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Comparative Sugar Transport by Crustacean Hepatopancreas and Intestine

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COMPARATIVE SUGAR TRANSPORT BY CRUSTACEAN HEPATOPANCREAS AND INTESTINE

By

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CERTIFICATE OF APPROVAL

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ABSTRACT

Glucose is transported in crustacean hepatopancreas and intestine by Na^+-dependent co-transport, while Na^+-dependent D-fructose influx has only been described for the hepatopancreas. It is still unclear if the two sugars are independently transported by two distinct cotransporter carrier systems. In this study lobster (*Homarus americanus*) hepatopancreas brush border membrane vesicles (BBMV) were used to characterize, in detail, the cation-dependency of both D-[^{3}H] glucose and D-[^{3}H] fructose influxes, while *in vitro* perfused intestines were employed to determine the nature of cation-dependent sugar transport in this organ. Over the sodium concentration range of 0-100 mM, both ^{3}H-D-glucose and ^{3}H-D-fructose influxes (0.1 mM; 1 min uptakes) by hepatopancreatic BBMV were hyperbolic functions of [Na^+], exhibiting K_m values of 2.30 ± 0.59 and 2.58 ± 0.95 mM, respectively. D-[^{3}H] glucose and fructose influxes by hepatopancreatic BBMV over a potassium concentration range of 15-100 mM were hyperbolic functions of [K^+], exhibiting K_m values of 9.85 ± 0.41 and 12.6 ± 0.80 mM respectively. Both sugars displayed significant ($p < 0.01$) Na^+/K^+-dependent and Na^+-independent uptake processes. Transepithelial 25 µM D-[^{3}H] glucose and D-[^{3}H] fructose fluxes across lobster intestine over a luminal sodium and potassium concentration range of 0 – 50 mM and 5-100 mM, respectively, were hyperbolic functions of luminal [Na^+] and [K^+]. As with hepatopancreatic sugar transport, transepithelial intestinal sugar transport exhibited both significant ($p < 0.01$) Na^+/K^+-dependent and Na^+-independent processes. Results suggest that both D-glucose and D-fructose are transported by a single carrier process in each organ with

sodium being the preferred cation for both sugars in the hepatopancreas, and potassium being the preferred cation for both sugars in the intestine.

INTRODUCTION

Dietary sugars (monosaccharides) are the main source of energy in living organisms and they have an important role in the proper functioning of organs (Brown, 2000; Wright et al., 2007). Most organisms use D-glucose, D-fructose and D-galactose as their main source of carbon (Walmsley et al. 1998). Cellular biological membranes allow the passage of small molecules and lipid soluble substances such as gases and small polar molecules through the lipid part of the membrane. Cell membranes are effectively impermeable to glucose, so movement of glucose into and out of cells must be mediated by protein transporters (Brown, 2000). The physiological mechanisms by which sugars are transferred across biological membranes to intracellular sites of metabolism have been extensively studied in mammals, leading to the finding of two distinctly different types of sugar transport proteins. The first transport system couples the transmembrane sugar transport to an existing electrochemical gradient such as that for sodium or protons (secondary active transport), whereas the second transport system uses the transmembrane concentration gradient of the substrate itself to drive its membrane transport (facilitated diffusion transport).

Facilitated diffusion transport involves the transport of certain ions and molecules across a biological membrane from an area of higher concentration to an area of lower concentration via carrier proteins. Since these ions and molecules move along the direction of their concentration gradients, energy is not required. Active transport, on the other hand, transports ions and molecules against the direction of their concentration gradients and thus requires the input of metabolic energy to function (Mader, 2010). There are two major types of active transport: primary and secondary active transport. Both involve transport of solutes

against an electrochemical gradient, but they differ in their source of energy. Primary active transport involves a carrier protein that directly hydrolyzes ATP to drive the transport process. Such carrier proteins are usually called ATPases. The Na^+-K^+ ATPase is an example of primary active transport whose function is to pump three sodium ions out of the cell in concert with pumping two potassium ions into the cell, thus maintaining an intracellular environment that has low sodium and high potassium. In addition, an electrical gradient across the plasma membrane is generated. In most cells, one-third to one-half of the total energy expended is used to run these ion pumps (Mader, 2010). Thus, the driving forces for actively transporting ions and molecules across the plasma membrane are both the concentration gradient and the membrane potential. Secondary active transport, however, utilizes energy stored in electrochemical gradients of ions to drive transport, which are established and maintained by the Na^+-K^+ ATPases. The transport of glucose is an example of secondary active transport in which the sodium exported from the cell by the Na^+-K^+ ATPase, is co-transported back into the cell, down its concentration gradient, using a glucose-specific transporter.

The SLC5 co-transporter gene family is a large family of 75kDa proteins consisting of several sodium-dependent glucose co-transporter proteins (e.g. SGLT) that transport glucose and other solutes such as galactose across biological membranes in conjunction with the cations Na^+ or H^+ using transmembrane ion gradients to drive the co-transported substrate into the cytosol of both eukaryotic and prokaryotic cells (Turk and Wright, 1997). Four SGLTs have had their transport functions illustrated experimentally: SGLT1, SGLT2, SGLT3, and SGLT4.

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Figure 1: SGLT 1 model. SGLT1 is a sodium-dependent co-transporter protein responsible for the uptake of dietary glucose from the gut. Sodium has to bind to the negatively charged co-transporter first to make the binding site for glucose available. Once glucose binds to its binding site, the co-transporter undergoes a conformational change releasing glucose into the cytosol first followed by Na^+. The co-transporter regains its negative charge and undergoes a conformational change returning to original state. From Wright et al. (2011).

SGLT1, whose mechanism of action is shown in Figure 1, is perhaps the best studied member and was the first to be cloned in 1987 (Hediger et al., 1987). SGLT1 functions as a high affinity, sodium-dependent glucose co-transporter and mainly participates in nutritional D-glucose and D-galactose absorption in the apical membrane of the mammalian intestine (Hediger et al., 1987). The SGLT1 transport protein consists of 14 transmembrane α-helices with both the N and C terminal facing the extracellular side of the membrane (Wright et al., 2007). SGLT2 is of low affinity, high capacity, and functions as a sodium-dependent glucose cotransporter as well but is mainly involved in renal reabsorption of glucose (Kanai et al., 1994), and SGLT 3 has been recently suggested to function as a glucose sensor rather than a glucose transporter (Diez-Sampedro et al., 2003). Recently, a cDNA clone of SGLT4 was isolated from a human small intestine cDNA library and was reported to act as a mannose/1,5-anhydro-D-glucitol/fructose (Man/1,5AG/Fru) transporter in the intestine and kidney (Tazawa et al., 2005).

The SLC2 gene family consists of 50 kDa proteins that use a separate mode of action than the SLC5 gene family. The SLC2 family transports glucose in a sodium independent manner (e.g. GLUT). Sugar molecules are transferred across a membrane, down the concentration gradient, by transport proteins that act as facilitated diffusion systems belonging to the GLUT family, whose mechanism of action is shown in Figure 2. Members of the GLUT family possess 12 transmembrane α-helices with both the N and C termini facing the intracellular side of the membrane (Brown, 2000). GLUT 2, located in the apical side of the intestinal epithelial cell, is the carrier protein responsible for the uptake of luminal glucose in the presence of high glucose concentrations.

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Figure 2: GLUT transport protein model. 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. The transporter undergoes a conformational change again to its original state. From Pessin and Bell (1992).

In addition, GLUT 2 is also present on the basolateral membrane and is responsible for bringing in both glucose and fructose from the blood into the cytosol and for efflux of both sugars from cytoplasm to blood (Kellett et al., 2000; Caccia et al., 2007). GLUT 5, however, is the known carrier protein responsible for luminal fructose uptake (Caccia et al., 2007).

While sugar transporters have been studied in detail in mammals, very little is known about the absorption of sugars in invertebrates. The first study describing a detailed model for carrier-mediated sugar transport across an insect epithelium was published in 2007 (Caccia et al., 2007). *Aphidius ervi* is a parasitic wasp that lays its eggs in the haemocoel of different species of aphids. The larval development of the insect occurs in the hemolymph of its host (Caccia et al., 2007). These authors used apical and basolateral fluxes of radiolabelled D-glucose and D-fructose, immunocytochemistry, and Western blot analysis to describe the sugar carrier proteins in larval parasitoid wasp (*Aphidius ervi,* Hymenoptera) midgut epithelial cells. The localization of SGLT1-like and GLUT5-like transporters were shown to be present on the intestinal brush-border membrane, while GLUT2-like proteins were present on the basolateral side of the gut and the brush-border membrane (Caccia et al., 2007), similar to the arrangement of these sugar carrier proteins in mammalian intestine (Uldry and Thorens, 2004).

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 believed to play a minimal role in nutrient absorption. The midgut includes the epithelial-lined intestine and a group of anterior and posterior diverticula that are all unchitinized and arise from embryonic endoderm (Wright and Ahearn, 1997). The

hepatopancreas is the largest diverticulum and is generally a large bilateral organ composed of a plethora of blind-ending epithelial-lined tubules in which the lumen is in fluid contact with the contents coming from the stomach and the intestine.

The hepatopancreas has several functions including synthesis and secretion of digestive enzymes, absorption of nutrients, secretion of emulsifiers, and storage of carbohydrates. The hepatopancreas epithelium is lined with at least four different cell types (Verri et al., 2001). The four cells types each have different structures thereby having different roles in digestive and absorptive functions (Wright and Ahearn, 1997; Verri et al., 2001). Embryonic cells (E-cells) are the smallest cells located at the distal tips of each hepatopancreatic tubule and are the only cells that give rise to the other three types of cells by differentiation processes (Al-Mohanna et al., 1985). Fibrillar cells (F-cells) are elongated cells and also found at the most distal part of each tubule. They have an extensive rough endoplasmic reticulum which suggests that they're involved in protein synthesis. They are also involved in transcellular nutrient absorption of sugars and amino acids (Verri et al, 2001; Finadra et al., 2006). Resorptive cells (R-cells) are the most abundant cell type and occur throughout the length of the tubule. During differentiation these cells store glycogen and then start lipogenesis processes (Conklin, 1995). Acid, alkaline and sugar phosphatases have been found at the brush border of R-cells, which suggests that R-cells could be involved in digestion and nutrient absorption of substrate molecules (Verri et al, 2001). R-cells are the primary site for storage and uptake of nutrients (Conklin, 1995). Blister cells (B-cells) are found in the proximal part of each tubule, they contain one large vacuole containing a variety of digestive enzymes that are released into the tubular lumen during entry of nutrients (Al-Mohanna and Nott, 1986; Conklin, 1995).

The crustacean intestine runs from the posterior portion of the pyloric foregut to the anterior part of the chitinized hindgut and is comprised of a single epithelial cell type possessing a microvillar apical border. The intestine is considered to be a scavenger organ, absorbing any leftover nutrients (Wright and Ahearn, 1997). A diagram of the digestive tract of *Homarus Americanus* illustrating the position of the intestine and hepatopancreas (mid-gut gland) and its cell types, is shown in Fig. 3.

Previous studies have shown glucose transport in both organs by facilitative transport and Na^+-dependent co-transport (Ahearn et al., 1985; Obi et al., 2011), while Na^+-dependent D-fructose influx has only been described for the hepatopancreas (Sterling et al., 2009). In neither organ have the details of ion-dependent sugar transport been elucidated and it is still unclear as to whether the two sugars are independently transported by two distinct co-transporter carrier systems or by the same transporter. Sodium or potassium gradients across brush border membrane vesicles of shrimp hepatopancreatic epithelial cells were each capable of driving the uptake of D-[^{3}H] glucose against transient concentration gradients in white shrimp (*L. setiferus)* (Simmons et al., 2012; Obi et al., 2013). In this shrimp, several hexoses (D-glucose, D-fructose, D-mannose) reduced labeled D-[^{3}H] glucose transport as potential competitive inhibitors (Obi et al., 2013). It was suggested that the sugar transporter in shrimp hepatopancreas physiologically resembles the mammalian SGLT4 sugar transporter (Tazawa et al., 2005).

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Figure 3: Diagram of the digestive tract of *Homarus Americanus* showing an epithelial-lined hepatopancreatic (mid-gut gland) tubule and intestine. Part of the intestine lies underneath the hepatopancreas and runs through the tail of the lobster. The hepatopancreas, also called the midgut gland, has several functions including synthesis and secretion of digestive enzymes, absorption of nutrients, secretion of emulsifiers, and storage of carbohydrates. The intestine is involved in the absorption of any leftover nutrients. From Conklin D. E 1995.

In the present study, lobster (*Homarus americanus*) hepatopancreatic brush-border membrane vesicles (BBMV) were used to characterize, in detail, the cation-dependency of both D-[^{3}H] glucose and D-[^{3}H] fructose influxes, while *in vitro* perfused intestines were employed to determine cation-dependent sugar transport in this organ. Results suggested that both D-glucose and D-fructose are transported by a single carrier process in each organ with sodium being the preferred cation for both sugars in the hepatopancreas, and potassium being the preferred cation for both sugars in the intestine.

MATERIALS AND METHODS

Magnesium precipitation technique to purify hepatopancreatic brush border membrane vesicles:

Live male lobsters (*Homarus americanus*), weighing approximately 500 g, were purchased from a commercial dealer (Fisherman's Dock, Jacksonville, Florida) and were maintained unfed at 15 °C for up to 1 week in an aquarium containing filtered seawater. Hepatopancreatic brush border membrane vesicles (BBMV) of fresh tissue removed from individual lobsters were produced using the $MgCl_2$ method of Kessler et al. (1978), as modified by Biber at al. (1981) for mammalian epithelia, and applied to crustacean hepatopancreas (Ahearn et al.,1985) as shown in Fig. 4.

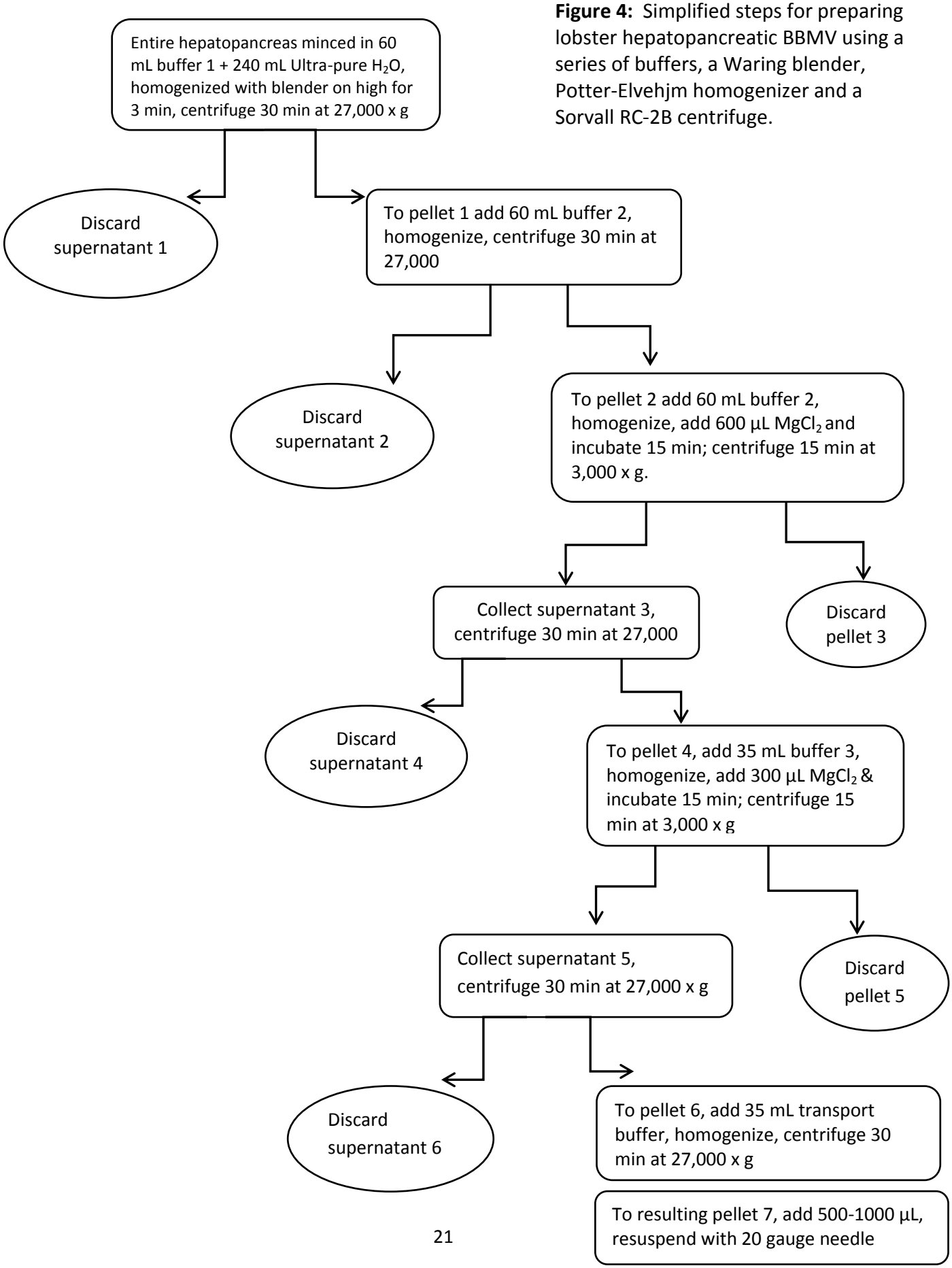
In this study, an entire hepatopancreas was homogenized for 3 min at high speed in a Waring blender in 60 mL ice-cold Buffer 1 (300 mM mannitol, 5 mM EGTA, 1 mM PMSF, and 12 mM Tris-HCl, pH 7.0) made hypotonic by adding 240 mL ultra-pure water. The homogenate was placed in 8 tubes and centrifuged at 27,000 x g for 30 min at 4°C. The resulting pellets were combined (pellet 1) and re-suspended in 60 mL ice-cold Buffer 2 (300 mM mannitol, 12 mM Tris/HCl, pH 7.0) and homogenized using 10 strokes of a Potter-Elvehjem tissue grinder at high speed. The homogenate was centrifuged at 27,000 x g for 30 min at 4°C. The resulting pellet (pellet 2) was re-suspended in 60 mL ice-cold Buffer 2 and homogenized. The homogenate to which 10 mM $MgCl_2$ was added was allowed to incubate on ice for 15 min followed by two centrifugations, the first at 3000 x g for 15 min at 4°C (discard pellet 3) and the second at 27,000 x g for 30 min at 4°C (save pellet 4). The resulting pellet (pellet 4) was resuspended in 35

mL of buffer 3 (60 mM mannitol, 5 mM EGTA, 12 mM Tris/HCl, pH 7.0) and the $MgCl_2$ was repeated on this mixture. Two centrifugations followed, the first at 3000 x g (discard pellet 5) and the second at 27,000 x g, retrieving pellet 6 from the second centrifugation. Pellet 6 was resuspended in 35 mL transport buffer (usually 200-300 mM mannitol, 12 mM Hepes, at pH 7.0) and homogenized. This was followed by centrifugation for 30 min at 27,000 x g. The resulting Pellet 7 was resuspended with a 20 gauge needle in enough transport buffer for experimentation, usually 500-1000 µL.

A small aliquot of this vesicle suspension was used to determine the amount of protein present by the BioRad protein assay (BioRad, Hercules, CA) at 595 nm using a DU 640 spectrophotometer (Beckman Coulter, CO). A standard curve was prepared using various concentrations of bovine serum albumin (BSA). BioRad dye (5 mL) was added to each sample and vortexed. The standards were tested using the Bradford-Stahl test, in a spectrophotometer, which yielded absorbances at 595 nm. Three 10 µL samples of the vesicles were tested against the standards. The absorbance values of the vesicles fell on the standard curve and were used to estimate protein concentrations of the vesicles.

Transport experiments were conducted at 23°C using BBMV produced by the method described above and the Millipore filtration technique developed by Hopfer et al. (1973). In these experiments 20 µL of BBMV were added to 180 µL of radiolabeled external medium containing D-[^{3}H]glucose, D-[^{3}H]fructose (American Radiochemical Corp., USA) and other chemical constituents specific to each experiment.

Figure 4: Simplified steps for preparing lobster hepatopancreatic BBMV using a series of buffers, a Waring blender, Potter-Elvehjm homogenizer and a Sorvall RC-2B centrifuge.



Incubation of vesicles with the radiolabeled nutrient was continued for time periods from 1 to 60 min after which a known volume of this incubation mixture (20 µL) was withdrawn and plunged into 2 mL ice-cold stop solution (generally an isotonic choline chloride medium with the inside buffer) to stop the uptake process. The resulting suspensions were rapidly filtered through Millipore filter paper (0.65 µm) to retain the vesicles and washed with another 3 mL of stop solution. Filters were then added to a Beckman scintillation cocktail and counted for radioactivity in a Beckman LS-6500 scintillation counter. Uptake values were expressed as ρmoles/mg of membrane protein per filter. Each experiment was conducted three times with 3 replicate samples per treatment using membranes prepared from different animals. The data were pooled for subsequent analysis. Values are means +/- SEM. Sigma Plot 10.0 curve fitting software (Systat Software, Inc. Point Richmond, CA, USA) was used to present data in figures and to obtain carrier-mediated influx constants.

Preservation and storage of lobster hepatopancreatic border membrane vesicles (BBMV):

The combined time for hepatopancreatic BBMV isolation and assay is often quite long and the ability to preserve a large quantity of vesicles permits many experiments on the same vesicle population and/or on the same day, thereby facilitating comparison of results. In a previous study, experiments were reported on the preservation of glucose transport and enzyme activities during freezing of BBMV from the intestinal epithelium of the warm-water euryhaline fish, *Oreochromis mossambicus* (Reshkin et al., 1988). Their study showed that the freezing of BBMV in liquid nitrogen preserved the properties of glucose transport found in fresh vesicles, including sodium-dependency, and overshoot characteristics. To assess the effect of

freezing on the transport activity of lobster hepatopancreatic BBMV, vesicles prepared from an individual lobster hepatopancreas were loaded with 300 mM mannitol, 12 mM Hepes/Tris at pH 7.0 and the resulting suspension was split into two. One half was placed into liquid nitrogen for 30 min and then stored in -80°C for at least 48 hours. The other half was used immediately to measure 0.1 mM D-glucose uptake over time periods of 15s, 1, 2, 5, 10, and 60 min of incubation in media consisting of either 150 mM NaCl, KCl or mannitol each with 12 mM Hepes/Tris at pH 7.0. The same experiment was repeated with the frozen half of the BBMV sample.

***In vitro l*obster intestinal perfusion technique:**

The part of the intestine used for each experiment was cut from 1 cm posterior to the stomach to about two-thirds of the length of the tail. This fraction of intestine was composed of midgut tissue only. *In vitro* transmural mucosal to serosal (MS) transport of D-[^{3}H]glucose and D-[^{3}H]fructose was studied using a perfusion apparatus as previously described (Ahearn and Maginniss, 1976) (Fig. 5). Isolated whole 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 an 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, USA) at a rate of 0.38 mL min^{-1} [a rate previously shown to provide constant transmural transport in lobster intestine for over 3 h of incubation without added oxygen at 23°C (Conrad and Ahearn, 2005)].

Transport time course experiments were conducted by adding concentrations of D-glucose or D-fructose, and NaCl or KCl to different 50 mL tubes (Falcon, Newark, N.J) containing physiological saline and D-[^{3}H]glucose or D-[^{3}H]fructose. Prior to the start of experimentation, triplicate aliquots of each experimental perfusate (200 µL) were collected from each Falcon tube 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 a total of 30 min. All experimental procedures were carried out at 23°C. Triplicate radioactive samples (200 µL) were collected from the serosal medium after passage across the intestine every 5 min for the duration of each experimental treatment. An equal amount of physiological saline was added to the serosal medium in order to maintain a constant volume in the bath.

The radioactive experimental samples collected were placed in 7 mL tubes containing 3 mL scintillation cocktail and counted for radioactivity. The mean background count was subtracted from each triplicate sample at each time point. Transmural MS rates were expressed in ρmol cm^{-2} min^{-1}.

Figure 5: A diagram of the perfusion apparatus used to measure D-[^{3}H]glucose and D-[^{3}H]fructose transport by the lobster intestine, *Homarus americanus* (Ahearn and Maginniss, 1976). The serosal medium represents the blood side while the mucosal medium represents inside the intestine.

RESULTS

HEPATOPANCREAS:

Effect of freezing on the sugar transport activity of lobster hepatopancreatic BBMV

To assess the effect of freezing on the transport activity of lobster hepatopancreatic BBMV, vesicles prepared from an individual lobster hepatopancreas were loaded with 300 mM mannitol, 12 mM Hepes/Tris at pH 7.0. Results in Fig 6A and B indicate the sugar uptake by both fresh and frozen vesicles in NaCl medium displayed an initial uptake overshoot followed by a slow return to similar equilibrium values at 60 min. The overshoot displayed in both the fresh and frozen vesicles was triple its respective equilibrium value. Sugar uptake by both fresh and frozen vesicles displayed no significant uptake overshoot in media containing potassium or mannitol. The similarity of these data suggest that lobster hepatopancreatic BBMV can be frozen and when used will yield similar uptake values as vesicles used immediately after protein isolation.

All vesicle data beyond this point have been obtained from frozen BBMV.

Increasing NaCl concentrations stimulate glucose and fructose influx by BBMV in a hyperbolic manner

To assess the effect of increasing NaCl concentrations on D-glucose and D-fructose influx kinetics by hepatopancreatic BBMV, vesicles were loaded with 200 mM mannitol, 12 mM Hepes/Tris at pH 7.0 and were incubated for 1 min in external media containing 0.1 mM D-[^{3}H]

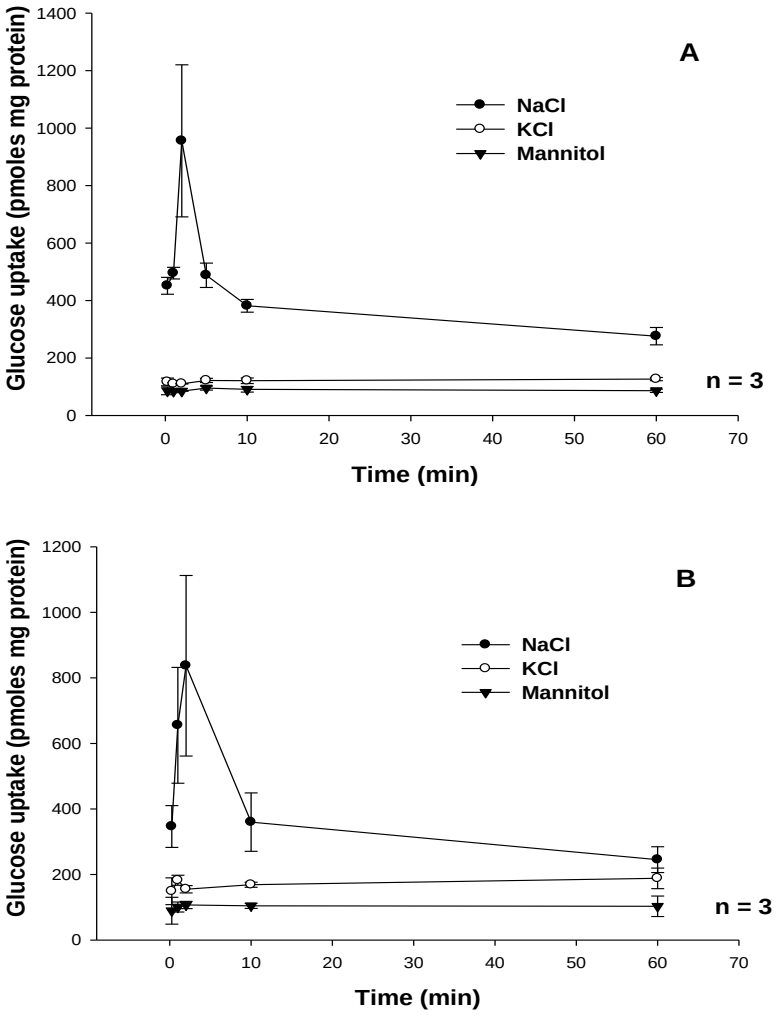


Figure 6: Frozen BBMV act in a similar manner to fresh BBMV. A) Time course of 0.1 mM D-[^{3}H] glucose uptake by fresh lobster hepatopancreatic brush-border membrane vesicles (BBMV) loaded with 300 mM mannitol, 12 mM Hepes/Tris at pH 7.0 and incubated in either 150 mM NaCl, KCl or mannitol each with 12 mM Hepes/Tris pH 7.0 for periods of time from 15s to 60 min. B) Time course of 0.1 mM D-[^{3}H] glucose uptake by frozen BBMV with same experimental conditions as A. The experiment was conducted three times (3 lobsters) with 3 replicates/treatment. Symbols are means ± 1 SEM.

glucose or D-[^{3}H] fructose, increasing concentrations of sodium (0, 1, 2.5, 5, 10, 25, 50, 100 mM NaCl) and 12 mM Hepes/Tris at pH 7.0. Results in Fig 7 and 8 displayed influx as a hyperbolic function of [Na^+] and followed the Michaelis-Menten equation for carrier-mediated transport:

$$J = J_{max} [Na^+] / (K_M + [Na^+]) \quad (1)$$

Where J is D-[^{3}H] glucose or D-[^{3}H] fructose influx (ρmol/mg protein x min), J_{max} is maximal glucose or fructose influx rate (ρmol/mg protein x min), K_M is the apparent affinity binding constant (mM), and [Na^+] is NaCl concentration in mM. Figures 7 and 8 indicate that NaCl was able to stimulate the uptake of both sugars in a saturable manner, indicating significant ($p < 0.01$) Na^+-dependent and Na^+-independent uptake processes. The resulting K_M values for glucose and fructose as shown in Table 1 were similar (2.30 ± 0.59 and 2.58 ± 0.95 mM, respectively). The maximal transport rates, however, were different, with transport being 26 times faster for fructose than for glucose.

Increasing KCl concentrations stimulate glucose and fructose influx by BBMV in a hyperbolic manner

There is not much research on the effect of potassium on sugar influx since adult lobsters are mostly carnivores, consuming little potassium in the foods they eat. This experiment was designed to demonstrate whether or not potassium was an effective symporter for glucose and fructose transport.

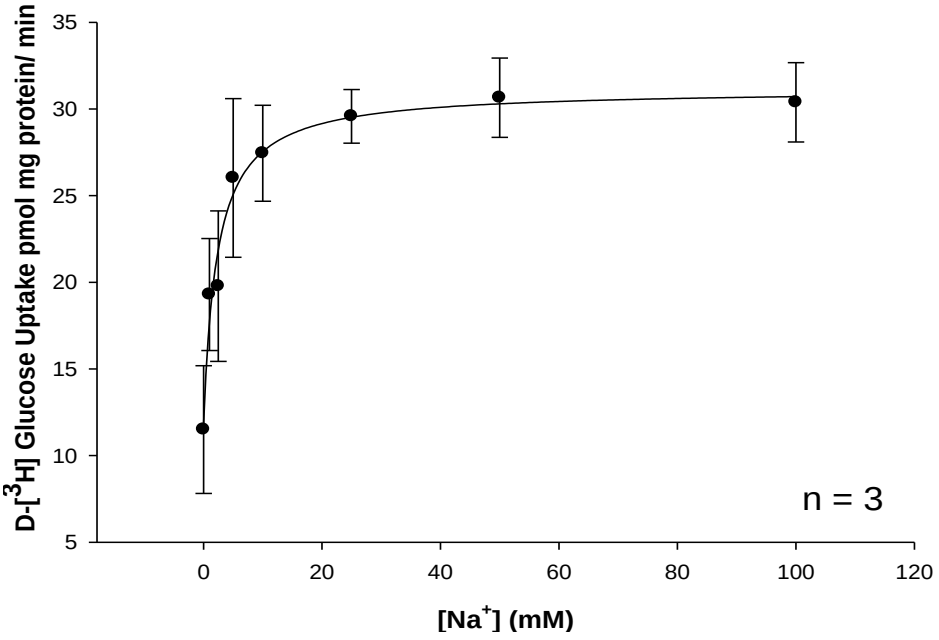


Figure 7: Increasing NaCl concentrations stimulate glucose transport in a hyperbolic manner. Effect of increasing Na^+ concentrations on 1 min 0.1 mM D-[^{3}H] glucose uptake in lobster hepatopancreatic BBMV. Vesicles were loaded with 200 mM mannitol, 12 mM Hepes/Tris at pH 7.0 and incubated in various NaCl concentrations (0, 1, 2.5, 5, 10, 25, 50, 100 mM), 12 mM Hepes/Tris at pH 7.0. The experiment was conducted three times with 5 replicates/treatment. Symbols are means ± 1 SEM. Curve fit lines and resulting kinetic constant values were obtained using Sigma plot 10.0 software. Kinetic constants are displayed in Table 1.

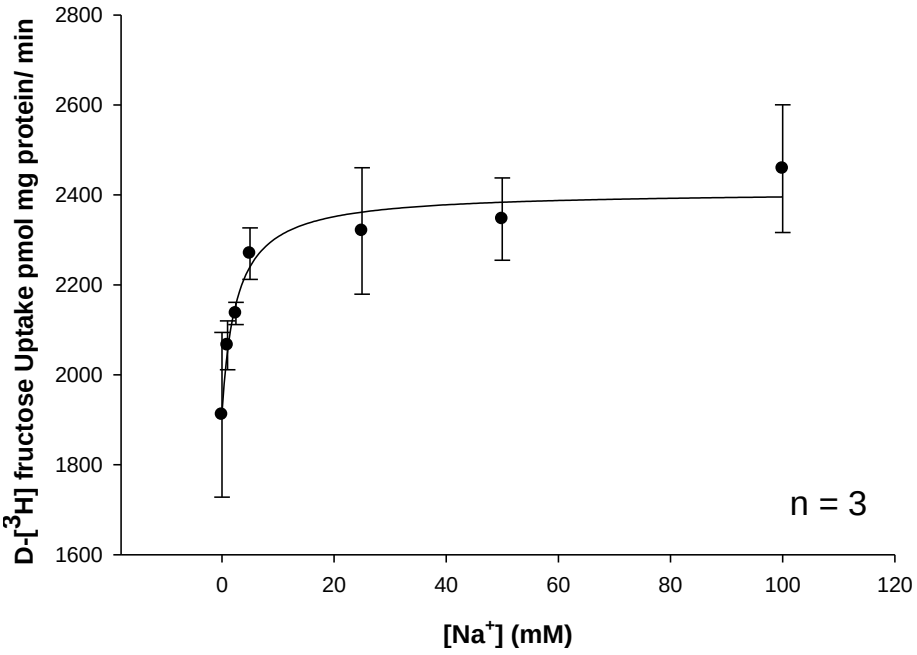


Figure 8: Increasing NaCl concentrations stimulate fructose transport in a hyperbolic manner. Effect of increasing Na^+ concentrations on 1 min 0.1 mM D-[^{3}H] fructose uptake in lobster hepatopancreatic BBMV. Vesicles were loaded with 200 mM mannitol, 12 mM Hepes/Tris at pH 7.0 and incubated in various NaCl concentrations (0, 1, 2.5, 5, 10, 25, 50, 100 mM), 12 mM Hepes/Tris at pH 7.0. The experiment was conducted three times with 5 replicates/treatment. Symbols are means ± 1 SEM. Curve fit lines and resulting kinetic constant values were obtained using Sigma plot 10.0 software. Kinetic constants are displayed in Table 1.

Figures 9 and 10 illustrate the effect of various potassium concentrations (5, 15, 25, 50 and 100 mM KCl) on 1 min uptake values for 0.1 mM D-[^{3}H] glucose and fructose by hepatopancreatic BBMV. Both D-glucose and D-fructose influx were hyperbolic functions of [K^+] and followed the Michaelis-Menten equation (1). Significant ($p < 0.01$) K^+-dependent D-glucose and D-fructose uptake processes were observed. The resulting K_M values for glucose and fructose influxes in potassium medium as shown in Table 1 appear to be similar (9.85 ± 0.4 and 12.6 ± 0.80 mM, respectively). The maximal transport rates, however, were different, with transport being 7 times faster for glucose than for fructose. Table 1 also suggests that the apparent cation-binding affinity was greater (lower K_M) in external NaCl than in KCl, indicating that Na^+ might be the "preferable ion" for stimulating hepatopancreatic BBMV glucose and fructose uptake in the American lobster.

Effects of D-fructose on the kinetics of D-[^{3}H] glucose influxes by BBMV in NaCl incubation medium

D-[^{3}H] glucose influx (1 min uptakes) as a function of external [D-glucose] was measured in NaCl incubation medium in the presence and absence of D-fructose (Fig. 11). Glucose influx under control conditions (lacking D-fructose) was a hyperbolic function of [D-glucose] and followed the Michaelis-Menten equation (1) for carrier-mediated transport, where J is unidirectional D-[^{3}H] glucose influx (ρmol/ mg protein × min), J_{max} is the maximal influx rate, K_M is an apparent affinity binding constant (mM), and [Glu] is the external sugar concentration (mM).

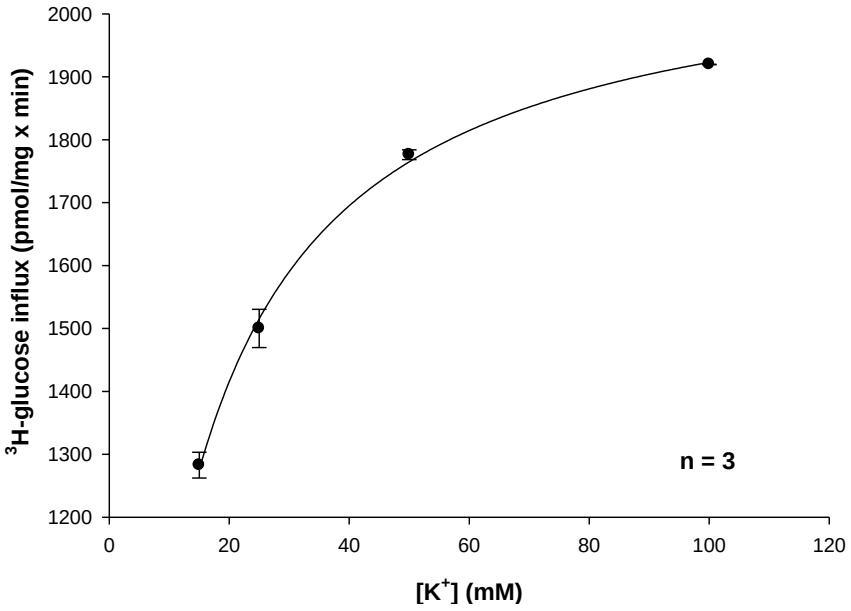


Figure 9: Increasing KCl concentrations stimulate glucose influx in a hyperbolic manner. Effect of increasing K^+ concentrations on 1 min 0.1 mM D-[^{3}H] glucose uptake in lobster hepatopancreatic BBMV. Vesicles were loaded with 200 mM mannitol, 12 mM Hepes/Tris at pH 7.0 and incubated in various KCl concentrations (15, 25, 50, 100 mM), 12 mM Hepes/Tris at pH 7.0. The experiment was conducted three times with 5 replicates/treatment. Symbols are means ± 1 SEM. Curve fit lines and resulting kinetic constant values were obtained using Sigma plot 10.0 software. Kinetic constants are displayed in Table 1.

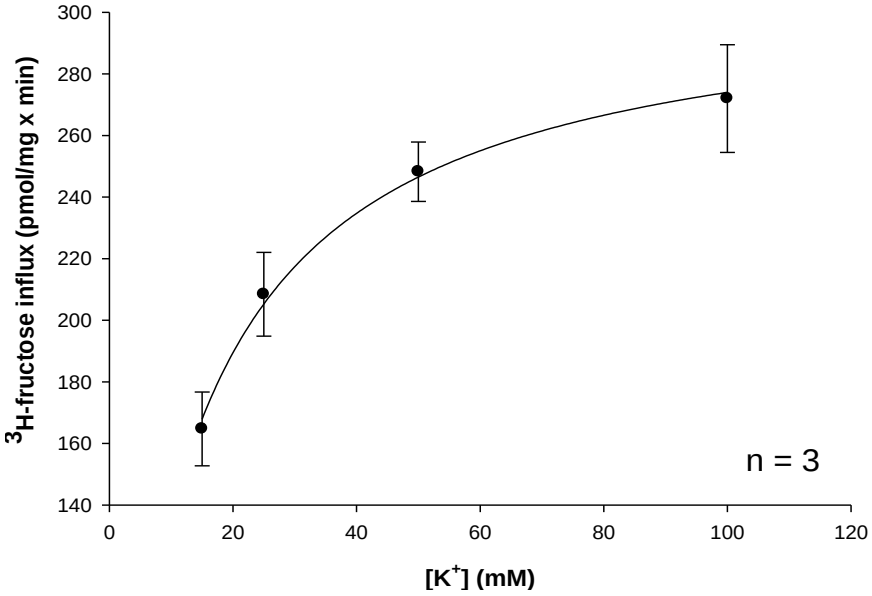


Figure 10: D-fructose displays K^+-dependent uptake processes in the hepatopancreas. Effect of increasing K^+ concentrations on 1 min 0.1 mM D-[^{3}H] fructose uptake by BBMV. Vesicles were loaded with 200 mM mannitol, 12 mM Hepes/Tris at pH 7.0 and incubated in various KCl concentrations (15, 25, 50, 100 mM), 12 mM Hepes/Tris at pH 7.0. The experiment was conducted three times with 5 replicates/treatment. Symbols are means ± 1 SEM. Curve fit lines and resulting kinetic constant values were obtained using Sigma plot 10.0 software. Kinetic constants are displayed in Table 1.

Table 1: Effect of increasing NaCl and KCl concentrations on D-[^{3}H] glucose or D-[^{3}H] fructose influx kinetic constants in lobster hepatopancreas.

Cation	Substrate	K_M (mM)	J_{max} (pmol/mg protein / min)
Na^+	D-[^{3}H] glucose	2.30 ± 0.59	19.3 ± 1.40
Na^+	D-[^{3}H] fructose	2.58 ± 0.95	492 ± 50.2
K^+	D-[^{3}H] glucose	9.85 ± 0.41	2112 ± 19.8
K^+	D-[^{3}H] fructose	12.6 ± 0.80	308 ± 5.01

Each experiment was conducted three times with 5 replicates/treatment. Symbols are means ± 1 SEM of three experiments. Concentrations of NaCl and KCl were; 0, 1, 2.5, 5, 10, 25, 50, 100 mM, and 15, 25, 50, 100 mM, respectively.

D-[^{3}H] glucose influx kinetics observed in the presence of 75 mM and 100 mM D-fructose in the external incubation medium are also displayed in Fig. 11 for comparison with influx under control conditions. In the presence of D-fructose, influxes of D-[^{3}H] glucose at each external [D-glucose] were lower than those occurring at the same [D-glucose] in the absence of D-fructose. A greater reduction was seen in 100 mM D-fructose than 75 mM D-fructose. Table 2 indicates that the addition of D-fructose to the external medium led to an increase in D-[^{3}H] glucose influx K_M , but had no effect on D-[^{3}H] glucose influx J_{max}. These results suggest that D-[^{3}H] glucose influx in NaCl incubation medium occurred by a carrier-mediated transport process that appeared to be competitively inhibited by D-fructose.

INTESTINE:

NaCl stimulates glucose and fructose transepithelial transport in a hyperbolic manner

To assess the effect of increasing sodium concentrations on D-glucose and D-fructose transepithelial transport, mucosal to serosal (MS) transport experiments over a 30 min time course at a variety of sodium concentrations (0,5, 10, 25, 50 mM) with 25 µM D-[^{3}H] glucose or D-[^{3}H] fructose were performed in triplicate (three animals) using perfused intestines. Slopes of the time course data were determined by linear regression analysis as shown by a representative graph in (Fig. 12A) and data curve fitting procedures, as shown in (Fig. 12B) using Sigma Plot 10.0 software.

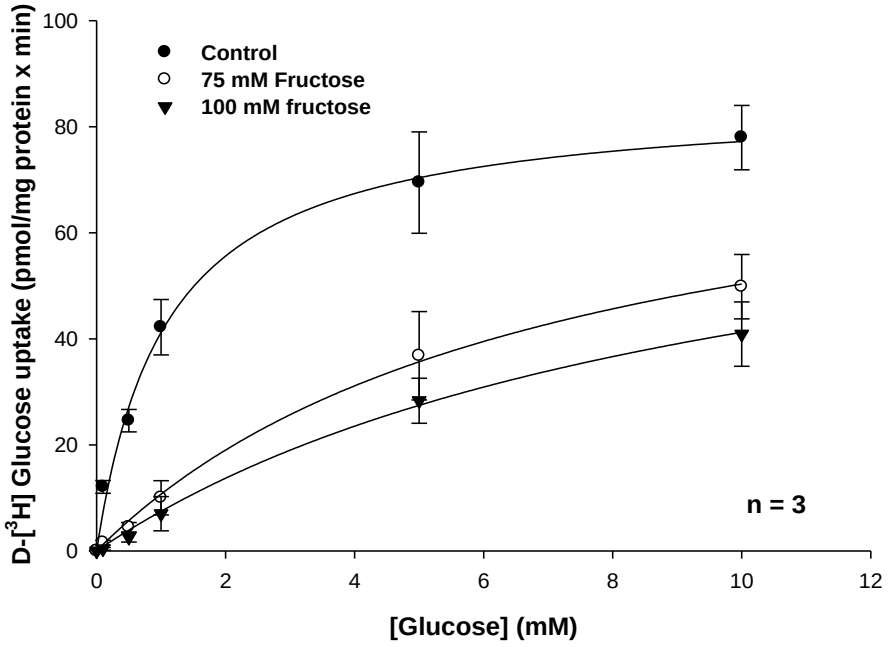


Figure 11: Effect of increasing D-[^{3}H] glucose concentrations (0.1, 0.5, 1, 5, 10 mM) on 1 min uptake in NaCl incubation medium in the presence and absence of various concentrations (75, 100 mM) of D-fructose. Vesicles were loaded with 310 mM mannitol, 12 mM Hepes/Tris at pH 7.0 and incubated in 100 mM NaCl, 12 mM Hepes/Tris at pH 7.0. Appropriate quantities of mannitol were added to external media to keep them osmotically equivalent to internal medium. The experiment was conducted three times with 5 replicates/treatment. Symbols are means ± 1 SEM. Curve fit lines and resulting kinetic constant values were obtained using Sigma Plot 10.0 software. Kinetic constants are displayed in Table 2.

Table 2: Kinetic constants showing the effect of increasing glucose concentrations on D-[^{3}H] glucose transport by BBMV in medium containing 100 mM NaCl in the presence and absence of various inhibitor concentrations.

[Inhibitor]	K_M (mM)	J_{max} (pmol/mg protein / min)
Control	1.07 ± 0.14	85.5 ± 3.25
75 mM Fructose	6.99 ± 0.94	87.5 ± 5.76
100 mM Fructose	10.1 ± 1.80	82.8 ± 8.47

Each experiment was conducted three times with 5 replicates/treatment. Symbols are means ± 1 SEM of three experiments. Glucose concentrations were; 0.1, 0.5, 1, 5, 10 mM.

Results in Fig. 12 and 13 show that increasing concentrations of luminal NaCl increased D-[^{3}H] glucose and D-[^{3}H] fructose transport in a hyperbolic manner, indicating significant ($p < 0.05$) sodium-dependent and sodium-independent uptake processes. Each data point represents the uptake slope ± SEM at each concentration of sodium. The resulting K_M values for glucose and fructose in sodium medium as shown in Table 3 appear to be quite similar suggesting that both sugars may be transported by a single carrier process in the intestine with a slightly lower binding affinity than in the hepatopancreas (Table 1 and 3).

KCl stimulates glucose and fructose transepithelial transport in a hyperbolic manner

In the intestine, MS transport experiments over a 30 min time course at a variety of potassium concentrations (5, 15, 25, 50 and 100 mM KCl) with 25 µM D-[^{3}H]glucose and D-[^{3}H]fructose were performed in triplicate (three animals each) (Fig 14 and 15). As in the hepatopancreas, results in Fig. 14 and 15 show that increasing concentrations of luminal KCl increased transepithelial D-[^{3}H] glucose and D-[^{3}H] fructose transport in a hyperbolic manner indicating significant ($p < 0.05$) potassium-dependent uptake processes. Each data point represents the uptake slope ± SEM at each concentration of potassium. The resulting K_M values for glucose and fructose in potassium medium as shown in Table 3 appear to be quite similar suggesting a single carrier process in the intestine. Table 3 also suggests that the apparent cation-binding affinity was greater (lower K_M) in external KCl than in NaCl, indicating that K^+ might be the "preferable ion" for stimulating glucose and fructose uptake in the intestine of the American lobster.

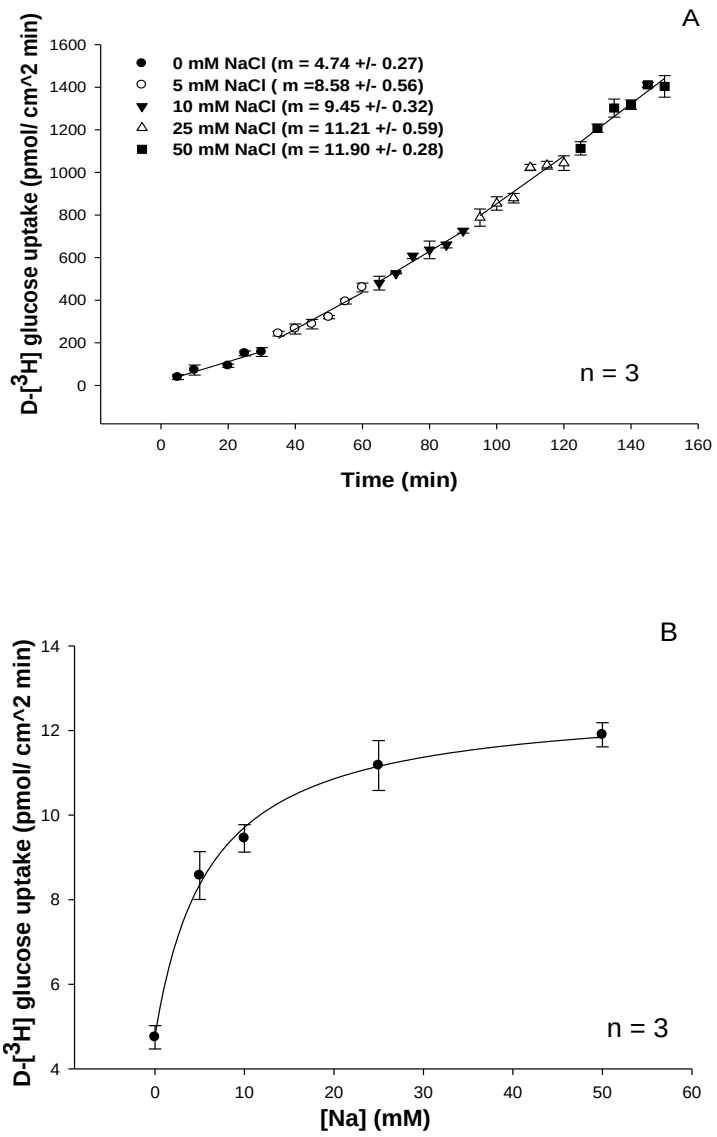


Figure 12: Effect of increasing sodium concentrations (0, 5, 10, 25, 50 mM) on intestinal mucosal to serosal (MS) transmural 25 µM D-[^{3}H] glucose transport. Each experiment was conducted three times with 3 replicates/treatment. Symbols are means ± 1 SEM. A) Time course data with slopes determined by linear regression. B) Slopes from Fig. 13A plotted against [Na]. Curve fit lines and resulting kinetic constant values were obtained using Sigma plot 10.0 software. Kinetic constants are displayed in Table 3.

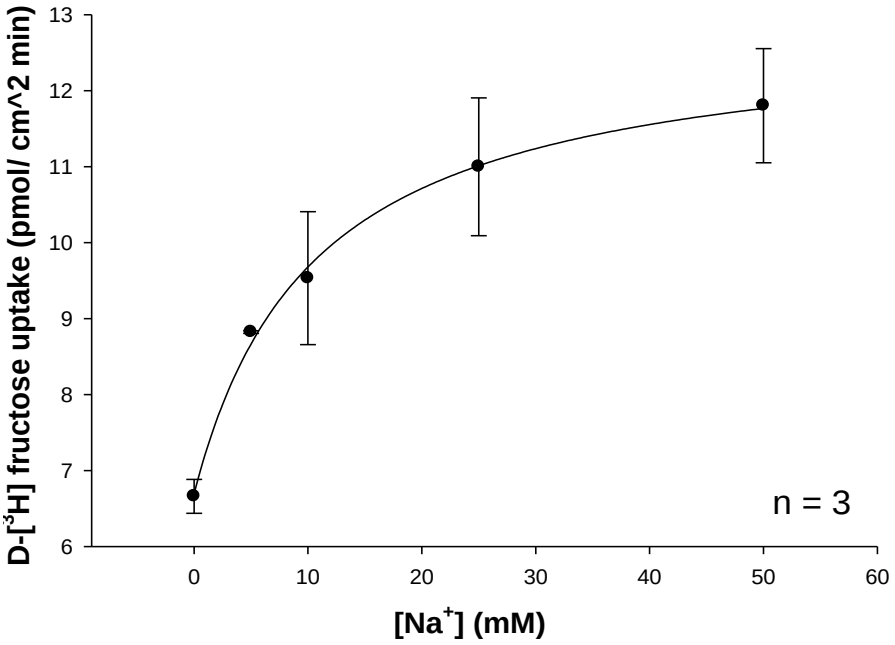


Figure 13: Increasing NaCl concentrations stimulate transepithelial fructose transport in a hyperbolic manner. Effect of increasing sodium concentrations (0, 5, 10, 25, 50 mM) on intestinal mucosal to serosal (MS) transmural 25 µM D-[^{3}H] fructose transport. Each experiment was conducted three times with 3 replicates/treatment. Symbols are means ± 1 SEM. Curve fit lines and resulting kinetic constant values were obtained using Sigma plot 10.0 software. Kinetic constants are displayed in Table 3.

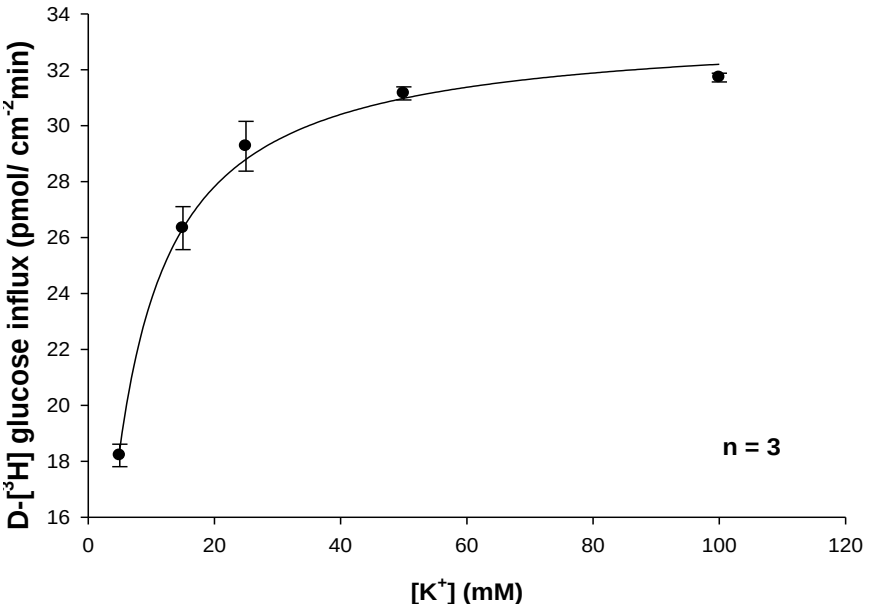


Figure 14: D-glucose displays K^+-dependent uptake processes in the intestine. Effect of increasing potassium concentrations (5, 15, 25, 50, 100 mM) on intestinal mucosal to serosal (MS) transmural 25 µM D-[^{3}H] glucose transport. The experiment was conducted three times with 3 replicates/treatment. Symbols are means ± 1 SEM. Curve fit lines and resulting kinetic constant values were obtained using Sigma plot 10.0 software. Kinetic constants are displayed in Table 3.

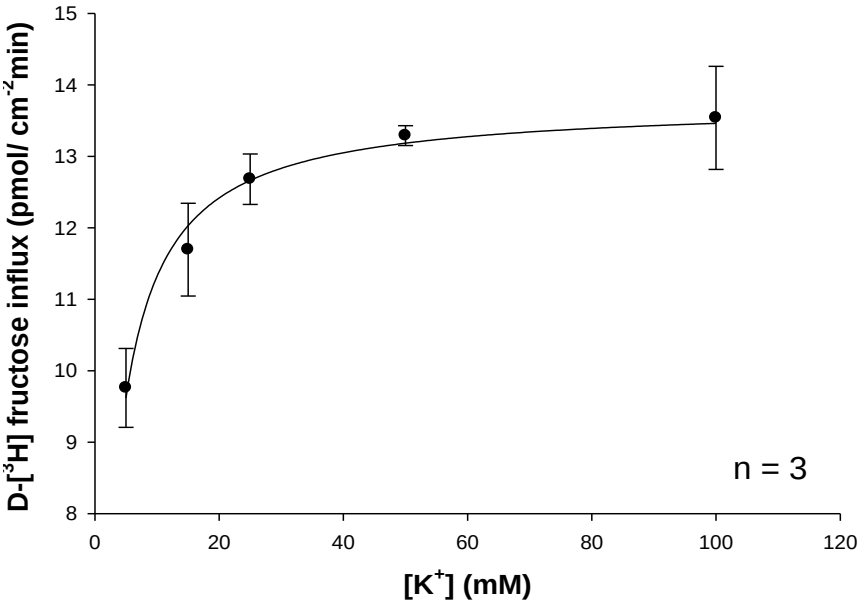


Figure 15: D-fructose displays K^+-dependent uptake processes in the intestine. Effect of increasing potassium concentrations (5, 15, 25, 50, 100 mM) on intestinal mucosal to serosal (MS) transmural 25 µM D-[^{3}H] fructose transport. The experiment was conducted three times with 3 replicates/treatment. Symbols are means ± 1 SEM. Curve fit lines and resulting kinetic constant values were obtained using Sigma plot 10.0 software. Kinetic constants are displayed in Table 3.

Table 3: Effect of increasing NaCl and KCl concentrations on D-[^{3}H] glucose or D-[^{3}H] fructose transepithelial transport kinetic constants in lobster intestine.

Cation	Substrate	K_M (mM)	J_{max} (ρmol/cm^2 × min)
Na^+	D-[^{3}H] glucose	6.05 ± 0.99	7.92 ± 0.38
Na^+	D-[^{3}H] fructose	10.5 ± 1.65	6.14 ± 0.29
K^+	D-[^{3}H] glucose	3.10 ± 0.24	33.5 ± 0.35
K^+	D-[^{3}H] fructose	2.12 ± 0.20	13.7 ± 0.15

Each experiment was conducted three times with 3 replicates/treatment. Symbols are means ± 1 SEM of three experiments. Concentrations of NaCl and KCl were; 0, 5, 10, 25, 50 mM, and 5, 15, 25, 50, 100 mM respectively.

Effects of D-fructose on the kinetics of transmural MS D-[^{3}H] glucose transport in KCl incubation medium

Transepithelial D-[^{3}H] glucose transport as a function of external [D-glucose] was measured in KCl incubation medium in the presence and absence of D-fructose (Fig. 16). Glucose influx under control conditions (lacking D-fructose) was a hyperbolic function of [D-glucose] and followed the Michaelis-Menten equation (1) for carrier-mediated transport. D-[^{3}H] glucose influx kinetics observed in the presence of 75 mM and 100 mM D-fructose in the mucosal medium are also displayed in Fig. 16 for comparison with influx under control conditions. In the presence of D-fructose, influxes of D-[^{3}H] glucose at each [D-glucose] $_{mucosal}$ were lower than those occurring at the same [D-glucose] in the absence of D-fructose. A greater reduction was seen in 100 mM D-fructose than 75 mM D-fructose. Table 4 indicates that the addition of D-fructose to the mucosal medium led to an increase in D-[^{3}H] glucose influx K_M , but had no effect on D-[^{3}H] glucose influx J_{max}. These results suggest that D-[^{3}H] glucose influx in KCl incubation medium occurred by a carrier-mediated transport process that appeared to be competitively inhibited by D-fructose in the intestine.

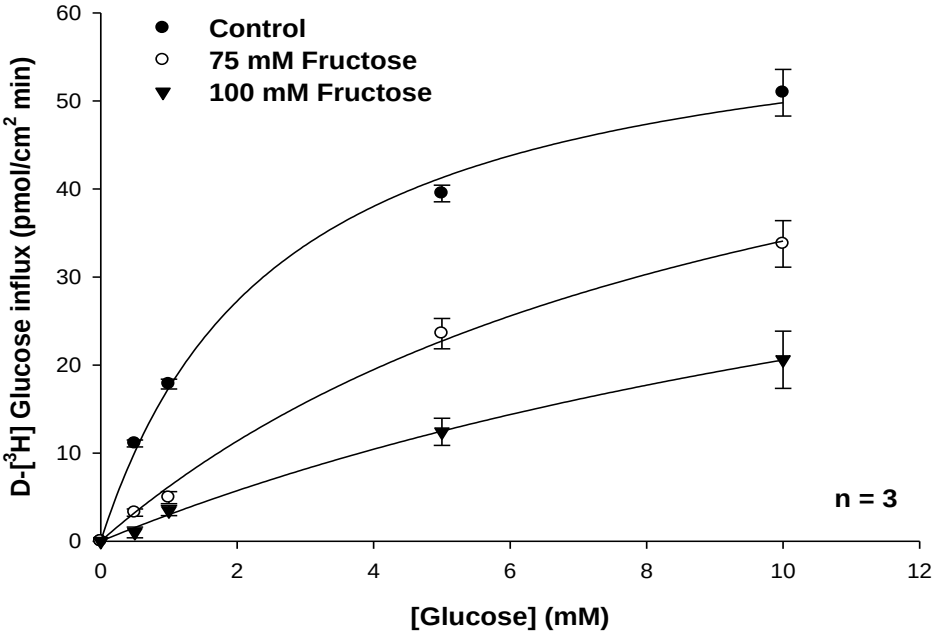


Figure 16: Effect of increasing mucosal D-[^{3}H] glucose concentrations (0.5, 1, 5, 10 mM) on transmural MS glucose transport in KCl incubation medium in the presence and absence of various concentrations (75, 100 mM) of D-fructose. The experiment was conducted three times with 3 replicates/treatment. Symbols are means ± 1 SEM. Curve fit lines and resulting kinetic constant values were obtained using Sigma Plot 10.0 software. Kinetic constants are displayed in Table 4.

Table 4: Kinetic constants showing the effect of increasing glucose concentrations on transmural MS D-[^{3}H] glucose transport in medium containing 100 mM KCl in the presence and absence of various inhibitor concentrations.

[Inhibitor]	K_M (mM)	J_{max} (pmol/mg protein / min)
Control	2.60 ± 0.34	62.7 ± 2.82
75 mM Fructose	9.98 ± 2.20	68.2 ± 8.57
100 mM Fructose	18.3 ± 4.40	58.3 ± 9.72

Each experiment was conducted three times with 3 replicates/treatment. Symbols are means ± 1 SEM of three experiments. Glucose concentrations were; 0.5, 1, 5, 10 mM.

DISCUSSION

The results of the present investigation suggest that both D-glucose and D-fructose are transported across the intestinal epithelium and hepatopancreatic brush border membrane of the American lobster (*Homarus americanus*), at least in part, by transport processes with properties apparently different from those of mammalian SGLT1.

The present research has examined the effects of a freezing preservation technique on lobster hepatopancreatic BBMV glucose transport characteristics. Previous investigations in which experiments the preservation of glucose transport and enzyme activities during freezing of BBMV from the intestinal epithelium of the warm-water euryhaline fish, *Oreochromis mossambicus*, showed that the freezing of BBMV in liquid nitrogen preserved the properties of glucose transport found in fresh vesicles, including sodium-dependency, and overshoot characteristics (Reshkin et al., 1988). Long-term storage and maintenance of functionality of a membrane system offers advantages over exclusive use of fresh protein preparations. The similarity in activity of frozen to fresh preparations greatly reduces experimental time and thus greatly increases productivity. Additionally, preservation of a single large vesicle preparation permits an increased number of experiments to be conducted on the same vesicle population, thereby reducing the variability created due to organismal and preparative differences. In this investigation, however, experiments were conducted with vesicles prepared from an individual lobster hepatopancreas and were repeated three times (3 lobsters). Rapid freezing in liquid nitrogen and storage in -80°C (at least 48 hours), in combination with slow, gentle thawing of the samples on ice, results in a sample preparation with essentially identical glucose transport

characteristics to those found in the fresh preparation. The overshoot displayed in both the fresh and frozen vesicle preparations was triple its respective equilibrium value. Glucose uptake by both fresh and frozen vesicle preparations displayed no significant uptake overshoot in media containing potassium or mannitol. The overshoot phenomenon was thus conserved and essentially unchanged in glucose transport by frozen vesicle preparations. The slow thawing seemingly results in less structural and functional change in membrane proteins, resulting in an improved preservation of original transport properties (Rudolph and Crow, 1985; Reshkin et al., 1988). Thus, slow thawing on ice may be an essential component for freeze-thaw vesicle preservation. All hepatopancreatic BBMV data in this investigation were obtained using at least 3 sets of pooled frozen BBMV preparations.

Glucose transport has been shown to be sodium-dependent in the invertebrate digestive tract (Ahearn and Maginniss, 1977; Ahearn et al., 1985; Blaya et al., 1998; Verri et al., 2001; Vilella et al., 2003). Glucose uptake in the absence of sodium was equilibrative (Ahearn et al., 1985). Sodium-coupled D-glucose transport by digestive tract epithelial cells was confirmed in the present study. While Na^+-dependent D-glucose influx has been extensively investigated, Na^+-dependent D-fructose influx has only been described in the hepatopancreas (Sterling et al., 2009). The observed sodium-dependent fructose influx by BBMV is in contrast to the mammalian paradigm, in which fructose uptake by BBMV involves a Na^+-independent GLUT-like facilitative transporter. Mucosal GLUT 5 is a known fructose transporter previously identified in the mammalian intestinal epithelial brush-border membrane (Miyamoto et al., 1994; Corpe et al., 2002). Increasing concentrations of sodium stimulated D-fructose influx by BBMV in a hyperbolic manner, similar to sodium-coupled D-glucose transport by BBMV. Similarity in K_M

values for Na^+-dependent D-glucose and D-fructose transport by hepatopancreatic BBMV (2.30 ± 0.59 and 2.58 ± 0.95 mM, respectively) suggest that they may be transported by a single carrier process with substrate specificities different from those demonstrated by mammalian SGLT1. The maximal transport rate, however, was different, with transport of fructose being 26 times faster than for glucose. The sodium-dependent fructose transport activity reported here can be compared with that of the mammalian SGLT4, which is known to transport fructose, in addition to glucose, in a sodium-dependent manner (Tazawa et al., 2005). D-fructose (75 and 100 mM) inhibits D-glucose influx in a competitive manner. Increasing [D-fructose] led to an increase in K_M (decrease in binding affinity) but no change in J_{max}. D-glucose and D-fructose appear to be competing for the same binding site on this sodium-coupled hexose transporter, and thus supporting the appearance of a carrier process that transports both D-glucose and D-fructose in a sodium-dependent manner in the hepatopancreas.

The ion-dependency of both D-glucose and D-fructose transport appeared due to the use of a co-transport protein that employed two simultaneous driving forces working together to power the transfer of the sugars from gut lumen to epithelial cytosol. These combined driving forces *in vivo* are the trans-apical sodium concentration gradient established and maintained by a basolateral ATP-dependent 3Na/2K-ATPase, and the transmembrane electrical potential difference created by the same primary active transport process. Both D-glucose and D-fructose influxes were hyperbolic functions of [potassium] and followed the Michaelis-Menten equation (1). Significant ($p < 0.01$) K^+-dependent D-glucose and D-fructose uptake processes were observed. The resulting K_M values for glucose and fructose in potassium medium appear to be similar (9.85 ± 0.4 and 12.6 ± 0.80 mM, respectively). The maximal

transport rates, however, were different with transport being 7 times faster for glucose than for fructose. Both D-glucose and D-fructose appear to be transported by a single carrier process with sodium and potassium as alternative driving forces. The apparent cation-binding affinity was approximately 5 times greater (lower K_M) in external NaCl than in KCl indicating that Na^+ might be the "preferable ion" for stimulating hepatopancreatic BBMV glucose and fructose uptake in the American lobster. While D-glucose transport using transmembrane gradients of either sodium or potassium as driving forces have been previously described for the Atlantic white shrimp (Simmons et al., 2012; Obi et al., 2013), sodium or potassium-coupled D-fructose transport by lobster hepatopancreatic BBMV has only been reported in the present investigation. The question, however, arises as to why increasing KCl concentrations stimulate sugar influx by BBMV in a hyperbolic manner as shown in the present investigation, while in a previous study by Ahearn et al.,(1985) a transmembrane potassium gradient was unable to result in a D-[^{3}H] glucose uptake overshoot in the same species. A tentative answer may be that potassium may be acting as an activator rather than a driver ion mediating D-glucose transport across the hepatopancreatic brush border membrane. By K^+ binding to cation-dependent sugar transporters it allows for more transporters to have an available binding site for sugar (see Fig. 1). Sugar transport is thus driven by the concentration gradient of the sugar itself, stimulating sugar transport without using a driving ion.

In addition to lobster hepatopancreatic BBMV, *in vitro* perfused intestines were employed to characterize the nature of cation-dependent D-glucose and D-fructose transport in this organ. Sodium-dependent D-glucose co-transport has been reported in both the hepatopancreas and intestine (Ahearn et al., 1985; Obi et al., 2011) while sodium-dependent D-

fructose co-transport has only previously been described for the hepatopancreas (Sterling et al., 2009). In the present study, however, sodium-dependent D-fructose influx by epithelial intestinal mucosal to serosal (MS) transport is reported. Increasing concentrations of sodium stimulate D-fructose influx by epithelial intestinal MS transport in a hyperbolic manner, similar to sodium-coupled D-glucose transport in the same organ. Similarity in K_M values for Na^+-dependent D-glucose and D-fructose influx by epithelial intestinal MS transport (6.05 ± 0.99 and 10.5 ± 1.65 mM, respectively) suggest that they may be transported by a single carrier process in the intestine. Furthermore, D-fructose (75 and 100 mM) inhibits D-glucose influx in a competitive manner. Increasing [D-fructose] led to an increase in K_M (decrease in binding affinity) but no change in J_{max}. D-glucose and D-fructose appear to be competing for the same binding site on this sodium-coupled hexose transporter, and thus supporting the appearance of a carrier process that transports both D-glucose and D-fructose in a sodium-dependent manner in the intestine also.

Potassium-dependency of glucose and fructose transport was investigated in the intestine as well. No previous studies have reported K^+-dependent sugar transport in the intestine except in the present investigation. In a recent study, D-glucose and D-fructose transport were examined in the lobster intestine but only sodium was used as the cation driving force (Obi et al., 2011). Both D-glucose and D-fructose influx by epithelial intestinal transport were hyperbolic functions of [potassium] and followed the Michaelis-Menten equation (1). Significant ($p < 0.01$) K^+-dependent D-glucose and D-fructose uptake processes were observed in the intestine also. The resulting K_M values for transmural MS transport of D-glucose and D-fructose in potassium medium appear to be similar (3.10 ± 0.24 and 2.12 ± 0.20 mM,

respectively). The maximal transport rates, however, were different with transport being 2.5 times faster for fructose than for glucose. Both D-glucose and D-fructose appear to be transported by a single carrier process with sodium and potassium as alternative driving forces. The apparent cation-binding affinity was approximately 5 times greater (lower K_M) in external KCl than in NaCl, indicating that K^+ might be the "preferable ion" for stimulating epithelial intestinal MS glucose and fructose influx in the American lobster. The magnitudes of the potassium concentration gradient or membrane potential across the epithelial luminal membrane *in vivo* is not known but even though the American lobster is mostly carnivorous, they do include some amount of marine algae in their diets (Conklin et al., 1995), which are high in potassium. An increase in luminal [potassium] would disturb the potassium equilibrium across the epithelial luminal membrane and result in an increased inwardly-directed electrochemical driving force for the uptake of potassium from lumen to epithelial cytosol (Obi et al., 2013). An estimation of the minimum luminal [K] needed to stimulate sugar uptake by an *in vivo* electrochemical driving force has been previously calculated using the potassium Nernst equation and was reported to be 10 mM (as occurs in seawater) (Obi et al., 2013).

In addition to cation-dependent glucose and fructose carrier processes observed in the hepatopancreas and intestine, sodium-independent glucose and fructose carrier processes appeared to be observed as well. In the absence of sodium (0 mM), there was still a considerable amount of glucose and fructose uptake by hepatopancreatic BBMV and transepithelial transport, suggesting the presence of lobster GLUT2-like and GLUT5-like transporters, respectively. In a recent study by Sterling et al., (2009) polyclonal antibodies to mammalian GLUT2 and GLUT5 were used to determine the localization of orthologous lobster

hepatopancreas proteins by western blot analysis. Both GLUT2 and GLUT5 were found in the hepatopancreas and intestine (Sterling et al., 2009). In addition, GLUT5-like transporters were localized to the brush border membrane of lobster intestine using immunohistochemical methods (Obi et al., 2011). Furthermore, western blot analysis using a rabbit anti-human SGLT4 antibody located an SGLT4-like protein in the lobster hepatopancreas (Sterling et al., 2009). These findings and those reported in the present investigation suggest that the sugar transporter ensemble for gastrointestinal absorption of glucose and fructose may be present, as previously described for mammalian epithelia, but also includes a co-transport process (SGLT-like) with properties apparently different from those of mammalian SGLT1.

Kinetic values of cation-dependent glucose and fructose transport in the hepatopancreas and intestine reported in this investigation are all displayed in Table 5 permitting comparisons between both organs. D-glucose and D-fructose appear to be transported by a single SGLT-like carrier process in each organ with Na^+ being the preferred (lower K_M) cation in the hepatopancreas and K^+ being the preferred (lower K_M) cation in the intestine. This cation preference in each organ may be due to [sodium] being depleted as it moves across the hepatopancreas (anterior organ) to the intestine (posterior organ) and potassium becoming more concentrated as it enters the intestine. Thus, in the hepatopancreas; Na^+ appears to be the preferred cation since it is in higher concentration than [K^+]; in the intestine, K^+ is the preferred cation since it may be in higher concentration than [Na^+]. The identity of this carrier that has the ability to transport D-glucose and D-fructose and be stimulated by either sodium or potassium in the lobster hepatopancreas and intestine is still unclear. It appears to be an SGLT-like carrier protein responsible for sugar transport in each

organ but whether it is a SGLT1-like or SGLT4-like co-transporter in the hepatopancreas or intestine remains unclear. It has been previously reported that SGLT1 does not use fructose at all (Ikeda et al., 1989; Wright and Turk, 2004), while SGLT4 has been reported to be selective for D-mannose, D-fructose and D-glucose (Tazawa et al., 2005). It appears the latter is a more suitable candidate for the identity of the sugar co-transport reported in the present investigation even though the potential role of potassium in mammalian SGLT4 function has not been reported.

Sugar absorption in the lobster digestive tract may occur sequentially as food travels from stomach to rectum, with transport characteristics being determined by substrate and ion concentration in each organ to fully remove glucose and fructose from the lumen. The apparent binding affinities for sugars in the lobster and prawn intestine are reported to be in the micromolar range (Ahearn and Maginniss, 1977; Obi et al., 2011), while in the hepatopancreatic epithelial cells these K_M values are much higher (Verri et al., 2001). In the hepatopancreas (anterior organ) luminal substrate concentrations are high and the brush border membrane 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. Similar arrangement of sodium-dependent sugar transporter apparent binding affinities is also observed along teleost fish digestive tract. 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). Also, the apparent binding affinity of the D-glucose transporter for sodium was at least two times higher in the pyloric caeca (anterior)

than in the upper intestine (posterior organ) (Ahearn et al., 1992). A similar trend in binding affinities for sodium is observed in the present investigation as well. Furthermore, in the mammalian proximal tubule, lower affinity sodium-dependent D-glucose transporters (SGLT2) displaying a higher sodium binding constant (high K_M) have been reported immediately after the glomerulus, followed by a high affinity SGLT1 transporter with a lower sodium binding constant (low K_M) (Turner and Moran, 1982).

Table 5: Kinetic Summary. Effect of increasing NaCl and KCl concentrations on D-[^{3}H] glucose or D-[^{3}H] fructose influx kinetic constants in lobster hepatopancreas and intestine.

		Na^+ / fructose	Na^+ / glucose	K^+/ fructose	K^+/ glucose
Hepatopancreas (BBMV)	K_M (mM)	2.58 +/- 0.95	2.30 +/- 0.59	12.6 +/- 0.80	9.85 +/- 0.4
	J_{max} (ρmol mg protein/min)	492 +/- 50.2	19.3 +/- 1.40	308 +/- 5.0	2112 +/- 19.8
Intestine	K_M (mM)	10.58 +/- 1.65	6.05 +/- 0.99	2.12 +/- 0.20	3.10 +/- 0.24
	J_{max} (ρmol/ cm^2min)	6.14 +/- 0.29	7.92 +/- 0.38	13.75 +/- 0.15	33.52 +/-0.35

Each experiment was conducted three times with 3-5 replicates/treatment. Symbols are means ± 1 SEM of three experiments. Concentrations of NaCl and KCl were; 5, 10, 25, 50 mM, and 5, 15, 25, 50, 100 mM respectively.

CONCLUSION

The following points can be concluded from this investigation:

1. Lobster hepatopancreatic BBMV can be frozen and when used yield similar uptake values as vesicles used immediately after protein isolation.
2. D-glucose and D-fructose transport displayed significant ($p < 0.01$) Na^+-dependent and K^+-dependent uptake processes in the hepatopancreas and intestine.
3. Similarity in K_M values for sodium and potassium-dependent D-glucose and D-fructose transport in each organ suggest that they may be transported by a single carrier process with substrate and ion specificities different from those demonstrated by mammalian SGLT1.
4. D-fructose inhibits D-glucose in a competitive manner in the hepatopancreas and intestine suggesting both sugars may be competing for the same binding site on this Na^+/K^+-coupled hexose transporter.
5. The apparent cation-binding affinity was greater (lower K_M) in external NaCl than in KCl suggesting sodium might be the “preferred ion” for stimulating hepatopancreatic BBMV glucose and fructose transport.
6. The apparent cation-binding affinity was greater (lower K_M) in external KCl than NaCl suggesting potassium might be the “preferred ion” for stimulating epithelial intestinal MS glucose and fructose transport in the American lobster.
7. Both sugars displayed apparent sodium-independent transport processes that remain to be described.

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