suggest a functional carrier-mediated disaccharide transport system in crustacean epithelium. An SCRT transporter was localized in the brush border membrane of the *Drosophila* hindgut and present data strongly suggests the presence of an SCTR-like transporter on the apical membrane of

crustacean intestine. Phloridzin and trehalose both were able to inhibit sucrose transport significantly indicating their binding to a transporter on the brush border membrane where sucrose was to bind for transepithelial transport. Figure 17 is a working model of intestinal sugar transport in the American lobster that incorporates results from the present investigation and those of other studies of this animal. The proposed transport model in *Homarus americanus* includes SCRT-like, SGLT1-like, and GLUT5-like carrier systems on the brush border membrane and a proposed SCRT-like transporter, Na/K ATPase, and GLUT2-like carrier complex on the basolateral membrane. A GLUT-2 like transporter was found on the basolateral membrane to assist glucose and fructose transport from inside the epithelial cell to the blood (Obi et al., 2011). Although TLC did not indicate transport of ^{14}C-glucose and ^{14}C-fructose originating from ^{14}C-sucrose, the monomers could be formed at a concentration needed to fuel the cells themselves, producing the 15% volatile fraction of activity initially associated with the disaccharide (Figure 15). Sucrase may be active inside the intestinal cell, but not at a fast enough pace to provide the monomers to the blood or even a substantial concentration of monomers to be detected through a radiolabelled substrate. Another exit process for sucrose from inside the crustacean epithelium is a suggested SCRT-like transporter on the basolateral membrane. The proposal of another SCRT-like transporter on the basolateral membrane is supported by phloretin being an effective inhibitor of the release of sucrose into the serosa in addition to the lack of monomers indicated through TLC (Figures 12 and 15).

The present data point toward an SCRT-like transporter on both the basolateral and apical sides of the intestinal epithelium. In the presence of phloretin, some ^{14}C-sucrose could pass out of the cell as volatile metabolites after being hydrolyzed inside the cell by sucrase or as an intact sucrose molecule. The amount of sucrose that was hydrolyzed by sucrase could be enough to

supply the intestinal tissue itself with needed energy. If the cell used a significant portion of sucrose for its own cellular respiration, the volatile metabolite produced may be in the form of $^{14}CO_2$. Data in Table 1 indicate about 15% of the sucrose transported into the cell was metabolized into CO_2.

The inhibition data, non-volatile fraction of metabolized radioactivity, and carrier-mediated kinetics suggest that ^{14}C-sucrose was transported from mucosa to serosa through both paracellular diffusion and transcellular transport. An estimate of the relative contributions of these two potential transmural pathways can be estimated at 0.1 mM ^{14}C-sucrose, the concentration used for all time course experiments here. Using the Michaelis-Menten equation (Equation 1) for 0.1mM sucrose, and substituting the values for K_M and J_{max} obtained from Figure 14, the carrier-mediated component of total transmural flux can be estimated below:

$$J = \frac{2.32\ \rho\text{mol cm}^{-2}\text{min}^{-1}[100\mu\text{M}]}{(15.82\mu\text{M} + [100\mu\text{M}])}$$

Therefore, the 0.1mM sucrose J_{max} = 120.19 ρmol cm^{-2} hr^{-1}. The SCRT-like transporter has a J_{max} = 139.2 ρmol cm^{-2} hr^{-1}.

In Figure 9, 0.1 mM total transmural sucrose transport amounted to 6.69 ρmol cm^{-2} min^{-1}, which is 401.4 ρmol cm^{-2} hr^{-1}; therefore, $\frac{120.19\ \rho\text{mol cm}^{-2}\text{hr}^{-1}}{401.4\ \rho\text{mol cm}^{-2}\ \text{hr}^{-1}} \times 100\% = 30\%$. Approximately 30% of the observed sucrose in the serosa was moved via transcellular transport. The remaining 70% of sucrose observed in the serosa is suggested to have moved through paracellular diffusion.

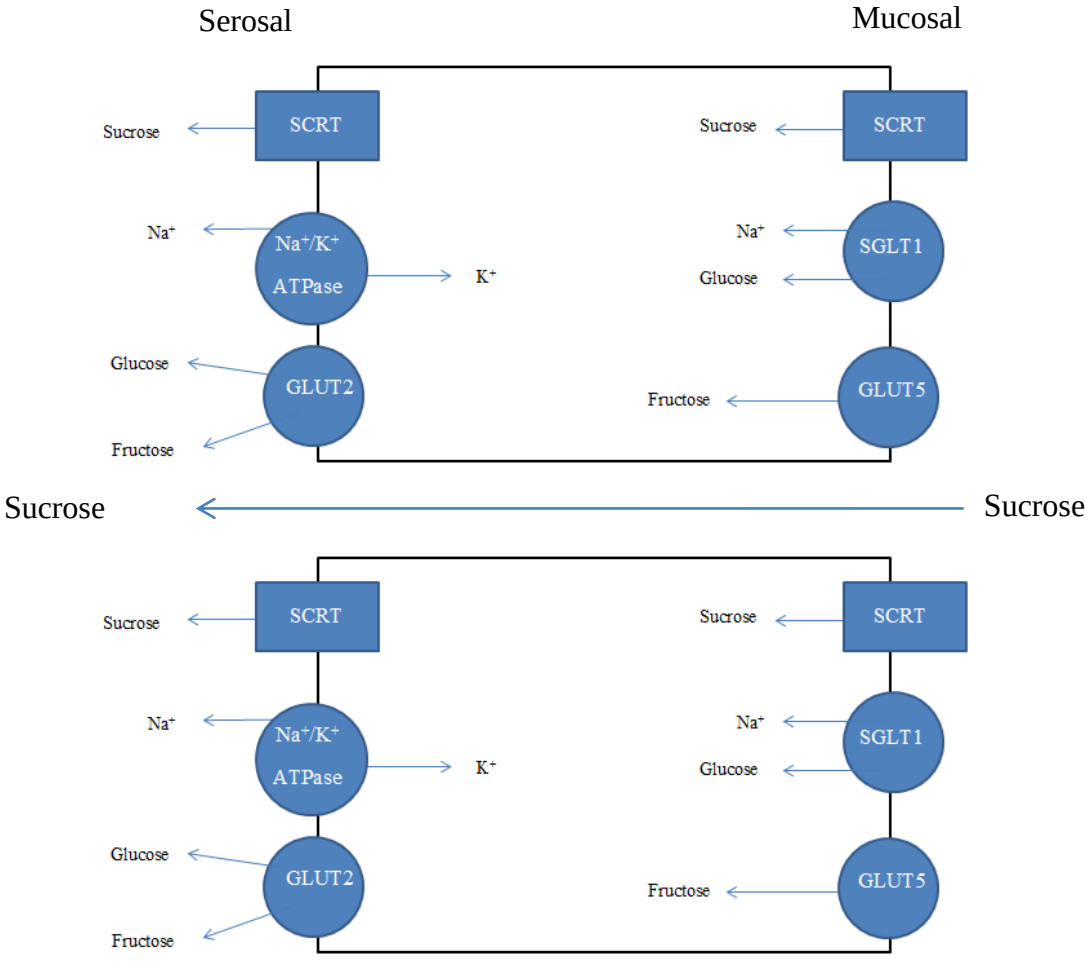


Figure 17 Suggested model of the proposed sucrose transport system alongside glucose and fructose transporters in lobster intestinal cells. The proposed model indicates active transport of the Na^+/K^+ ATPase, the secondary active transporter (SGLT1), fructose transport by GLUT5-like, transport of glucose and fructose to the blood by GLUT2-like, and the suggested transport of sucrose from the lumen to the blood by an SCRT-like transporter. Paracellular sucrose transport is also suggested to contribute to total transmural transport.

One possible candidate for this residual transport process might be a crustacean analog of the *Drosophila* SCRT disaccharide carrier system. It is possible that BOTH hydrolysis of

disaccharides to monosaccharides followed by their individual transports may be present on the same tissue as is a SCRT-like disaccharide transport protein transferring the complex carbohydrate across the intestine intact. Also, the presence of the GLUT5-like protein from previous research indicated the brush border localization of this fructose transport protein to provide support for evolutionary transporter conservation of epithelial D-glucose and D-fructose transport by digestive organ systems (Obi et al., 2011). Additional experiments are needed to clarify this situation. An SCRT transporter or SCRT-like transport system is expected to be present in *H.americanus* since trehalose is an available sugar and utilized by many invertebrates (Meyer et al, 2011). It is possible that both hydrolysis of disaccharides to monosaccharides followed by their individual transports may be present on the same tissue as is a SCRT-like disaccharide transport protein transferring the complex carbohydrate across the intestine intact.

Phylogenetic Evaluation

A phylogenetic analysis of a sucrose transporter (SCRT) in *Drosophila melanogaster* identified the protein as a sister to a clade comprising SLC45A1, SLC45A2, and SLC45A4 (Figure 2). The SCRT was termed by FlyBase as Slc45-1 because it shows a significant similarity to members of the human SLC45 family (Vitavska and Wieczorek, 2013). Fig 2 indicates the apparent phylogenetic relationship of the animal proteins not only with the plant counterparts, but also with those found in bacteria and fungi. The highest resemblance with SCRT was found for SUC3 from *Arabidopsis thaliana*, with a similarity of 38% and an identity of 20%. With respect to vertebrates, proteins with high similarities were found among the human SLC45 family sharing between 32% to 47% of similar and 18% to 27% of identical amino acids with SCRT (Lalonde and Frommer, 2012). SCRT was localized in the apical membrane of hindgut epithelial cells of *D. melanogaster* which suggests a role in transmembrane and/or

transepithelial sucrose transport (Meyer, 2011). Since molecular phylogeny reflects the evolutionary process, it provides the most reliable guide to the structure, function, and mechanism of biological macromolecules (Saier, 2000). Although it is very difficult to predict the functional characteristics of sugar transporters based on amino acid sequences because of the subtle difference that exist between various transport families, the amino acid sequence can indicate a general function exists between sugar transporters in a major facilitator superfamily (Kikuta, 2012).

In summary, the SCRT-like transporter that this investigation suggests to be present in crustacean intestinal cells can, like plant SCRT, utilize several disaccharides as substrates. The *Homarus americanus* is very unlikely to come into direct contact with sucrose as table sugar in its habitat. However, trehalose, maltose, and other disaccharides are found in numerous marine plants that the lobster would use as food in larval and post-larval life stages (Sainte-Marie, 2002). Halophilic marine cyanobacteria are found in the lobster's habitat and are part of the lobster larval diet. These cyanobacteria can accumulate sucrose and trehalose which would be digested and could potentially use the lobster SCRT transporter (Mackay, 1984).

As an adult, the lobster's diet consists mainly of crabs, mussels, clams, starfish, sea urchins, and marine worms. Particularly mussels, clams, and starfish can have large stores of glycogen in the hepatopancreas, pyloric caeca, and gonads. As a lobster ingests these animals, α-amylase in the lobster hepatopancreas would be able to break the glycosidic bonds of the glycogen stores of mussels, clams, and starfish into polysaccharides and monosaccharides some of which could be absorbed by the hepatopancreas and intestine by the processes described in Figure 17. Results of the current study, for the first time, provide evidence for the presence of a novel sugar transport protein in the animal digestive tract that has not been functionally described previously. Future

studies will be directed at the potential of hepatopancreatic epithelial cells to exhibit an isoform of this intestinal transport system with kinetic properties that may be adapted to higher concentrations of dietary disaccharides.

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