Glucose transport into everted sacs of the small intestine of mice

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Background

The intestinal epithelium has many functions, including serving as a barrier against invasion from the environment, digestion and absorption of nutrients, and water and electrolyte/solute transport (4, 8). Not surprisingly, absorption of ions (i.e., Na\(^+\)), solutes (i.e., glucose), and water are important functions performed by epithelial cells of the small intestine. Many transport proteins (ion channels, facilitated transport carrier proteins, and primary and secondary active transport proteins) work in concert to perform absorption. Consequently, if any transport protein involved in the absorptive process is compromised, then this can result in clinical conditions, such as GGM (1, 32–34).

Glucose is a crucial energy source, and it is important in many cellular processes (5). The major site of glucose absorption in the body is the epithelial cells of the small intestine, whereas reabsorption of glucose is carried out by epithelial cells of the proximal tubule of the nephron. Based on studies using the isolated guinea pig small intestine, Riklis and Quastel (21) first reported that active transport of sugars was dependent on the presence of Na\(^+\), and, at international meetings in 1960, Robert Kellogg Crane (1919–2010) (9–11) introduced the “cotransport hypothesis,” which provided a possible mechanism in epithelial cells of the small intestine whereby glucose absorption was coupled with Na\(^+\) transport. Since Crane’s introduction of the cotransport hypothesis, many research groups have examined coupled Na\(^+\)-glucose absorption in a variety of tissues. In brief, Bhler and Crane (7) demonstrated, in the hamster small intestine, that Na\(^+\) was the only cation tested that could result in active sugar transport. Furthermore, they reported that Na\(^+\) transport was localized to the brush border of the epithelium, which was the same region of the intestinal cell that was demonstrated as the route of transport for sugar (12), providing additional experimental evidence for the proposed coupled Na\(^+\)-sugar mechanism. Schultz and Zalusky (25, 26) were the first to use the short-circuit current technique, developed by Ussing (29), to examine the electrical properties of the mammalian intestinal (rabbit ileum). Specifically, Schultz and Zalusky demonstrated, via electrophysiological and radioisotopic experiments, that the addition of sugars to the mucosal solution bathing the epithelium resulted in a rapid increase in the transmural potential difference (voltage), short-circuit current, and rate of active Na\(^+\) transport across the epithelium.

In 1987, Crane’s prediction of a Na\(^+\)-coupled glucose transporter was validated when Wright and colleagues cloned a Na\(^+\)-glucose cotransporter from the rabbit intestine (16). This was a pioneering achievement and represented the first time a transport protein had been cloned. With the introduction of the HUGO Gene Nomenclature Committee (http://www.genenames.org/), which standardized the name of various human genes, the transport protein that Wright and coworkers...
cloned was named SGLT1 (for sodium-glucose transporter 1), which is the product of the SLC5A1 gene (33, 34, 36).

For further information about Dr. Crane, students are directed to the following website: http://the-aps.org/mm/Membership/Living-History/Crane and Hamilton (15). Additional information about the SLC gene family can be obtained from the following Genetics Home Reference website: ghr.nlm.nih.gov/geneFamily/slc. Wright and coworkers (34) have recently published an excellent comprehensive review of human Na+/glucose cotransporters to which the reader is directed for further information regarding SGLT1 and other members of the SGLT gene family. Briefer review articles on this gene family are available (33, 35, 36).

The classical model of glucose absorption (Na+/glucose cotransport) by epithelial cells of the small intestine that has evolved from Crane’s initial proposed model is shown in Fig. 1 (11). The entry step of glucose across the apical membrane is via SGLT1 (SLC5A1), which is inhibited by phloridzin (Fig. 1) (13). SGLT1 is an example of a secondary active transport protein, which uses the electrochemical gradient for Na+ entry into the cell to accumulate glucose in the cell above its equilibrium concentration. The electrochemical gradient for Na+ is generated and maintained by basolateral Na+/K+-ATPase [Na+ pump (27)], which is blocked by ouabain (14). Na+ transported into the cell with glucose exits the cell across the basolateral membrane by the Na+ pump, which transports three Na+ for the exchange of two K+ with the expenditure of ATP. As both the Na+ and K+ are transported against their electrochemical gradients and this is directly coupled to the expenditure of ATP, the Na+ pump is an example of a primary active transport protein. K+ is recycled back across the basolateral membrane via K+ channels, aiding in the maintenance of the negative membrane potential of the epithelial cell, which contributes to the electrochemical gradient for Na+ entry across the apical membrane (Fig. 1). Glucose transported into the cell and accumulated by SGLT1 is carried across the basolateral membrane via glucose transporter 2 (GLUT2; SLC2A2), which transports glucose, galactose, or fructose (3). GLUT2 is an example of a facilitated transport carrier protein, as the function of GLUT2 is dependent solely on the concentration gradient of the sugars across the basolateral membrane of the epithelial cell (3). The transepithelial transport of Na+ contributes to a charge separation across the epithelium, providing a driving force for Cl− absorption via the paracellular pathway. The absorption of Na+, Cl−, and glucose generates a slight osmotic gradient across the epithelium, providing a driving force for water absorption via both transcellular and paracellular pathways (Fig. 1).

Learning Objectives

After completing this 3-h laboratory exercise, the student will be able to:

1. Prepare the everted sac preparation of the small intestine of the mouse.
2. Describe the cellular mechanism of Na+ coupled glucose transport of the small intestine.
3. Explain how the function of SGLT1 is dependent on the presence of Na+ in the bathing solution.
4. Describe the net flux of Na+ -glucose cotransport by the small intestine.
5. Describe the effect of phloridzin on the overall process of Na+ -glucose cotransport by the small intestine.
6. Explain the physiological basis of the symptoms of GGM.

METHODS

Prerequisite Student Skills

Students should have a basic skill set that includes:

1. Knowledge of experimental design.
2. Formulation of hypotheses and appropriate use of controls.
3. Physical dexterity for handling mouse jejunal tissue.

Prerequisite Student Knowledge

Students should have a basic knowledge of:

1. The different segments of the small intestine.
2. The structure of an epithelial cell.
3. The membrane transport proteins that are crucial for the Na+ -glucose absorptive process.
4. The pharmacological blockers used in conducting experiments testing the cellular model of Na+ -glucose cotransport by the jejunum.

Prelaboratory Questions

Before the laboratory session, students are asked to prepare for the laboratory exercise by answering a series of prelaboratory preparation questions. The questions and answers are provided in the RESULTS AND DISCUSSION.

Class Groups

Generally, there are two laboratory rooms with 24 students/room performing this laboratory exercise per laboratory day. The laboratory exercise is scheduled for 3 h. Students work in pairs, and each pair of students prepares an everted sac. The experimental conditions that the students use for a given everted sac are described below. There is normally one academic staff member and one teaching assistant per laboratory room.

Animals and Tissue Preparation

All experiments are performed on the jejunum of male Swiss-Webster mice (20–35 g) or a similar strain of mouse. [Note that we have only conducted experiments with Swiss-Webster mice. We presume that other strains of mice would be appropriate, and it should be noted that rat and hamster preparations have been used for research.
Table 1. Basic formulation for the Ringer solutions used in this laboratory exercise

<table>
<thead>
<tr>
<th>Salt</th>
<th>Stock, mM</th>
<th>Required Amount, mM</th>
<th>Milliliter per 1 liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl*</td>
<td>1,000</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>KCl</td>
<td>1,000</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1,000</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1,000</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>HEPES-Tris</td>
<td>300</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td>Glucose</td>
<td>500</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td>7.4</td>
</tr>
</tbody>
</table>

*For the Na⁺-free Ringer solution, all Na⁺ is replaced with N-methyl-D-glucosamine.

preparations (31). The Otago University Institutional Animal Ethics Committee under the guidelines of the National Animal Ethics Advisory Committee of New Zealand approved all of experimental protocols used in this study (ET-7/08 and ET-14/11). Adopters of an activity are responsible for obtaining permission for human or animal research from their home institution. For a summary of the “Guiding Principles for Research Involving Animals and Human Beings,” please see www.the-aps.org/mmPublications/Ethical-Policies/Animal-and-Human-Research.

Organisms should have access to tap water and food ad libitum until used. Mice are euthanized by cervical dislocation, and the intact isolated jejunum (cut into 6-cm lengths) are placed in ice-cold NaCl Ringer solution (50 ml) composed of (in mM) 150 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES-Tris, and 10 glucose (pH 7.4) and gassed with O₂ (see Table 1). All solutions contain 10 mM glucose. Jejuna required for the Na⁺-free protocol (Table 2) are placed in ice-cold Na⁺-free Ringer solution (50 ml). Normally, the jejuna are isolated 30 min before the beginning of the laboratory so that the students can begin preparing the everted sacs immediately after a short introduction to the laboratory exercise. The isolation of the small intestine is shown in a movie (see below). All chemicals are purchased from Sigma-Aldrich (St. Louis, MO).

Everted Sac Technique

Originally, Wilson and Wiseman (31) developed the everted sac preparation using the intestine of the hamster and rat to examine solute (glucose and methionine) transport across the intestinal epithelium. The everted sac preparation is simple to prepare and robust, and the small volume contained within the sac (serosal solution) allows for the rapid accumulation of a transported solute (31). Sanford (23) discussed the advantages of the everted sac preparation, which include that the fluid absorption into the sac can be determined gravimetrically, the volume bathing the mucosal surface of the epithelium can be very large so that the concentration of substances the mucosal bath are not appreciably altered, and, finally, that the substances transported can be determined in both the “gut wall” or “small volume” of the fluid bathing the serosal surface; thus, one can determine if the substances are being transported against their concentration gradients. Alam and colleagues (2) have explored the limitations and applications of the everted sac technique in a recent review to which readers are directed.

Everted Sac Solutions and Experimental Protocols

The tools and equipment necessary for the laboratory exercise are shown in Figs. 2 and 3 and Table 2. Students use either a control protocol in which the everted sacs are incubated with normal NaCl Ringer solution (Table 1) inside and outside of the sac or one of three experimental protocols (Table 2), which include the following: 1) incubating sacs that have Na⁺-free Ringer solution inside and outside of the sacs, 2) incubating sacs with normal NaCl Ringer solution inside the sacs and in normal NaCl Ringer solution plus phloridzin (100 μM) outside the sacs, or 3) incubating sacs with normal NaCl Ringer solution with ouabain (2 mM) within the sacs and normal NaCl Ringer solution outside the sacs. It is important that the students use the correct Ringer solutions for the preparation and incubation of the everted sacs; otherwise, interpretation of data will be compromised. Therefore, each pair of students is assigned a given protocol (Table 2) that states which serosal solution to fill their everted sac and the mucosal solution in which they incubate their everted sac. The Kᵢ of phloridzin for SGLT1 is between 5 and 10 μM (18); therefore, a concentration of 100 μM phloridzin is used to assure almost complete block. In the whole animal, phloridzin can affect the central nervous system and increase feeding behavior and pulmonary alveolar function, among a number of side effects (13). Further information about phloridzin can be found in a recent review by Ehrenkrantz and colleagues (13).

Preparation of the Everted Sacs

The following are instructions given to the student detailing the preparation of the everted sacs.

Fig. 2. Equipment used for preparing the everted sac. This equipment includes a petri dish, forceps, scissors, a razor blade, thread, a 1-ml syringe with needle and short length (4 cm) of polyethylene tubing, plastic Pasteur pipettes, Eppendorf tubes, small plastic test tubes, a glucometer with test strips, a notched glass rod, an indelible marking pen, and a plastic tray.
Step 1. Before starting the experiment, watch the movie (the movie will be provided to instructors who implement this laboratory practical) demonstrating the technique. Once you have viewed the movie, ensure that you are wearing a laboratory coat and gloves and check with the instructor that you have the correct Ringer solutions for your assigned protocol.

Step 2. Add sufficient ice-cold Ringer solution to the petri dish to half fill the dish.

Step 3. Next, ask the instructor for a piece of jejunum. This will be provided in a 100-ml beaker containing ~50 ml of Ringer solution. Carefully transfer the piece of jejunum from the beaker with forceps to the petri dish (Fig. 2) containing either ice-cold NaCl or Na⁺-free Ringer solution. Remember that forceps will damage the intestine, so when you pick up the jejunum with forceps, only hold it by either end.

Step 4. Take care at all times to ensure that the intestinal epithelium is kept moist with ice-cold Ringer solution. Also, when handling the jejunum, always moisten your fingers with Ringer solution first.

Step 5. Locate the glass rod that you are going to use to ever the sac and note that it has a rounded end with a constriction a few millimeters from one end (Fig. 4A). Moisten the glass rod with the appropriate Ringer solution and slip the end of the intestine over the glass rod until the intestine extends just past the constricted region of the rod (Fig. 4B). Next, tie a loop of moistened thread around the intestine and glass rod at the point of constriction. A double-loop knot is desirable to prevent slippage. Make certain that the intestine is tied tightly onto the glass rod and then trim the thread on either side of the knot to leave 1–2 cm. Note the color of thread used as this will allow you to identify your jejunal sac.

Step 6. Now, gently push the intestine over the knot and rod so that the intestine doubles over on itself (Fig. 4C, top). It is important that this is done carefully as the intestine is very fragile and, as it is everted, the mucosal surface of the intestine is exposed (Fig. 4C, bottom). Therefore, it is very important that you do not touch the mucosal surface, as that will damage the epithelial tissue. Continue this until the entire intestinal segment is turned inside out. When you have completed evertting the intestine, if it hangs over the end of the glass rod, trim it off with a pair of scissors so that the everted jejunum is slightly longer than the glass rod.

Step 7. Next, use a razor blade to cut through the intestine just to the right of the knot formed to tie the intestine onto the glass rod (Fig. 4D). Then, with your moistened fingers on the glass rod, push the intestine off the glass rod into the petri dish from the end opposite to where the knot was tied (Fig. 4D, bottom).

Step 8. Now, cut the everted intestine so that it is a 3–4 cm-long segment. You will use this piece of intestine to make a sac. Remember, the mucosal surface is now exposed, so try not to touch the tissue with your fingers.

Step 9. To form a sac, firmly tie off one end of the everted intestine with a double-loop knot of moistened thread, as described above (Fig. 4E).

Step 10. Completely fill a 1-ml plastic syringe with the appropriate Ringer solution. Attach the needle to the syringe and fill the tubing.
Table 3. Materials required for each pair of students (except for water baths)

<table>
<thead>
<tr>
<th>Materials</th>
<th>Number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scissors</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Forceps</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Syringe (1 ml) with 18-gauge needle (1 in.)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Plastic tubing (4 cm × 1.4 mm outside diameter)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Three-way luer lock valve</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Petri dish (8.5 cm)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ice bucket</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Notched glass rod (11 cm × 0.4 cm, notch 1 cm from end)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Spool of colored thread (cotton)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Shaking water bath (37°C) for the entire laboratory</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Plastic test tubes (8 cm)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Eppendorf tubes (1.5 ml)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pasteur pipette (3-ml volume)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Beaker (100 ml)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Razor blade</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Glucometer</td>
<td>1</td>
<td>Advantage type-8 (catalog no. 800767, Boehringer Mannheim)</td>
</tr>
<tr>
<td>Glucometer test strips</td>
<td>8</td>
<td>Accu-Chek Advantage II Test Strips (code 676, Roche Diagnostic)</td>
</tr>
<tr>
<td>Indelible pen</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tubing for connecting air lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td></td>
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</tbody>
</table>

making sure there are no air bubbles inside the syringe or tubing. Depending on your protocol, you should have filled the syringe with one of the following solutions:

A. Control experiment [NaCl Ringer solution plus glucose (10 mM)]

B. Na⁺-free experiment [Na⁺-free Ringer solution plus glucose (10 mM)]

C. Phloridzin experiment [NaCl Ringer solution plus glucose (10 mM)]

D. Ouabain experiment [NaCl Ringer solution plus glucose (10 mM) and ouabain (2 mM)]

Step 11. Insert the plastic tubing of the syringe gently into the opposite end of the piece of intestine that is tied. Then, tie the intestine firmly to the tubing with a piece of moistened thread (Fig. 4F).

Step 12. Once the sac is firmly tied to the tubing, slowly fill the everted sac (Fig. 4G). When filled with the correct amount of solution (~1 ml), the everted sac will have a slightly distended, “sausage-like” appearance, showing the fuzzy mucosal surface. If the sac does not “inflate” or slowly “deflates” over a minute or so after filling, check that the knots are tied firmly. If the knots are loose, use another piece of thread to tie the sac more firmly. If the knots are not loose and the sac still deflates, this suggests that it has been damaged and should be replaced with another piece of jejunum.

Step 13. When you are sure the sac is not damaged, tie it off with a second moistened thread just in front of the end of the plastic tubing (Fig. 4G). Then, cut between the two knots and trim the threads to ~1–2 cm (Fig. 4H). Take particular care to ensure that the sac is completely sealed by the thread. If tied properly, the sac should retain its distended, sausage-like appearance. If this appearance is lost, it will be necessary to get another piece of tissue. The everted sac can now be handled by holding the threads and without touching the delicate mucosal surface.

Step 14. Transfer the everted sac to the correct incubation beaker (Fig. 3A) filled with the appropriate Ringer solution (50 ml) that is being warmed in the shaking water bath (the temperature of the solutions within the incubation beakers should be 37°C; Fig. 3B). Make sure you record the number of the beaker in which you have placed the everted sac. Below are the solutions present within the incubation beakers in the water baths (for example, see Fig. 3C):

A. Control experiment [NaCl Ringer solution plus glucose (10 mM)]

B. Na⁺-free experiment [Na⁺-free Ringer solution plus glucose (10 mM)]

C. Phloridzin experiment [NaCl Ringer solution plus glucose (10 mM) and phloridzin (100 µM)]

D. Ouabain experiment [NaCl Ringer solution plus glucose (10 mM) and ouabain (2 mM)]

Step 15. Record the time that you added the sac to the beaker and incubate it for at least 90 min. Make sure that the solution in the beaker is bubbling at a sufficient rate so that the everted sac is gently agitating in the bath solution throughout the incubation period.

Measurement of the Glucose Concentration

Once the everted sacs have incubated for at least 90 min, retrieve your everted sac and determine the glucose concentration of the solution within the sac (serosal solution) and the incubating bath solution (mucosal solution). The following are instructions to determine the glucose concentration.

Step 1. Remove your everted sac from the incubation bath. Cut one end of the everted sac and drain the contents of the sac into a clearly labeled plastic test tube. This is the serosal solution.

Step 2. Also collect a 1-ml sample of the incubation solution from the beaker in which your everted sac was incubated and transfer the sample to another clearly labeled plastic test tube. This is the mucosal solution.

Step 3. Measure the glucose concentration of the mucosal and serosal solutions using a glucometer (Fig. 2). The glucometer is accurate between 5 and 30 mM (note that this may vary depending on the glucometer purchased). When determining the glucose concentrations with the glucometer, use the undiluted solutions but do not throw away the sample remaining, as you may have to dilute the sample to obtain an accurate measurement.

Step 4. Turn the glucometer on and make certain that the code number on the glucometer matches the code number on the test strip container (specific directions may vary according to the type of glucometer purchased). After this, remove one test strip from the container and place it in the test strip slot on the side of the glucometer. The glucometer will indicate on the screen when it is the appropriate time to add the solution to the test strip (this may differ depending on the type of glucometer purchased).

Step 5. Using a Pasteur pipette, carefully place a drop of either the mucosal or serosal solution into the indented part of the strip (from the side of the strip, not the top). It is important to ensure that the entire square is covered with solution. If done correctly, the yellow mesh will darken slightly. After ~40 s, the glucose concentration (in mM) should appear on the display window. Repeat this measurement two additional times with the sample, using a new test strip each time, and calculate the average glucose concentration for the sample.

Step 6. If a “HI” reading is shown on the glucometer for a sample, this indicates that the glucose concentration of that sample is higher than 30 mM. Therefore, to obtain an accurate measurement, you will have to dilute the sample before determining the glucose concentration. In the first instance, dilute the sample 1:1 with water before determining the glucose concentration and repeat the measurements. If you have to dilute the sample before determining the glucose concentration, remember to correct for the dilution of the original sample when recording the result. For
example, if you made a 1:1 dilution of a sample, multiply the measured glucose concentration by 2 to correct for the dilution factor. Depending on how high the glucose concentration is within the sac, after correcting for dilution(s), the glucose concentration may be as high as 100 mM.

**Step 7.** After you have made three successful measurements of glucose and calculated the average glucose concentrations for both the mucosal and serosal solutions, record the average values of glucose concentration of the samples on the whiteboard (or Excel spreadsheet) and place the results in the data table (Table 4) in your laboratory book.

**Step 8.** At the end of the experiment, dispose of the test strip(s) by removing the strip from the glucometer and placing it in a designated waste bucket. Then, turn off the glucometer.

**Questions Related to the Experimental Protocols**

After all data are collected and averaged, students should be able to answer a series of questions based on the results of the experiments they have performed. The questions and answers are provided in the **RESULTS AND DISCUSSION**.

**Safety Considerations**

In this laboratory, students should wear laboratory coats and gloves during all procedures of the laboratory. Some students will be using solutions that contain either phloridzin or ouabain. Those students who have the ouabain protocol should wear protective eyewear. According to the material safety data sheets provided by Sigma-Aldrich for both of these chemicals, phloridzin and ouabain can cause...
skin and eye irritation. In the event that a student does have skin contact with either solution containing either chemical, the student should wash their hands immediately with soap and water. If a student has contact in their eyes with a solution containing ouabain, the student should first check and remove contact lenses and then flush their eyes with water for a few minutes for phloridzin or at least 15 min for ouabain.

Troubleshooting

Students may experience a variety of problems while working through the practical part of this laboratory exercise. Listed below are a series of potential problems and solutions.

*Problem 1.* If a student tries to fill the everted sac with Ringer solution and the sac does not inflate, this usually indicates that 1) the sac has a hole or 2) one or both of the knots tied at either end of the sac is too loose. In either case, the student will need to prepare another everted sac. As noted above, if the sac slowly deflates in the first minute after filling, that too usually indicates that the sac is damaged or the knots are not tied firmly enough.

*Problem 2.* If a control sac bathed in normal NaCl Ringer solution with glucose does not accumulate glucose at a concentration above that of the bathing solution, this generally results from 1) the sac not being filled sufficiently (it is important that the sac has a distended, sausage-like appearance after filling), 2) the sac was not agitated sufficiently during the incubation period, or 3) the temperature of the incubation solution was not held at 37°C.

*Problem 3.* If the glucose concentration of the everted sacs containing ouabain or everted sacs bathed in phloridzin increases to a level above that of the bathing solution, this generally results from 1) the glucose concentration of the bathing solution was not held at 37°C, 2) the sac was not agitated sufficiently during the incubation period, or 3) the temperature of the incubation solution was not held at 37°C.

*Problem 4.* Students may be concerned that they will damage the intestine by tying the knots too tightly. However, in our experience, this is generally not a problem; the greater problem is not tying the knots tight enough and the sac leaks.

Statistical Analysis

Data are presented as mean ± SD. Data were analyzed with two-way ANOVA with multiple measures followed by a Bonferroni post hoc test using Graphpad Software Prism (version 5, La Jolla, CA). n equals the number of tissues used for a given protocol. A total of 8 mice were used for every laboratory group of 24 students. The level of significance was chosen as P = 0.05. P values are reported in the text.

RESULTS AND DISCUSSION

Over 850 second-year physiology students at the University of Otago have completed this laboratory exercise since 2008. The overall objective of this laboratory was for the students to gain a better understanding of the cellular mechanism of the coupled transport of Na+ and glucose using the intact mouse jejunum as a model. To understand this mechanism, students used the everted sac technique to test three tenets of the cellular model of Na+-glucose cotransport, which are 1) the Na+ dependence of glucose absorption, 2) the dependence of glucose absorption on SGLT1, and 3) the role of the Na+ pump in glucose absorption by SGLT1. Additionally, students performed an internet search on a case study of GGM to aid their understanding of the basic physiological function of the small intestine and the pathological complications of GGM. The case study is provided below in *Wider Educational Applications*.

Normal Glucose Transport Function and Na+ Dependence of SGLT1

Initially, students prepared everted sacs and incubated the sacs with prescribed Ringer solutions within the sac and in the bath (Table 2). All Ringer solutions contained 10 mM glucose at the beginning of the experiments. It was hypothesized that if active glucose absorption was dependent on Na+-coupled glucose transport, the everted sacs filled with normal NaCl Ringer solution and incubated in NaCl Ringer solution would have normal Na+-glucose cotransport function and, thus, would accumulate glucose inside the everted sac (Fig. 5, NaCl control). As shown in Fig. 5, the serosal solution of the everted sacs had a significantly higher (P < 0.001) glucose concentration (58.1 ± 25.7 mM, n = 91; hatched bars) than the mucosal bath solution (9.6 ± 1.7 mM, n = 90; solid bars). Next, students hypothesized that in Na+-free Ringer solution, SGLT1 would not be able to function and, as a result, glucose would not be accumulated within the sacs. To test the Na+ dependence of SGLT1, students prepared everted sacs that were filled with Na+-free Ringer solution, and the sacs were incubated in Na+-free Ringer solution. As shown in Fig. 5 (Na+-free conditions; hatched bars), under these conditions, the glucose concentration of the serosal solution was not different from the mucosal bath solution (solid bars; 10.6 ± 4.1 and 9.7 ± 1.5 mM, respectively, P > 0.05, n = 61 for both conditions). These data clearly demonstrate that the glucose absorption is active and is dependent on Na+.

Inhibition of the Function of SGLT1 by Phloridzin

Phloridzin was originally isolated from apple trees and has been used as a pharmaceutical tool for over 150 yr (13).
Phloridzin acts to reduce intestinal glucose absorption and produce renal glucosuria through the inhibition of SGLT1 (13). Therefore, students hypothesized that glucose transport into the everted sac would be reduced in the presence of phloridzin compared with glucose transport by control sacs containing NaCl Ringer solution. This hypothesis was tested by incubating everted sacs containing normal NaCl Ringer solution in a bathing solution of normal NaCl Ringer solution that contained phloridzin (100 μM). As shown in Fig. 5 (NaCl + phloridzin), everted sacs incubated in phloridzin exhibited a markedly lower (P < 0.001) serosal glucose concentration (19.6 ± 9.6 mM, n = 57; hatched bars) than NaCl control sacs (58.1 ± 25.7 mM). There was a slight but significant increase (P < 0.01) in glucose concentration compared with sacs incubated with phloridzin in the mucosal bathing solution (10.9 ± 1.2 mM, n = 57; solid bars). These data clearly demonstrate that phloridzin reduces glucose transport via SGLT1 and that the transport of glucose is dependent on the function of SGLT1.

Effect of Inhibition of the Na⁺ Pump on Glucose Absorption

The cardiac glycoside ouabain has been used as an inhibitor of the Na⁺ pump for >60 yr (14). The proper function of the Na⁺ pump is critical in maintaining the electrochemical gradient for Na⁺ entry into cells that is used by many Na⁺-coupled secondary active transport proteins, including SGLT1. Students hypothesized that inhibition of the Na⁺ pump by ouabain would result in a reduced Na⁺ electrochemical gradient; hence, the amount of glucose absorbed would be reduced compared with the glucose absorbed by sacs not containing ouabain. To examine the effect of ouabain on glucose absorption, everted sacs were filled with NaCl Ringer solution containing ouabain (2 mM), and the sacs were incubated in normal NaCl Ringer solution. Under these conditions (Fig. 5, NaCl + ouabain), the glucose concentration of the contents of the sacs was 16.1 ± 8.0 mM (n = 59; hatched bars), which was slightly increased (P < 0.01) compared with the glucose concentration of the mucosal bath solution (10.1 ± 2.1 mM, n = 61; solid bars) but considerably lower (P < 0.001) than the glucose concentration accumulated in control sacs bathed in NaCl Ringer solution (Fig. 5, NaCl control). A possible explanation for the accumulation of glucose within the sacs in the presence of ouabain is that there are a number of isoforms of the Na⁺ pump and there is evidence of varying sensitivities of the inhibition of rodent Na⁺ pump function by ouabain (6, 19). Therefore, incomplete inhibition of the Na⁺ pump may be the reason for these results.

Overall, the students’ experiments clearly demonstrated that Na⁺-glucose cotransport function of the jejunum 1) was dependent on Na⁺, 2) required function of SGLT1, and 3) required proper function of the Na⁺ pump.

Questions for the Prelaboratory Preparation

Question 1. What are the names and functions of the three regions of the small intestine?

Question 2. If the mucosal Na⁺ concentration of the small intestine was decreased, what would happen to the rate (increase or decrease) of transcellular glucose absorption? Explain your answer.

Question 3. Explain how Na⁺-K⁺-ATPase (Na⁺ pump) is important for the absorption of glucose by the small intestine?

Answers to the Prelaboratory Preparation Questions

Questions should be answered by students before they come to the laboratory. These questions should have assisted the students as they prepared to test components of the cellular model of Na⁺-glucose cotransporter absorption of the small intestine.

Answer to question 1. The small intestine is composed of three sections: the duodenum, jejunum, and ileum. The duodenum receives the acid chyme (partially digested food) from the stomach and secretions from the pancreas that contribute digestive enzymes and bicarbonate that aid in the neutralization and breakdown of chyme. The jejunum is the middle segment of the small intestine. This segment is important in secretion and absorption, including Na⁺ and glucose absorption. The terminal end of the small intestine is the ileum. This segment is responsible for the absorption of vitamin B₁₂ and bile salts, with the latter being important in the digestion and absorption of dietary fat within the small intestine.

Answer to question 2. SGLT1 requires both Na⁺ and glucose to function properly; therefore, if the mucosal Na⁺ concentration was reduced, this would decrease the rate of absorption of glucose across the apical membrane. This is because a reduction in the Na⁺ concentration in the mucosal solution would reduce the electrochemical gradient for the entry of Na⁺ across the apical membrane of the epithelial cell (Fig. 1).

Answer to question 3. The Na⁺ pump is the key transport protein that establishes and maintains the Na⁺ concentration gradient across the epithelial cell membrane. SGLT1 is a secondary active transport protein and, therefore, is crucially dependent on the electrochemical gradient for Na⁺ for the proper function of this cotransporter.
Questions Related to the Experimental Protocols

Question 1. For the Na⁺ Ringer control sacs, explain why there was an increase in the serosal glucose concentration within the everted sac?

Question 2. Would there be a maximum concentration of glucose that could be generated within the everted sac or would the concentration continue to increase as long as the sac is incubated? Explain your answer.

Question 3. Explain how the presence of Na⁺-free Ringer solution reduced the absorption of glucose into the everted sac?

Question 4. Explain how phloridzin altered the accumulation of glucose into the everted sac?

Question 5. Explain how ouabain decreased the absorption of glucose into the everted sac?

Answers to the Questions Related to the Experimental Protocols

Answer to question 1. Students should remember that at the beginning of the experiments, all of the Ringer solutions in all these experiments contained 10 mM glucose. The average concentration of the glucose in the mucosal solution was 9.6 ± 1.7 mM (Fig. 5, NaCl control). However, the concentration of glucose within the everted sac was 58 ± 25.7 mM (Fig. 5). So, there was an approximately fivefold increase in the glucose concentration within the sac due to the combined action of apical SGLT1, basolateral GLUT2, the Na⁺ pump, and K⁺ channels (Fig. 1).

Initially, the everted sac is set up with equal concentrations of glucose in the mucosal and serosal solutions (Fig. 1). Under these conditions, in the absence of an active transport, the passive fluxes of glucose across the epithelium, which occur via the paracellular pathway, would be equal and opposite; therefore, there would be no net change in glucose concentration, as follows:

\[ J_{MS}^{\text{passive}} = - J_{SM}^{\text{passive}} \]

where \( J_{MS}^{\text{passive}} \) is mucosal to serosal passive flux and \( J_{SM}^{\text{passive}} \) is serosal to mucosal passive flux. However, in the presence of SGLT1, active cellular transport of glucose occurs from the mucosal solution to the serosal solution. Hence, the total mucosal to serosal flux is the sum of the passive paracellular flux (\( J_{MS}^{\text{passive}} \)) and active cellular flux and is greater than the passive serosal to mucosal flux (\( J_{SM}^{\text{passive}} \)) via the paracellular pathway. As a result, there is net flux of glucose from the mucosal to serosal solution and the concentration of glucose in the everted sac increases, as follows:

\[ J_{MS}^{\text{total}} = J_{MS}^{\text{passive}} + J_{MS}^{\text{active}} > J_{SM}^{\text{passive}} \]

where \( J_{MS}^{\text{total}} \) is total mucosal to serosal flux and \( J_{MS}^{\text{active}} \) is active cellular flux. Although there is a net mucosal to serosal flux of glucose, the concentration of glucose in the mucosal solution does not change appreciably because the amount of glucose in the mucosal solution is much greater than the amount transported.

Answer to question 2. The glucose concentration will not increase indefinitely, and there are two possible reasons for this: one is related to the properties of the paracellular pathway for solute movement across the epithelium and the other is related to the cellular mechanism of glucose absorption. First, the increase in serosal glucose concentration will result in an increased passive serosal to mucosal flux of glucose via the paracellular pathway. This will continue to increase as the glucose concentration increases until eventually a point is reached when the serosal to mucosal concentration gradient is such that the passive serosal to mucosal flux via the paracellular pathway equals the total mucosal to serosal flux of glucose. At this point, the system is in a steady state, and the glucose concentration will not increase further. It should be recognized that any damage to the intestinal epithelium creates a second nonphysiological “paracellular” pathway for glucose flux. Therefore, in the presence of damage to the epithelium, the passive flux of glucose will occur through both the paracellular pathway and damaged regions of the epithelium. As a result, in the presence of tissue damage, the passive flux will be greater for any given concentration gradient, and a steady state, where the passive serosal to mucosal flux equals the total mucosal to serosal flux, is reached at a lower serosal concentration.

The second reason that the serosal glucose concentration will not continue to increase indefinitely relates to the cellular mechanism of glucose transport. As noted above (Fig. 1), glucose absorption is a two-step process; it is transported across the apical membrane via SGLT1 and exits across the basolateral membrane, primarily via GLUT2. The entry of glucose across the apical membrane via SGLT1 is a secondary active transport process and is driven by the electrochemical gradient for Na⁺. However, the exit of glucose from the cell across the basolateral membrane is passive transport (e.g., facilitated transport carrier protein) and is dependent on the glucose concentration gradient from the cell to the serosal solution. Therefore, it is possible that the concentration of glucose in the serosal solution reaches a point where there is no longer a passive driving force for the facilitated diffusion of glucose from the cell in to the serosal bath.

Answer to question 3. SGLT1 protein is dependent on the presence of Na⁺ within the mucosal solution. Therefore, the incubation of everted sacs in Na⁺-free Ringer solution will prevent the transport of glucose across the apical membrane, limiting the absorption of glucose. The students’ data support this as there was no difference between the glucose concentration of the mucosal solution (bathing Na⁺-free Ringer solution) or the serosal solution when the sacs were incubated in Na⁺-free Ringer solution (Fig. 5, Na⁺-free).

Answer to question 4. Phloridzin is an inhibitor of SGLT1 (13). One might expect that there would be no transport of glucose once an everted sac was incubated in NaCl Ringer solution containing phloridzin. But, indeed, the glucose accumulated within the sacs incubated in NaCl Ringer solution with phloridzin (Fig. 5, NaCl + phloridzin) was greater than the glucose concentration of mucosal NaCl Ringer solution in which the sac was bathed. However, the glucose within the sacs was less than the glucose accumulated within the control sacs incubated in normal NaCl Ringer solution (Fig. 5). These data clearly demonstrate that phloridzin reduces the absorption of glucose by SGLT1.

Answer to question 5. Students determined that incubating everted sacs with NaCl Ringer solution containing ouabain greatly reduced the amount of glucose transported into the sac (Fig. 5, NaCl + ouabain) compared with everted sacs incubated in normal NaCl Ringer solution (Fig. 5). However, the
accumulated glucose incubated in the sacs in the presence of ouabain was greater than the glucose concentration of the mucosal NaCl Ringer solution (Fig. 5). With ouabain inhibiting the Na\(^+\) pump, Na\(^+\) exiting the cell is reduced; additionally, there was still Na\(^+\) and glucose entry via SGLT1 across the apical membrane of epithelia cells. This transport scenario results in increasing the cellular concentration of Na\(^+\), thus reducing the driving force for Na\(^+\) entry into the cell, and reduced transport by SGLT1. If the pump is completely inhibited by ouabain, the active transport of glucose will cease once the electrochemical gradient for Na\(^+\) is dissipated. The fact that there was some accumulation of glucose within the sac suggests that the Na\(^+\) pump was not completely inhibited. The end result is less Na\(^+\) and glucose transported across epithelial cells.

### Inquiry Applications: Other Possible Experiments

As we developed this student laboratory, we were limited to the number of experiments that could be successfully completed in a 3-h laboratory session. However, there are a number of additional experiments that can be conducted to test other aspects of Na\(^+\)-glucose cotransport function of the mouse jejenum. These experiments include the following:

1. Examination of the time course of glucose transport by incubating everted sacs in NaCl Ringer solution for varying times (15, 30, 45, 60, and 90 min) to determine the rate of glucose accumulation within the everted sac.

2. Demonstrating the O\(_2\) dependence of Na\(^+\)-glucose cotransport function by incubating everted sacs in NaCl Ringer solution and bubbling everted sacs with O\(_2\) or N\(_2\).

3. Examining the effects of a high-carbohydrate diet (high-carbohydrate mouse chow or high-glucose water) on the glucose entry into the cell, and cellular transport models and physiology that explains the symptoms of GGM. This case study can be completed during the 90-min period that the everted sacs are incubating. In the authors’ experience, there is ample time for students to conduct a literature search and have small-group discussions about the GGM case during the 90-min period that the everted sacs are incubating.

4. Demonstrating the role of basolateral GLUT2 in glucose absorption through the action of phloretin.

5. Demonstrating inhibition of SGLT1 by a high concentration of galactose.

6. Demonstrating inhibition of SGLT1 by a high concentration of lactose.

7. Demonstrating inhibition of SGLT1 by a high concentration of galactose.

### Wider Educational Applications

**SGLT and oral rehydration therapy.** What is the significance of understanding the mechanism of glucose absorption? Sometimes, the impact of a scientific advance is not appreciated until that advance explains a treatment of a clinical pathology. A case in point is the Na\(^+\)-glucose cotransport hypothesis (11). Secretory diarrhea leads to an increase fluid loss by the body, primarily by increased intestinal secretion. Infectious diarrheas, including cholera, have claimed the lives of hundreds of millions of people over the past few centuries, many of whom were children under the age of 5 yr (28). In 1982, it was estimated that >1 billion cases of diarrhea were reported annually, of which nearly 5 million deaths were of children (28). However, although the World Health Organization reported that 1.7 billion cases of diarrhea occurred in 2013, there were <800,000 deaths of children of 5 yr and under (30). The key to the reduction in deaths has been the introduction of oral rehydration therapy (ORT). SGLT1 is the “heart” of the treatment of diarrhea with ORT. Currently, the formulation of the ORT solution is NaCl (45 mM), glucose (75 mM), KCl (20 mM), and trisodium citrate (dihydrate, 10 mM). Therefore, when individuals with diarrhea are administered ORT solution, the ingested glucose and sodium enter the small intestine, and the activity of SGLT1 is stimulated, which increases the transepithelial absorption of Na\(^+\), Cl\(^-\), and glucose, resulting in increased absorption of water by the small intestine (Fig. 1).

Individuals with diarrhea can recover in a matter of hours to days. Students are referred to an enlightening review by Schultz (24), in which he presents a concise historical perspective of ORT. Schultz (24) reminds the reader that “…ORT does not cure any disease! It simply provides an effective, safe, and inexpensive way to replace fluid lost by diarrhea and maintain normal fluid balance once losses are replenished. It staves off the lethal consequences of severe hypovolemia [low blood volume], namely circulatory collapse or shock.” It is difficult to appreciate the impact of Crane’s hypothesis has had on medical science; however, it has been quoted that “…the discovery that sodium transport and glucose transport are coupled in the small intestine, so that glucose accelerates absorption of solute and water, was potentially the most important medical advance this century [20th century]…” (17). Students interested in further information about ORT are referred to Ruxin (22) and Rao (20).

**GGM case study.** The following case study was developed from studies by Abad-Sinden and coworkers (1) and Wimberely and colleagues (32). The information provided in the case study was chosen to highlight the various symptoms that a young patient suffering from GGM would exhibit. Students are encouraged to engage in a literature search using a number of databases, including PubMed, Ovid Medline, Web of Science, and Google Scholar, to obtain research articles and reviews to aid their understanding of the symptoms of GGM and cellular transport models and physiology that explains the symptoms of GGM. This case study can be completed during the time that the everted sacs are incubating. In the authors’ experience, there is ample time for students to conduct a literature search and have small-group discussions about the GGM case during the 90-min period that the everted sacs are incubating.

Baby Jane weighed 3.4 kg (75th percentile) at birth, her blood Na\(^+\) was 142 mM, and she adapted well to breast feeding. At 1 mo of age, she was readmitted into the hospital, and her weight was only 3.2 kg (10th percentile). She had diarrhea, was very dehydrated and malnourished, and had hypernatremia (blood Na\(^+\) concentration of 166 mM) and mild glucosuria (glucose in the urine).

Tests were performed on Baby Jane, and it was demonstrated that her intestinal amino acid transport and function of Na\(^+\)-K\(^+\)-ATPase were normal. Additionally, her renal function suggested normal renal-concentrating ability. Interestingly, shifting Baby Jane from breast feeding to non- or low-carbohydrate-based liquids reduced the diarrhea within hours.
Questions related to the GGM case study. QUESTION 1. Which transporter is affected in GGM?

QUESTION 2. Baby Jane is dehydrated because she is suffering from diarrhea. Explain why malabsorption of glucose and galactose results in diarrhea?

QUESTION 3. Explain how her blood Na⁺ concentration was elevated?

QUESTION 4. Based on your knowledge of the mechanism of glucose reabsorption by the nephron, explain why Baby Jane has glucosuria.

Answers to questions related to the GGM case. ANSWER TO QUESTION 1. The transporter that is affected in GGM is SGLT1. This transport protein is located within the small intestine and the proximal straight tubule of the nephron (34). There are now ~30 reported mutations of SGLT1 protein (34, 35) that result in mistrafficked SGLT1, nonfunctional proteins residing in the apical membrane, reduced production of SGLT1, or early degradation of SGLT1 (34).

ANSWER TO QUESTION 2. Baby Jane is experiencing an osmotic diarrhea. An osmotic diarrhea results from the presence of an excess of nonabsorbable osmotically active solutes in the intestine. In Baby Jane’s case, the nonabsorbable solutes are glucose and galactose. Normally, these sugars would be absorbed via SGLT1. However, since SGLT1 is defective in GGM, these sugars are not absorbed and remain in the lumen, along with an osmotically equivalent volume of water. It is this water that results in Baby Jane’s diarrhea.

ANSWER TO QUESTION 3. Although SGLT1 is defective in patients suffering from GGM, there are other mechanisms present in the intestine that absorb Na⁺ from the intestinal lumen. Consequently, patients suffering from GGM are still able to absorb Na⁺, Cl⁻, and associated water from their meals; it is just the glucose, galactose, and associated water that they are unable to absorb. When this reduced water absorption is combined with continued insensitive loss of water, mainly through respiration but also sweating, plus increased urinary water loss associated with the glycosuria, Baby Jane experiences a net loss of water in excess of Na⁺ compared with the proportion normally found in the blood and extracellular fluid. The result is an increase in plasma Na⁺ concentration or hypernatremic dehydration.

ANSWER TO QUESTION 4. Reabsorption of the filtered glucose in the nephron is a two-step process. The bulk of the filtered glucose is reabsorbed in the proximal convoluted tubule by SGLT2 (34), a second isoform of the Na⁺-glucose cotransport that is not affected in GGM. However, reabsorption of the filtered glucose is completed in the proximal straight tubule, and this involves SGLT1 (34), which is affected by GGM. Therefore, for individuals with GGM, 100% of the filtered glucose is not reabsorbed by the nephron; thus, glucose remains in urine, resulting in mild glycosuria, and this glucose reabsorption involves SGLT2.

Conclusions

In summary, in the present article, we present a laboratory exercise in which students test various components of the basic cellular model of Na⁺-glucose cotransport of the small intestine using a reliable mouse intestinal everted sac preparation. Based on comments from the course evaluation, students realized that preparing an everted sac was very challenging and rewarding. The experimental protocols allowed the students to test the Na⁺ dependence of SGLT1, the inhibition of SGLT1 by phlorizin, and the effects of ouabain on overall glucose absorption by the small intestine.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.L.H. and A.G.B. conceived and designed research; K.L.H. and A.G.B. performed experiments; K.L.H. analyzed data; K.L.H. interpreted results of experiments; K.L.H. prepared figures; K.L.H. drafted manuscript; K.L.H. and A.G.B. edited and revised manuscript; K.L.H. and A.G.B. approved final version of manuscript.

REFERENCES


