A guide to Ussing chamber studies of mouse intestine

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Submitted 10 November 2008; accepted in final form 27 March 2009

The Ussing chamber provides a valuable, time-proven method for the measurement of electrolyte, nutrient, and drug transport across epithelial tissues. The method was developed over 50 years ago by the Danish biologist Hans H. Ussing as a means to understand the phenomenon of active NaCl transport. Active transport, i.e., the capacity of an epithelium to move ions or nutrients against an electrical and/or concentration gradient, had been previously demonstrated by isotopic tracer experiments. Frog skin was used by Ussing and colleagues as a model system because it had the capacity to move NaCl from the skin surface into the interstitium against more than a 100-fold concentration difference. However, a major difficulty was distinguishing the movement of ions actively transported by epithelial cells from the passive movement of ions through paracellular, i.e., intercellular, pathways. Ussing solved this problem by developing an experimental system whereby dissected frog skin separated two halves of a chamber, each of which superfused the frog skin with identical electrolyte solutions of the same volume. Thus paracellular ion movements driven by the passive forces of transepithelial concentration and osmotic and hydrostatic gradients were eliminated. The passive transepithelial driving force created by the spontaneous electrical potential across the epithelium was eliminated by clamping the potential to zero with an external current passed across the epithelium. This current, known as the short-circuit current (Isc), is equivalent to the algebraic sum of electrogenic ion movement by active transport (i.e., when using conversion with the Faraday constant). Thus, by eliminating transepithelial diffusion forces (osmotic and electrochemical gradients), the movement of ions as measured by isotopic tracers or the Isc in the Ussing chamber resulted from active transport. Observations from this system led to the Koefoed-Johnsen-Ussing two-membrane model for epithelial transport in which the apical membrane is permeable to Na+ and the basolateral membrane is permeable to K+. This paradigm served as the foundation for our present model of transepithelial transport in which primary active transport by Na+/K+ ATPase in the basolateral membrane provides the electrochemical gradient for secondary active transport by Na+ channels (as well as Na+-coupled cotransporters/antporters) at the apical membrane. For more information regarding the genius and discoveries of Hans Ussing, see the Hans Ussing Memorial issue of the Journal of Membrane Biology, issue 184, 2001. Today, the Ussing chamber method has been applied to virtually every epithelium in the animal body, including the reproductive tract, exocrine/endocrine ducts, intestine, airway, eye, and choroid plexus. Furthermore, the method has been extensively used for studies of cultured epithelial cells (primary cells and cell lines) where tight junction integrity maintains apical and basolateral membrane polarity.

Ussing chamber studies of intestinal mucosa have provided many of the key observations that moved our understanding of transepithelial transport processes toward a molecular basis. The laboratories of Peter Curran, Michael Field, and Stanley Schultz trained a generation of scientists, most notably in studies of rabbit ileum, which precisely defined the transport mechanisms that exist in many epithelial tissues. Together, with observations from model tissues (amphibian epithelia, shark rectal gland), studies of intestinal mucosa have been instrumental in elucidating the well-known processes of elec-
trogenic Cl\(^-\) secretion, electrogenic Na\(^+\)-coupled glucose absorption, and electroneutral NaCl absorption. The \(I_{sc}\) and transepithelial voltage potential (\(V_t\)) measurements in Ussing chamber studies have shown that electrogenic Cl\(^-\) secretion is not only important to normal digestive physiology but also serves as the target for enterotoxic and inflammatory-mediated secretory diarrhea. On the basis of these studies, it is now known that the process involves the activities of the Cl\(^-\) channel cystic fibrosis transmembrane conductance regulator (CFTR) at the apical membrane and the Cl\(^-\) uptake mechanisms of the Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter NKCC1 and the Cl\(^-\)/HCO\(_3\)-exchanger AE2 at the basolateral membrane (8, 20, 41, 53). Loss of CFTR in the genetic disease cystic fibrosis compromises hydration of the mucus and debris on epithelial surfaces, which leads to the disease manifestations of intestinal obstruction, pancreatic insufficiency, and failure of the pulmonary mucociliary apparatus (57). Bioelectric measurements in Ussing chambers established the process of Na\(^+\)-coupled glucose transport as the prototype for Na\(^+\)-coupled nutrient absorptive mechanisms, and the eventual identification of the molecular entity SGLT1 has resulted in the discovery of a family of hexose transporters that support viability of a variety of cell types (for review, see Ref. 59). Electroneutral NaCl transport (also known as coupled NaCl absorption) does not exhibit rheogenic properties, which was disadvantageous for \(I_{sc}\)-dependent studies in Ussing chambers and thus was first defined in perfused intestinal preparations (50). However, isotopic flux measurements in Ussing chambers led to a broader understanding of the characteristics and regulation of coupled Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)\(-\) exchangers (4, 7, 35, 42). Molecular identification of the exchanger families has led to pharmacological and knockout (KO) mouse studies that indicate that electroneutral NaCl absorption principally involves the coupling of the Na\(^+\)/H\(^+\) exchanger NHE3 (5, 12, 39) to the Cl\(^-\)/HCO\(_3\)-exchanger DRA (55). The central point to be made from these examples is that Ussing chamber studies revealed the characteristics and relative importance of transport processes in the intestinal epithelium, which were eventually identified at the molecular level. The contemporary investigative techniques of patch-clamp and fluorescence microscopy are used to define the molecular physiology of these proteins through the advantages of isolation and amplification in heterologous expression systems. However, the application of these methods to native intestinal epithelial cells is strongly limited by technical demands. As our understanding of the molecular interactions of transporters is refined, the methodology of the Ussing chamber will continue to provide a “gold standard” in the application of this knowledge to the physiological complexities of healthy and diseased intestinal mucosa.

Genetically manipulated mouse models have provided an important confluence between our molecular descriptions of transporters and their functions in native intestinal epithelium. Transgenic and gene-targeted mice are used to elucidate the activity of a transporter in both overexpression and loss-of-function (KO) experiments. Studies of transporter KO mice reveal previously unrecognized functional or regulatory properties of other transporters. For example, studies of the CFTR KO mouse intestine demonstrated \(I_{sc}\) changes indicative of cAMP regulation of Na\(^+\)-coupled glucose absorption, an effect that is normally obscured by the large \(I_{sc}\) response induced by CFTR activation (18). However, serendipitous findings such as these illustrate the need for investigations of genetically manipulated mice to ensure that functional or anatomical compensatory changes are not responsible for observational data before assigning specific roles to the targeted transporter. The CFTR KO mouse was developed in 1992 and was among the first genetically modified models of a major human genetic disease, i.e., cystic fibrosis (48). To this day, the CFTR KO mouse continues to be a valuable reagent for the investigations of cystic fibrosis and a variety of other diseases that invoke alterations in CFTR function. However, when the CFTR KO mouse was developed in 1992, there were less than 20 publications identified by MEDLINE search describing the normal transport physiology of the murine gastrointestinal tract. Despite the fact that the mouse intestinal tract demonstrates many similarities in function to more traditional models of intestinal physiology such as rat and rabbit, the specificity of molecular manipulations requires more exacting descriptions of comparative intestinal function and protein (e.g., transporter) physiology/pharmacology in the mouse. Since resources for these types of investigations are limited, the information will have to be gleaned from the control studies of various research efforts.

The importance of understanding the normal intestinal physiology of the mouse may gain wider acceptance with increasing efforts to generate “humanized” murine models in which human genes are inserted against the background of a mouse homolog KO (62). Although conceptual and technical difficulties of humanized mice are foreseen, these models provide an alternative approach to understanding physiological function that is not possible with studies in heterologous expression systems. On the basis of these considerations, the intersection of molecular and integrative physiology in these genetically manipulated mouse models will likely require the application of the Ussing chamber method. Although the method is sometimes criticized for its “black box” limitations (27), the negative is far outweighed by the instances where scientific deductions resulting from Ussing chamber studies have provided an accurate paradigm for discoveries of a molecular nature. Thus the Ussing chamber will continue to provide a useful method for integrating our knowledge of transporter molecules into the complex physiological functions of an intestinal epithelium.

**Description of the Method**

All animal experiments and protocols used to demonstrate the Ussing chamber method were approved by the University of Missouri Animal Care and Use Committee.

The basic design of a classical Ussing chamber is illustrated in Fig. 1. Although the design is specific for the classic technique, the basic principles described herein also apply to new equipment designs. The intestinal section is opened and orientated as a flat sheet to separate the two halves of the chamber. In Fig. 1, A and B, the intestinal preparation (red arrow) is situated vertically such that the mucosal membrane (also referred to as the apical or luminal-side membrane) is facing one chamber half, whereas the serosal membrane (also referred to as the basolateral, nutrient, or blood-side membrane) is facing the other half-chamber, thus separating the solutions that independently bathe each chamber half. The reservoirs above each chamber are water jacketed to enable warming the superfusate to body temperature for mammalian intestine. Typically, a CO\(_2\)/HCO\(_3\)-buffered Ringer solution...
like Krebs bicarbonate Ringer (KBR) is used as the superfusate (see Standard protocol and reagents). Oxygen and carbon dioxide tension are maintained in a physiological buffer by injection ports positioned such that they also provide gas lift circulation in the tubing that leads from each chamber half. For mammalian physiological buffers, the gas is a mixture of 95% O₂ and 5% CO₂ (also known as carbogen), which oxygenates the solutions to a fairly high level of PO₂ (>400 mmHg) that is necessary to overcome the lack of hemoglobin delivery by arterial blood supply. Carbogen also provides a PCO₂ approximately equivalent to venous blood, which maintains the HCO₃⁻ buffer at the physiological pH 7.4. The gas lift circulation is typically set at rates that reduce the unstirred layer at either side of the mucosa without damaging the tissue. A rule of thumb is to set the gas lift so that the individual bubbles of gas are just barely discernible to the naked eye. Needle valves (cat. no. 06393-70, Cole-Parmer) situated in the gas line before entering the chamber port are very helpful in minimizing variability in gas lift rates. In this type of Ussing chamber design, it is important to level the two reservoirs so that the hydrostatic pressure on each side is identical. Ballooning of the mucosa into one chamber during an experiment indicates an imbalance in either solution height or rates of gas lift. Volumes of superfusate that are 5 ml or less are used for mouse intestine because of limitations in the surface area and tissue strength of the intestinal preparation.

Under the above conditions, the intestinal mucosa or epithelial monolayer will exhibit a spontaneous transepithelial voltage potential (Vᵢ), which for mouse intestine is typically in the range of 1–20 mV. By current convention, the serosal bath serves as ground, and normal murine intestine will typically have a negative Vᵢ (e.g., −2 mV). The Vᵢ is measured by a potentiometer and electrodes (typically calomel half-cells or Ag-AgCl) that are connected by salt bridges to each chamber half. Inside the chamber, the bridge ends are located in close proximity to the mucosa on the mucosal and serosal sides of the intestinal sheet. Salt bridges are used both for convenience and to prevent exposure of the epithelium to the toxic effects of Ag from Ag-AgCl electrodes. The salt bridges for the Vᵢ-sensing electrodes typically consist of 3% agar melted in 3 M KCl solution that is congealed in the tubing. With the exceptions of protons and hydroxyl ions, K⁺ and Cl⁻ are the major physiological electrolytes with high and nearly equivalent electric mobilities in aqueous solution, which, in an agar bridge, provides an electrical conduit with low resistance (19, 32). If 3 M KCl salt bridges are used, the diameter of the bridge can be small (e.g., 0.4–1.2 mm), which minimizes KCl contamination of the bathing physiological buffers. As shown in Fig. 1, B and C, a Vᵢ-measuring circuit is established from the potentiometer of the voltage clamp to a calomel electrode (in 3 M KCl) connected to the chamber via a 3 M KCl salt bridge across the mucosa in KBR to a second 3 M KCl salt bridge and calomel electrode connected to the potentiometer. Another circuit is used to pass current (i.e., the Iₑ) across the epithelium for the purposes of clamping the spontaneous Vᵢ to zero. The Iₑ-passing electrode can also be used to briefly clamp the intestinal preparation to a defined voltage for the purpose of measuring the transepithelial conductance Gₑ or its reciprocal, the transepithelial resistance Rₑ (see below, Standard protocol and reagents). The magnitude of the Iₑ necessary for voltage clamping is determined from Vᵢ and the series resistance of the circuit plus mucosa, and it is applied continuously during an experiment by an automatic voltage clamp (see Equipment). To minimize circuit resistance, the Iₑ is typically passed from paired Ag-AgCl electrodes on each side of the mucosa to the chamber by salt bridges of larger diameter (3 mm ID). If larger salt bridges are used, they should be composed of 3% agar melted in the same physiological buffer (e.g., KBR) used for bathing the mucosa to avoid alterations in ionic composition of the superfusate. The Iₑ-passing electrode salt bridges are
situated externally to the $V_I$-sensing bridges. As shown in Fig. 1, $B$ and $C$, an $I_{sc}$-passing circuit is established from the voltage clamp to a Ag-AgCl electrode in KBR connected to the chamber via a KBR salt bridge across the mucosa in KBR to a second KBR salt bridge and Ag-AgCl electrode connected to the automatic voltage clamp. Any complete interruption in these circuits will be immediately obvious, resulting in loss of $V_I$ measurement or the ability to voltage clamp the preparation. However, more subtle effects can result from poor connections in the circuit, which can be due to small bubbles on the salt bridge ends, broken agar in the salt bridges, slippage of the agar within the salt bridges (the gravity-dependent end of the bridge should contain a narrowing to avoid slippage), deteriorating solder between the Ag-AgCl electrode and the conduit wire, and poor connections at the head stage of the voltage clamp.

The application of the Ussing chamber technique to mouse intestine required miniaturization of the chamber dimensions. To reduce circuit resistance, the optimal geometry of the superfusion compartment of the half-chamber is a cone with the apex at the $I_{sc}$ bridge expanding to the tissue aperture. However, a cylindrical bore with a diameter equivalent to the tissue aperture suffices for smaller chambers used with mouse intestine. As shown in Fig. 2, one technique is to affix (or “mount”) the mouse intestine across the aperture of the half-chamber by impaling the edges of the intestinal mucosa on a circle of pins outside the chamber aperture (see PREPARATION OF INTESTINAL MUCOSA below in Standard protocol and reagents). The pins enter opposing blunt end holes when the two half-chambers are assembled and secured by the peripheral force of thumbwheel-driven screws at the end of each half-chamber (see white arrow, Fig. 1). We have found that the optimal pin circle diameter and aperture diameter for small intestinal sections from adult mice to be 8 mm and 5.5 mm, respectively. This arrangement minimizes stretching/tearing at the pins and “edge damage” of the mucosal preparation (see Troubleshooting). We also employ Parafilm that is cut to form gaskets about the aperture to minimize mucosal edge damage in our acrylic chambers. The gaskets are held in place by modest application of stopcock grease. The chambers in our laboratory have a circular aperture suffices for smaller chambers used with mouse intestine. As shown in Fig. 2, one technique is to affix (or “mount”) the mouse intestine across the aperture of the half-chamber by impaling the edges of the intestinal mucosa on a circle of pins outside the chamber aperture (see PREPARATION OF INTESTINAL MUCOSA below in Standard protocol and reagents). The pins enter opposing blunt end holes when the two half-chambers are assembled and secured by the peripheral force of thumbwheel-driven screws at the end of each half-chamber (see white arrow, Fig. 1). We have found that the optimal pin circle diameter and aperture diameter for small intestinal sections from adult mice to be 8 mm and 5.5 mm, respectively. This arrangement minimizes stretching/tearing at the pins and “edge damage” of the mucosal preparation (see Troubleshooting). We also employ Parafilm that is cut to form gaskets about the aperture to minimize mucosal edge damage in our acrylic chambers. The gaskets are held in place by modest application of stopcock grease. The chambers in our laboratory have a circular aperture, but even greater surface area can be achieved with an elliptical aperture. However, the elliptical aperture requires a longer section of unblemished intestinal mucosa, thereby increasing the possibility that Peyer’s patches in small intestinal preparations or inadvertent mucosal damage from the tissue preparation will be included in the exposed surface area. Moreover, a circular aperture reduces edge damage by minimizing the edge length to aperture area ratio.

Equipment. Several vendors carry Ussing chamber equipment and accessories. Water-jacketed reservoirs, acrylic chambers, and voltage clamps for classical Ussing chamber applications are available from Physiologic Instruments (San Diego, CA), Warner Instruments, a subsidiary of Harvard Apparatus (Hamden, CT and Holliston, MA), and World Precision Instruments (Sarasota, FL). The acrylic chambers used in our laboratory were custom designed at the University of Missouri Scientific Instrument Shop. Two useful features for the chambers are the use of guideposts for fitting the two chamber halves together and luer fittings to provide leak-free connections for the salt bridges and reservoir perfusion tubing. Custom chamber design and production is also available commercially at PlexiCraft (Iowa City, IA; formerly Jim’s Instrument Shop). Calomel electrodes can be purchased from a variety of sources. Our laboratory uses calomel half-cells manufactured by Radiometer America (www.radiometeramerica.com). Tubing for salt bridges can also be obtained from a variety of vendors and can be fitted with plastic luer for easy connections to the Ussing chamber.

Navicyte (SDR Clinical Technology, Sydney, Australia; distributed by Harvard Apparatus) and Physiologic Instruments also manufacture modular systems in which the reservoir and gas lift are made integral with the tissue chamber within the same acrylic block (see Fig. 3). Heating to body temperature is made possible by conduction from a surrounding metal block into which each chamber is secured. With the Physiologic Instruments system, the intestinal mucosa is mounted on pins in a “slider” that fits into a space between the two halves of the chamber. Multiple slider designs are available with apertures optimized for specific tissues and also for securing various cell culture preparations.

Standard protocol and reagents. SUPERFUSATE. The superfusate that bathes both sides of the intestinal preparation in a Ussing chamber experiment is typically a physiological Ringers-type solution. Although several recipes are available, our laboratory routinely uses KBR, which has the following composition (in mM): 115 NaCl, 25 NaHCO₃, 2.4 K₂HPO₄, 1.2 CaCl₂, 1.2 MgCl₂, 0.4 KH₂PO₄, at pH 7.4 when gassed with 95% O₂-5% CO₂ at 37°C. In most studies, 10 mM glucose is added to the serosal bath to provide an energy substrate and 10 mM mannitol is added to the mucosal bath to maintain osmotic balance across the mucosa. Identification of transporter activity sometimes requires symmetrical changes in ionic concentration of the KBR, which typically involves substitution with ions that are impermeable to cellular ion transport processes such as N-methyl-D-glucamine⁺ for Na⁺ and isethionate⁻ or gluconate⁻ for Cl⁻. Organic anions used for Cl⁻ substitution are known to reduce free Ca²⁺ concentration, and gluconate⁻, in particular, causes more than a 10-fold decrease in free Ca²⁺ in Ringers solutions (52). Typically, an additional 4–12 mM of a Ca²⁺ salt (calcium gluconate, CaCl₂, CaSO₄) is added to superfusate solutions containing gluconate⁻ to maintain physiological levels of free Ca²⁺. Special care must also be taken when altering the HCO₃⁻ concentration of the solution,
which is routine for some Ussing chamber studies (see pH stat in Special Uses of the Ussing Chamber). Removing or altering the HCO₃⁻ concentration requires a corresponding change in the PCO₂ to maintain pH at 7.4. For CO₂/HCO₃⁻-buffered media like KBR, these concentrations are calculated using the Henderson-Hasselbalch equation: \[ [\text{HCO}_3^-] = 10^{\text{pH} - pK \times [\text{CO}_2]] \text{, where } [\text{CO}_2] = \text{PCO}_2 \times \text{CO}_2 \text{ solubility coefficient. The } pK \text{ and CO}_2 \text{ solubility coefficient must be adjusted for ionic strength, temperature, and pH. For example, standard cell culture solutions containing other buffers (HEPES) and/or lower levels of HCO}_3^- \text{ will be acidic (pH } \approx 5.5-6.5) \text{ if gassed with 95% O}_2\text{-5% CO}_2. \text{ Hence oxygenation of these solutions often requires gassing with 100% O}_2.}

ELIMINATION OF ELECTRICAL BIAS. The standard protocol for Ussing chamber studies involves steps to eliminate bias in the electrical measurements, and this is performed during set up by operating the chamber in the absence of an intestinal preparation. Thus the Ussing chamber apparatus is assembled without mucosa, and the reservoirs and chambers are filled with superfusate. All bubbles near bridge ends or interfering with chamber circulation are removed, and any leaks in the system are secured. After the superfusate warms to 37°C, bias in the electrical measurements is eliminated by “zeroing.” First, the voltage difference between the two \( V_t \) sensing electrodes is nullified by application of an offset voltage, thus ensuring that voltage clamping to 0 mV applies only to the \( V_t \). Second, the resistance of the superfusate must be compensated so that it is not included in the determination of the \( G_t \) (or \( R_t \)). Most automatic voltage clamps include the feature of “fluid resistance compensation.” The magnitude of compensation will vary with the electrolyte content of the solution, and the distance between the \( I_{sc} \)-passing salt bridge ends. Hence the zeroing procedure will need to be repeated if a salt bridge is replaced or dislodged before the experiment. It is advisable to readjust the offset and fluid resistance compensation setting after \( \sim 10-15 \) min since electrode drift and fluid junction potentials (see Troubleshooting) require time to achieve equilibrium. After zeroing is completed, the voltage clamp circuitry is set to standby mode, and the intestinal preparation is mounted in the chamber with fresh superfusate.

INTESTINAL PREPARATION. Following euthanasia, the murine intestine is removed by sharp dissection. Care must be taken to cut but not pull the intestine from its mesenteric attachment to avoid damaging the epithelium where the arterial-neral network penetrates the intestinal musculature to the submucosa. The intestinal section is opened longitudinally along the mesenteric attachment remnant so that the antimesenteric mucosa, which is likely to undergo less excision damage, will be situated in the aperture of the chamber. The seromusculature layers (serosa, longitudinal, and circular smooth muscle) are relatively thin in the mouse intestine, so some studies use whole-thickness mouse intestine for Ussing chamber studies to preserve the intramural neuromuscular activity and avoid additional pharmacological treatments that minimize this activity. There are two important considerations when using this approach. First, the seromusculature layers present a significant diffusion barrier to experimental drugs/isotopes and to nutrients/oxygen, which reduces the viability of the intestinal preparation. An excellent investigation of the effect of seromusculature layer on the viability of rat intestine in the Ussing chamber (as determined by isotopic Na⁺ flux and glucose addition) has been provided by Binder and Rawlins (3). Second, whole-thickness intestinal preparations undergo rhythmic neuromuscular contractions that produce corresponding changes in the \( V_t \) and thus \( I_{sc} \) by physiological means.

The focus of our laboratory has been to isolate as much as possible the properties and regulation of ion transport by the epithelium. Therefore, before mounting in the Ussing chamber, we prepare the murine intestine by both pharmacological treatment and seromusculature “stripping” to minimize the influence of the intrinsic neuromuscular system. Seromusculature stripping removes the serosa (visceral peritoneum) and the longitudinal/circular muscle layers of the intestinal wall, leaving only the underlying submucosal elements, remnants of muscle, and the epithelium (see SEROMUSCULAR STRIPPING). The submucosal damage from seromuscular stripping induces phospholipase C or A2 activity, resulting in liberation of arachidonic acid and subsequent eicosanoid generation (46). In particular, prostanooid exposure has variable but well-documented effects on coupled NaCl absorption and CFTR activation attributable to stimulation of intracellular cAMP and Ca²⁺ mobilization (6). Therefore, it is useful to incubate freshly excised intestinal sections in ice-cold, gassing KBR containing 1 \( \mu \)M indomethacin for 10 min before seromuscular stripping. The incubation allows blockade of tissue cyclooxygenases,
which prevents exposure of the mucosa to prostanoids elaborated during seromuscular stripping. If cyclooxygenase activity is desirable during an Ussing chamber experiment, e.g., activation secondary to infectious agents, then prostanoid exposure during muscle stripping and treatment with cyclooxygenase inhibitors can be avoided by washing the stripped mucosal preparation in a series of baths before mounting.

**SEROMUSCULAR STRIPPING.** Seromusculature stripping can be achieved by a variety of methods such as scraping the serosal side of the intestine with the edge of a glass slide or by pulling a taut thread longitudinally between the mucosa and muscle layers. However, we have found less damage to the mucosa occurs when the seromuscular layers are bluntly dissected under a dissection stereomicroscope. As shown in Fig. 4A, the intestinal section (typically 1 cm in length) is pinned mucosal side down to a plate containing cured Sylgard (~0.5 cm thick). The intestinal section is kept cold during dissection either by prechilling the plate or covering the section with ice-cold KBR. Intestinal sections should only be handled at the tissue edges (using fine forceps). Bottom lighting of the stereomicroscope is essential to inspect the intestinal section for damage (or the presence of Peyer’s patches in small intestinal sections) and for dissection of the seromuscular layer, especially during the learning stages of the procedure. As shown in Fig. 4, B and C, the seromuscular layer is “scored” or cut with a scalpel blade, and the edge of the layer is reflected along the longitudinal axis of the intestine with the use of fine forceps. After completion of seromusculature stripping, the mucosa is typically mounted on the pins of Ussing half-chamber under the stereomicroscope (Fig. 2). Once the assembled Ussing chamber is secured by the pressure clamps, superfusion is initiated. Typically, we include tetrodotoxin (0.1 μM) in the serosal bath to eliminate residual neural activity. The effect of tetrodotoxin requires ~20 min, during which the $I_{sc}$ decreases to a steady-state level.

**SHORT-CIRCUIT CURRENT.** The $I_{sc}$ of an intestinal preparation is a summation of all ionic currents across the epithelium: $I_{sc} \approx I_{Na^+} + I_{K^+} + I_{Cl^-} + I_{HCO_3^-} - I_{K^+}$ ($I_{K^+}$ is typically an outward current). For murine small intestine, the $I_{sc}$ under these conditions is slightly negative ($\sim -30 \mu A/cm^2$), which reflects the balance of a basal $I_{Cl^-} + I_{HCO_3^-}$ secretory current primarily attributable to CFTR activity and a smaller $K^+$ secretory current.

**TRANSEPITHELIAL CONDUCTANCE.** Calculation of the $G_t$ using Ohm’s law requires measurement of the $I_{sc}$ and $V_t$ of the preparation. Determination of $G_t$ is performed at intervals either by briefly unclamping the mucosal preparation to read the spontaneous $V_t$ ($G_t = \frac{I_{sc}}{V_t}$) or by pulsing a small command voltage, e.g., 5 mV, in voltage clamp mode and recording the resulting change in the $I_{sc}$ ($G_t = \Delta I_{sc} / 5 \text{ mV}$). The latter technique is more accurate for preparations with low resistance or displaying low $V_t$, i.e., the small intestine.

The $G_t$, or its reciprocal $R_t$, is a useful measure of the integrity of the intestinal preparation, so it is important to discuss $G_t$ (and $R_t$) in relation to the paracellular pathway across the intestinal epithelium. In “leaky” epithelium like the intestine, the ionic conductance through the paracellular pathway, in contrast to the transcellular pathway, accounts for >90% of the total transepithelial ionic conductance $G_t$ (14). Hence changes in $G_t$ can signal untoward effects on the tissue integrity. Unlike tight epithelia (26), changes in the transcellular conductance (e.g., activation of a channel) are usually difficult to discern in Ussing chamber experiments of the intestine. Ion conductance through the paracellular pathway of the epithelium is limited by both the tight junctional complex and the relative apposition of the basolateral membranes of adjacent epithelial cells, which determines the volume of the surrounding aqueous column, i.e., the lateral intercellular space (LIS). In addition to $G_t$, measurements of paracellular flux are
often used to estimate intestinal permeability under a variety of conditions. Typically, hydrophilic solutes (mannitol, dextran) that do not undergo transcellular transport are included in both the serosal and luminal baths (equal concentrations), and a trace amount of labeled solute (e.g., [3H]mannitol) is introduced on one side. After an equilibration period of 20–30 min, the labeled solute will reach a steady-state rate of flux into the opposite bath, which can be sampled. Hydrophilic solutes of differing sizes are used to probe the permeability of the paracellular pathway. Although a limit to the size of the paracellular pathway in polarized intestinal cell lines has been estimated at ~9.0 Å, there is evidence from studies of small intestinal preparations indicating a second pathway for much larger probes (e.g., inulin, ~5,000 molecular wt) but at 100–1,000 times lower permeability than that for mannitol (28). In general, the paracellular flux of a smaller hydrophilic solute probe, e.g., [3H]mannitol, will change in parallel with measured changes in \( G_t \), but one should not assume that this is always the case. Subtle changes in solute permeability can occur without corresponding changes in \( G_t \) (for example, see Ref. 31).

Regulation of the molecular elements of the tight junction is presently an area of avid research interest (for reviews, see Refs. 51, 56, and 61), and it is known that many pathological conditions can induce disruption of the tight junctional complex, which is often accompanied by epithelial shedding. In an Ussing chamber experiment, an insult to tissue integrity can be measured in real time by the \( G_t \). However, under many pharmacological and physiological conditions encountered by the epithelium, rapid changes in \( G_t \) can also result from alterations in LIS volume. Significant reductions in NaCl and water absorption cause “collapse” of the LIS, i.e., loss of LIS volume and closer apposition of the lateral cell surfaces, which decreases \( G_t \) (or increases \( R_t \) ) (49). Similarly, as shown in Fig. 5, rapid induction of CFTR-mediated anion secretion in the intestine will collapse the LIS, decrease \( G_t \), and, interestingly, limit the magnitude/duration of the anion secretory response (perhaps because of temporary dehydration of the LIS volume) (15). In contrast, the absence of CFTR prevents acute collapse of the LIS and changes in \( G_t \) during cAMP treatment. Although altered \( G_t \) can signal disruption of the epithelium, it is important to recognize that \( G_t \) also reflects physiological regulation of LIS volume, and, therefore, interpretation of changes in \( G_t \) often requires supporting studies (typically histological or ultrastructural evidence).

Troubleshooting.

EDGE DAMAGE. Edge damage refers to the extrusion into the chamber compartment of a small portion of the crushed mucosa along the outer diameter of the aperture when the two half-chambers are pressure clamped together. Since the crushed mucosa is a shunt pathway between the chamber halves, the actual \( V_t \) is artificially reduced and the \( G_t \) is artificially increased (as well as the “apparent” paracellular movement of isotopes/reagents). An excellent examination of this artifact in Ussing chamber experiments was provided by Dobson and Kidder, who showed that the effect on these electrical parameters of the mucosa increased proportionally with a reduction in aperture surface area, i.e., the ratio of edge damage surface area to the exposed surface area increased with smaller apertures (11). Thus the aperture size providing the largest exposed surface area of the intestinal mucosa is optimal.

CONTAMINATION. One of the more serious but often overlooked problems in Ussing chamber studies is drug or reagent contamination. The Ussing chambers are designed for reuse and are constructed of plastic polymers. Drugs, particularly those dissolved in DMSO vehicle and, to a lesser extent, ethanol, can penetrate plastic surfaces. Although less a problem with the hard plastics, e.g., acrylics like Plexiglas used for the chamber, any soft plastic or latex tubing used to connect the reservoirs to the chamber are particularly troublesome and should be changed after each use. To minimize contamination when drugs using DMSO vehicle are used, the acrylic chambers should be soaked in soapy water (10–15 min), rinsed, and soaked briefly in a 20% DMSO aqueous solution for 5 min before a final rinsing. Acrylics should not be cleaned with alcohols or other organic solvents because damage may result. Glass reservoirs can remain attached to the support stand but should be thoroughly washed, rinsed with 70% ethanol, and rinsed with distilled water. Another source of contamination is the reuse of agar salt bridges, where the drug or isotope can penetrate ~1 cm along the length of the bridge end during a typical experiment. Thus agar salt bridges are changed after use (the solidified agar is easily ejected from tubing by application of compressed air).
The effects of drug contamination can be insidious and, unfortunately, are often recognized only after a number of experiments yield measurements of basal transport that do not match past studies. Forskolin, ionomycin, and EIPA are among the drugs that we have found to be particularly troublesome with regard to contamination. Figure 6A shows a representation of the changes in the basal and stimulated $I_{sc}$ that occurred in normal mouse intestine during a series of experiments evaluating forskolin effects. In one group (black line), the Ussing chamber system was properly cleaned as above, whereas, in the second group (red line), the soft plastic tubing between the reservoirs and Ussing chambers was not changed (only washed and rinsed between experiments). Note the elevated baseline and reduced response to forskolin treatment in the second group. Another source of contamination is sequestration of reagents in small cracks or “crazing” of the inner chamber surface of the acrylic chambers that occur with repeated use. Routine inspection of the chamber under a dissection microscope enables tracking of changes in the integrity of the chamber surface.

**Drug Response.** One of the more unanticipated aspects of Ussing chamber studies of native intestine are differences in reagent concentrations that are necessary to achieve the same effect reported for cell expression systems. For example, drug dosages to elicit transport changes in native intestine are sometimes one to three orders of magnitude greater than that reported for responses in expression systems. The reason for this difference is multifactorial. First, the native intestine has many diffusion barriers at the epithelial surfaces. On the apical membrane, both the glycocalyx at the cell surface and goblet cell secretions present a mucous barrier that limits diffusion from the bath, both by enhancing the unstirred layer effect and by binding certain compounds. Accumulation of mucus on the apical surface can often be observed macroscopically during an Ussing chamber experiment. The observable mucus layer can be removed near the end of an equilibration period (i.e., before the experiment) by applying a gentle stream of superfusate to the apical surface to dislodge the mucus followed by refreshing the apical solution. Mucolytic agents such as DL-dithiothreitol (100 μM for 5 min) can also be used, but, as a sulfhydryl reagent, control studies are necessary to ensure relevant protein function is not affected. On the basolateral membrane, the submucosa, composed of collagen and cellular elements such as fibroblasts, also presents a structural diffusion barrier that enhances the unstirred layer and by binding certain compounds. Accumulation of mucus on the basolateral surface may require a longer period of equilibration, especially if the villous epithelium is targeted. The basolateral diffusion barrier is further limiting when the seromuscular layer is left intact although this effect can be lessened by tetrodotoxin treatment (43). Figure 6C shows a representation of the difference in the $I_{sc}$ responses of seromuscular-striped and -unstripped mouse intestine to blockade of stimulated Cl$^{-}$ secretion by the diuretic bumetanide. Bumetanide acts by blocking NKCC1, the Na$^{+}$/K$^{+}$/2Cl$^{-}$ cotransporter that is located at the basolateral membrane in murine intestinal epithelium. Note that maximal blockade of NKCC1, as denoted by the decrease in the forskolin-stimulated $I_{sc}$, occurs at 5 min in the stripped preparation but is only 40% of maximal blockade at 5 min in the unstripped preparation.

Second, the ultrastructural and microscopic architecture of the intestine also contributes to the alterations in drug responses by the epithelium. The orderly epithelial cell layer of the native intestine often produces greater responses in trans-
port function (e.g., \(I_{sc}\) response to forskolin) compared with polarized cell monolayers simply because of the number of available epithelial cells within the same gross surface area. However, compared with cell monolayers, the microvilli of the brush border are tightly compacted, and the interdigitation of adjacent basolateral membranes is more complex, which slows the diffusion of compounds, especially to the extracellular domains of membrane proteins. The microscopic architecture of the crypt-villus arrangement in native intestine also can alter drug action, which is typically not encountered in studies of polarized cell monolayers. Diffusion of compounds to the apical membrane of crypt epithelial cells from the luminal solution or to the basolateral membrane of the villous epithelium from the basolateral solution is reduced relative to the opposite condition (i.e., diffusion to the basolateral membrane of the crypt epithelial cell from the basolateral solution and to the apical membrane of the villous epithelial cells from the luminal solution). If drug action elicits tachyphylaxis or receptor desensitization, then the magnitude of the response to a given dose may be artificially reduced because of slow diffusion. Stripping of the seromuscular layer lessens the effect of the microscopic architecture, apparently by widening the crypt openings and villous bases. An example of this effect came from preliminary studies evaluating the effects of uroguanylin and Escherichia coli heat-stable toxin (STa) on intestinal Cl\(^-\) and HCO\(_3\)\(^-\) secretion in mice (22). STa acts almost exclusively on apical membrane guanylate cyclase C receptors to stimulate intracellular cGMP levels and activate CFTR-dependent anion secretion, which is principally located in the crypt epithelium. Initial studies using unstripped intestinal preparations yielded inconsistent, moderate \(I_{sc}\) responses to luminal STa treatment, whereas rapid, sustained \(I_{sc}\) responses to luminal STa were elicited in seromusculature-stripped intestinal preparations (L.L. Clarke, unpublished observations).

A third factor to be considered in the alteration of drug responses is the composition of ionic buffer solutions that are typically employed in Ussing chamber studies of intestine. The presence of CO\(_2\)/HCO\(_3\)\(^-\) buffering has an important influence on drug action not only through its buffering and chaotropic properties but also through its contribution to the total buffering capacity of the epithelial cell (44). Because of the difficulties in sustaining CO\(_2\) tension in cell culture preparations (e.g., carbogen gassing), buffers such as HEPES, which do not contribute to the cell’s buffering capacity, are used in most studies of cell expression systems. Thus reagents may elicit abrupt nonphysiological changes in cell pH that go undetected when added to superfusate buffered without CO\(_2\)/HCO\(_3\)\(^-\). The effect of using CO\(_2\)/HCO\(_3\)\(^-\) buffering vs. HEPES buffering on reagent action can be demonstrated using intestinal preparations mounted in a modified Ussing chamber system for intracellular pH (pHi) measurement (see Future Uses below). For example, H\(^+\)-dipeptide transport via the transporter Pept1 is an electrogenic process that stimulates the \(I_{sc}\) in jejunal preparations (58). Figure 7A shows pHi measurements of intact murine villous epithelium during treatment with luminal application of the inert dipeptide gly-sar. At the same extracellular pH, gly-sar treatments elicit rapid acidification of the villous epithelium bathed in HEPES-buffered Ringers solution but has no effect on pHi in villous epithelium bathed in KBR solution containing CO\(_2\)/HCO\(_3\)\(^-\) buffer. The ionic strength of a physiological Ringers solution is also a factor when comparing drug effects on native intestine with those reported for cell systems. For example, previous studies have shown that physiological levels of Na\(^+\) significantly alter the dose of EIPA required for blockade of Na\(^+\)/H\(^+\) exchangers in native epithelium (parotid duct) compared with cell studies where EIPA dose-response relationships were performed in solutions with low Na\(^+\) concentration (33). Similarly, the effect of the anion transport inhibitor DIDS is more effective at blockade of the Cl\(^-\)/HCO\(_3\)\(^-\) exchanger putative anion transporter-1 when applied in buffers containing low Cl\(^-\) concen-
trations (∼5 mM) compared with physiological Ringers solutions with ∼120 mM Cl⁻ concentration (16, 44, 45).

**Preparation Viability.** Upon removal from the animal, the ex vivo intestinal preparation incubated in Ringers solution has limited viability. One can reasonably expect a strongly viable mouse intestinal preparation for up to 3 h in an Ussing chamber. Viability decreases with longer time periods or during treatment with certain reagents. The magnitude of the $G_i$ (or $R_i$) is one real-time indicator of viability as tissue integrity deteriorates and can be useful in discriminating between mechanisms of action and nonspecific effects of reagents. For example, as shown in Fig. 7B, cadmium (Cd²⁺, 100 mM), which has been shown to block CLC-2 chloride channels (63), was added to the luminal bath solution to evaluate the role of CLC-2 in the residual $I_{sc}$ exhibited by CFTR KO small intestine (9). If one was monitoring only the $I_{sc}$ (dark line), application of Cd²⁺ was found to rapidly reduce the $I_{sc}$, consistent with Cl⁻ channel blockade. However, a repeating command voltage was imposed across the intestine as a means to monitor changes in the $G_i$. As shown by the increased magnitude of the resulting $I_{sc}$ deflections, Cd²⁺ also induced a rapid increase in $G_i$ indicating an untoward effect on tissue viability. Both an inability to reverse the Cd²⁺ effect by washout and the subsequent absence of an $I_{sc}$ response to luminal glucose application (activating Na⁺-coupled glucose transport) confirmed loss of preparation viability. In addition to experimental time, an important consideration is the age of the mice that are used for the intestinal preparations. Mice at 6 wk of age have achieved ∼90% of their growth potential; therefore, adult mice between 2 and 4 mo of age are used for most of our studies. After 5 mo of age, we have found significant decreases in the magnitude of ion transport processes, which includes cAMP-activated anion secretion, basal NaCl absorption, and gastric HCl secretion. Thus control and treatment mice should be matched not only for sex but also age, and preferably as littermates or siblings.

**Liquid Junction and Diffusion Potentials.** It is sometimes desirable to use solutions of different ionic composition on each side of the mucosa in an Ussing chamber experiment, e.g., to maximize the chemical gradient for an ion channel or to perform pH stat experiments in which the luminal bath is devoid of HCO₁⁻ (see pH stat). These experiments should be carefully considered since there are both technical and conceptual difficulties that are encountered that necessitate a number of assumptions. Significant imbalances in the major electrolytes (Na⁺, Cl⁻, K⁺) between the luminal and basolateral bath results in voltage measurement artifact. Under this condition (e.g., replacement of luminal Cl⁻ with an impermeant anion like gluconate⁻), the measured voltage potential (apparent $V_i$) is a combination of the true $V_i$, the bi-ionic diffusion potential created by the imbalanced ion moving through the leaky paracellular pathway ($V_p$) and the liquid junction potential ($V_{lp}$). $V_{lp}$ is the voltage potential resulting from the different mobilities of ions at the interfaces between different solutions (e.g., the junction of the 3 M KCl salt bridge on the side of ion substitution). Voltage clamping to 0 mV yields an $I_{sc}$ that is not a direct measure of active transport but includes the effect of clamping $V_p$ and $V_{lp}$, both of which may change over time as ions contaminate the second solution. Thus the $I_{sc}$ under this condition has little meaning unless efforts are made to differentiate the different voltage components (for example see Ref. 2). Some useful information from the apparent $V_i$ or $I_{sc}$ can be gleaned if acute experiments of agonist or antagonist actions are studied, e.g., activation of CFTR and measurement of the acute change in $I_{sc}$ ($\Delta I_{sc}$) in the absence of luminal Cl⁻. However, these studies require the assumption that the agonist-antagonist does not alter the permselectivity of the paracellular pathway to the imbalanced ion, or, if the experiment is internally controlled (e.g., by comparing wild-type and KO mouse intestine), that the ionic permselectivity of the paracellular pathway across the intestine of each group is identical.

Recent investigation into the molecular nature of the tight junctional complex has spawned an interest in measurements of the bi-ionic diffusion potential $V_p$, i.e., where the concentration of either the cation or anion of a salt is the same on each side of the mucosa, but the concentration of the counter ion on one side is different because of substitution (e.g., isethionate⁻ for Cl⁻). These experiments can be used to estimate changes in permselectivity of the paracellular pathway, which is largely determined by a family of cell adhesion proteins known as claudins (28). Rows of claudins on the surface of each epithelial cell meet to form the strands of the tight junctional barrier. The expression of different members of the claudin family largely determines the size, charge, and conductance properties of the pathway through the tight junctions of epithelia. Furthermore, it is known that naturally occurring mutations in claudins can result in disease, e.g., mutations in claudin-16, which is located in tight junctions of the thick ascending limb of the loop of Henle, result in urinary magnesium loss and hypomagnesia (28). Experiments to determine $V_p$ are best performed when the superfusate contains only one anion and one cation of equal charge (e.g., NaCl). The change in $V_i$ ($\Delta V_i$) measured immediately after imposition of a transepithelial concentration gradient can be used to estimate the junctional permeability ($P$) of the cation relative to the anion, or vice versa. For NaCl, the apparent junctional selectivity ($A$) for Na⁺ relative to Cl⁻ (i.e., $P_{Na⁺}:P_{Cl⁻}$) is described by the equation $\Delta V_i = A \ln (c_2/c_1)$, where $c$ is equal to the Na⁺ concentration before ($c_1$) and after ($c_2$) imposing the Na⁺ concentration gradient and $A$ is equal to $(RT/F)(P_{Na⁺} - P_{Cl⁻})/(P_{Na⁺} + P_{Cl⁻})$, where $R$ is the gas constant, $T$ is the absolute temperature (°K), and $F$ is the Faraday constant (38). For quantitative measurements of $V_p$, efforts to minimize or estimate the true $V_i$ may be necessary, which often requires transporter blockade or transporter KO mice. Additionally, correction must be made for liquid junction potentials. The magnitude of $V_{lp}$ typically falls between 2 and 12 mV for most physiological solutions (32). For Ussing chamber experiments, the $V_{lp}$ can be estimated by placing a short 3 M KCl salt bridge between the half-chambers at the solution interface (instead of the mucosa). During measurement of $V_i$, a switch is made from identical solutions on each side to the test solution on one side of the central bridge. The $\Delta V_i$ is an empirical estimate of the $V_{lp}$ and can be compensated in subsequent experiments by appropriating altering the offset voltage function of the voltage clamp. Early but elegant examples of experiments measuring bi-ionic diffusion potentials across epithelia are given in Refs. 34 and 37.

**Physical Imbalances.** As discussed previously, the $G_i$ of an intestinal preparation is largely a measure of paracellular conductance, which is limited by both the tight junctional complex and the relative volume of the lateral intercellular space LIS. The intestinal epithelial LIS is altered by physical imbalances
that demonstrate “sideness” with regard to the effect on $G_t$ (15). Hyperosmotic conditions not only induce cell shrinkage but have different effects on $G_t$ depending on whether the mucosal or serosal side of the epithelial preparation is exposed to a hypertonic solution (47). As shown by the experiment on murine intestine depicted in Fig. 8A, hypertonicity of the luminal bath abruptly collapses the LIS and decreases $G_t$, whereas hypertonicity of the basolateral bath distends the LIS and moderately increases $G_t$ (electron micrograph of changes in LIS morphology for the latter effect is shown in Ref. 15). Thus a fortunate “valve” effect is created in which extracellular fluid loss via the paracellular pathway is minimized during luminal hypertonicity of the intestine, whereas gain of extracellular fluid can result from basolateral hypertonicity, i.e., dehydration. Small hydrostatic pressure imbalances also affect $G_t$. As shown in Fig. 8B, a hydrostatic pressure difference of $<3$ mmHg on the basolateral side of the murine intestinal mucosa increases $G_t$. This latter effect is consistent with alterations in submucosal Starling forces that accompany conditions resulting in filtration secretion diarrhea. Thus any departure from the balanced physical conditions of an Ussing chamber experiment requires consideration of LIS dynamics in the interpretation of data, especially with regard to studies addressing the barrier function of the intestine.

**Special Uses of the Ussing Chamber**

**Isotopic flux measurements.** One of the most useful aspects of the Ussing chamber is the capability for isotopic or fluorescent tracer flux measurements. Flux studies measure the steady-state rate of transfer of an electrolyte or other substrate across the epithelium from the luminal bath to the basolateral bath or vice versa. These measurements provide an undeniable standard that serves as the starting point for investigation into the mechanism of transfer or its alterations by pharmacological or pathological action. Most studies employ isotopic tracers due to the desirable properties of specificity and sensitivity of measurement. However, the safe handling and clean up of radioactive materials adds a laborious aspect to their use. Present interests focused on intestinal permeability in disease have resulted in an increasing number of studies that use fluorescent tracers often of specific size or composition to probe the permeability characteristics of the paracellular pathway (16, 23).

Isotopic flux measurements of murine intestinal mucosa involve the basic set up and principles of the Ussing chamber as described above. Bidirectional flux studies measure isotopic flux from the mucosal bath to the serosal bath ($J_{ms}$) and vice versa ($J_{sm}$), which enables the calculation of net flux using the equation: $J_{ms} - J_{sm} = J_{net}$. Thus net absorption of an isotope is indicated by $+J_{net}$, whereas net isotope secretion is indicated by $-J_{net}$. Since different isotopes of the same ion or substrate are often not available, bidirectional flux studies involve measuring the unidirectional flux (i.e., $J_{ms}$ or $J_{sm}$) on different intestinal preparations from the same mouse. Since the $G_t$ of intestinal preparations is >90% paracellular conductance, significant bias in the determination of $J_{net}$ occurs if one of the unidirectional measurements is performed on a preparation with a significantly greater $G_t$ compared with the opposite unidirectional measurement. Thus a critical aspect is that the mucosal preparations must be paired on the basis of similar $G_t$. For large intestine, 25% difference in $G_t$ as a paired preparation is acceptable, whereas for the leakier small intestine a 15% difference in $G_t$ is used for pairing. However, small intestine studies are advantageous in that a greater number of preparations can be studied from the same mouse (e.g., 3 $J_{ms}$ and 3 $J_{sm}$ preparations), which increases the likelihood of obtaining at least one pair from each mouse.

The sequence of a typical isotopic flux experiment in which $^{22}$Na and $^{36}$Cl tracers are used is shown in Fig. 9. The sequence begins after seromuscular-striped mucosal preparations are mounted in the Ussing chambers for ~20 min to allow TTX blockade of neural function (if desired). First, $G_t$ is measured and each preparation is assigned either $J_{ms}$ or $J_{sm}$ direction in an alternating pattern from the preparation with lowest $G_t$ to the one with highest $G_t$. Since the $G_t$ of a preparation may change during an experiment (especially if two flux periods with an intervening treatment are performed), assignment of this pattern yields more successful pairs by the end of the experiment. Second, before isotope addition, a sample is taken from the “sink” side...
(e.g., the serosal bath in a Jms measurement) to serve as a “blank” for background radiation determination in counting and to ensure that that the chamber is not contaminated with isotope from previous use. After the blank sample, isotopes are added to the “source” side (e.g., the mucosal bath in a previous use). After the blank sample, isotopes are added to the “source” side (e.g., the mucosal bath in a previous use).

Approximately 0.5–1.0 μCi/ml provides a specific activity in the source bath of murine intestinal preparations to ensure that initial samples taken from the sink side will have sufficient counts/min (cpm) to be at least 10× above background radiation. The source bath should contain “cold” ion or substrate to “source” side (e.g., the mucosal bath in a previous use). After the blank sample, isotopes are added to the “source” side (e.g., the mucosal bath in a previous use).

To determine the length of the equilibration period for the flux measurement, we use for transepithelial flux calculation was described by Schultz and Zalusky (40). The unidirectional flux across each preparation in μeq/cm² of intestinal surface area·h is calculated from the equation: 

\[ J_{\text{ms}} = V \times (S_2 - S_1 \times \text{dil})/ \text{specific activity} \times \text{surface area} \times \text{time}, \]

where V is the volume of sink superfusate (in ml); S1 and S2 are the dpm/ml of samples taken at the beginning and end of the flux period, respectively; dil is the dilution of the sink superfusate resulting from sample fluid replacement; specific activity is the specific activity of the isotope in the source bath (dpm/μeq); surface area is the area of exposed mucosa, i.e., aperture area in cm²; and time is the length of the flux period in hours. During a flux period, Isc and Gi are recorded and each averaged. The mean Gi is used to make the final pairing of the unidirectional fluxes after an experiment where all preparations are from the same mouse. When an electrolytic ion transport process is the subject of study, the magnitude of mean Isc can be compared with the rate of ion flux by converting current to flux units. The movement of an equivalent of univalent ions (eq) is approximately equal to the flow of one faraday of electrons (95,485 electrons).

Thus Isc = μA/cm² = μA (μC/s) × 1/cm² × 1/F (μC/μeq) × 3,600 s/h = μeq (μmol/cm²·h), which simplifies to Isc = μA/cm² × 0.0373 = μeq (μmol/cm²·h).

If 22Na and 36Cl tracers are used simultaneously in a flux study, then it is necessary to correct the dpm of β-emitting 36Cl for 22Na spillover (FNa spillover, i.e., fraction of 22Na spillover). The 22Na spillover results from γ-radiation from 22Na that activates the scintillation cocktail of the sample and, therefore, must be subtracted from the β cpm of the sample to achieve an accurate measurement of 36Cl. The scintillation spectrum of 22Na spillover overlaps the scintillation spectrum for β emission by 36Cl. The FNa spillover can be minimized by reducing the amount of 22Na used in the study and by focusing the acquisition window settings of the liquid scintillation counter for the 36Cl β emission spectrum. However, the residual FNa spillover still introduces a significant error in the 36Cl measurement. Correction requires determination of FNa spillover by measuring both the β and γ dpm of a 22Na standard, i.e., a known quantity of 22Na, in liquid scintillation cocktail. The FNa spillover is calculated by dividing the β dpm by the γ dpm of the 22Na standard. All flux samples (plus a 36Cl standard for the liquid scintillation counter) are measured for γ and β dpm. The γ dpm of each

 periods for flux measurements are more accurate (36), so we typically use a 30-min period. To avoid hydrostatic pressure differences across the mucosa during a flux period, samples taken from the sink side are replaced with identical volumes of bath medium but require that the flux calculation be corrected for dilution of the end sample. Alternatively, samples from both the source and sink sides can be taken and not replaced, provided that sufficient superfusate is maintained and the flux calculation is corrected with each sample. Since flux experiments often require 2–3 h, it is necessary to place a glass or plastic condenser over the reservoir to minimize evaporative water loss. Depending on the treatment and the viability of the preparation, time control experiments are often necessary to ensure accurate accounting of treatment effects.

The final step is calculation of isotopic flux from the sample measurements. The method we use for transepithelial flux calculation was described by Schultz and Zalusky (40). The unidirectional flux across each preparation in μeq/cm² of intestinal surface area·h is calculated from the equation: 

\[ J_{\text{ms}} = V \times (S_2 - S_1 \times \text{dil})/ \text{specific activity} \times \text{surface area} \times \text{time}, \]

where V is the volume of sink superfusate (in ml); S1 and S2 are the dpm/ml of samples taken at the beginning and end of the flux period, respectively; dil is the dilution of the sink superfusate resulting from sample fluid replacement; specific activity is the specific activity of the isotope in the source bath (dpm/μeq); surface area is the area of exposed mucosa, i.e., aperture area in cm²; and time is the length of the flux period in hours. During a flux period, Isc and Gi are recorded and each averaged. The mean Gi is used to make the final pairing of the unidirectional fluxes after an experiment where all preparations are from the same mouse. When an electrolytic ion transport process is the subject of study, the magnitude of mean Isc can be compared with the rate of ion flux by converting current to flux units. The movement of an equivalent of univalent ions (eq) is approximately equal to the flow of one faraday of electrons (95,485 electrons).

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Fig. 9. Flow chart for performing 22Na/36Cl flux studies of murine intestine.
sample is used not only for the determination of the $^{22}\text{Na}$ activity but is also multiplied by the $I_{\text{Naspill}}$, and this quantity is subtracted from the $\beta$ dpm to yield actual $^{36}\text{Cl}$ activity. With conditions optimized for our scintillation counter and technique, we find that the $I_{\text{Naspill}}$ is $\sim 0.15$. A comparison of this method with other methods for simultaneous measurement of $^{22}\text{Na}$ and $^{36}\text{Cl}$ in aqueous samples has been provided by Rangachari and McWade (36).

$pH$ stat. The pH stat technique is used to measure the flux of $\text{H}^+$ (acid) or $\text{HCO}_3^-$ (base) across the intestinal mucosa in the Ussing chamber. Typically, the practice of this method involves the removal of buffers ($\text{HCO}_3^-$, $\text{K}_2\text{HPO}_4$, and $\text{KH}_2\text{PO}_4$ in KBR) from either the mucosal or serosal bath, but one should be mindful that this violates one of the basic principles of the Ussing chamber, i.e., balanced solution composition across the mucosa. The reason for using a buffer-free solution (e.g., saline) is that the elaboration of $\text{H}^+$ or $\text{HCO}_3^-$ into the bath medium on the side of interest must be accurately measured to achieve continuous titration at a certain pH value, i.e., clamping pH typically at pH 7.0 or 7.4. This deviation from the classical Ussing chamber method requires several considerations that can be illustrated by a description of the pH stat technique used to measure the secretory flux of $\text{HCO}_3^-$ ($J_{\text{sm} \text{HCO}_3^-}$) across murine small intestine. In the measurement of $J_{\text{sm} \text{HCO}_3^-}$, the removal of $\text{HCO}_3^-$ from the mucosal bath establishes a concentration gradient (25 mM) since the serosal bath typically contains standard KBR plus glucose gassed with 95% $\text{O}_2$-5% $\text{CO}_2$, pH 7.4. Fortunately the permeability of the paracellular pathway to $\text{HCO}_3^-$ is not significantly affected by moderate differences in the $G_o$, such as duodenal preparations (10 mS/cm$^2$) (53). An important factor in these measurements is the requirement to constantly displace $\text{CO}_2$ solubility ~1.1 g/kg water, 37°C) from the unbuffered solution with a less soluble gas to minimize the pH effects from spontaneous hydration of $\text{CO}_2$. Thus the luminal solution is vigorously gassed with 100% $\text{O}_2$ solubility ~0.03 g/kg water, 37°C) or 80% $\text{N}_2$ solubility ~0.01 g/kg water, 37°C)-20% $\text{O}_2$ mixture (simulating air) to minimize the partial pressure of $\text{CO}_2$ in solution. If measurements of $\text{HCO}_3^-$ secretion in the absence of transepithelial gradients of $\text{CO}_2$/HCO$_3^-$ are desired, the reader is referred to an in vitro method developed by Feldman et al. (13).

The flux of acid or base equivalents is estimated by the rate of base or acid addition, respectively. For example, $J_{\text{sm} \text{HCO}_3^-}$ into the unbuffered luminal bath is determined by number of acid equivalents of titrant (e.g., 5 mM HCl) necessary to maintain the pH at 7.4 during a timed flux period. Clamping to a predetermined pH value can be accomplished using an automatic titration burette system (e.g., Radiometer America PHM290 pH stat controller/ ABU900 autoburette) or manually with a pipette. An important consideration is that pH measurement of real-time changes in acid or base secretion is delayed by the time necessary to distribute the secreted equivalents or the titrant additions throughout the volume of the chamber. As shown in Fig. 10A, even automated titration results in a series of pH excursions about the target pH attributable to mixing the necessary to distribute secreted $\text{HCO}_3^-$ or added titrant. Hence single time points (e.g., “peak” secretion) may misrepresent the data since pH stat algorithms cannot accurately predict titrant addition during rapid changes in acid-base secretion, and calculation of the flux rates from short time periods further amplifies the magnitude of this error. pH measurement also lags behind the instantaneous measurements of the $I_{\text{sc}}$, which can result in an artificial incongruity between acute $I_{\text{sc}}$ and $J_{\text{sm} \text{HCO}_3^-}$ responses. An example of incongruity between the time course of the $I_{\text{sc}}$ and electrogenic $J_{\text{sm} \text{HCO}_3^-}$ during an acute response of murine duodena to cAMP stimulation is shown in Fig. 10B. Therefore, measurement of acid-base flux should be performed during a steady-state flux period. We typically perform 30-min basal flux periods, allowing 15–20 min for agonist-antagonist responses to attain a steady-state before a second 30 min flux period. However, multiple flux periods for pH stat of native intestine are often problematic because of the accumulation of mucus at the mucosal surface that affects the action of some drugs. In these instances, accumulated mucus on the mucosal surface is removed before a flux period, allowing enough time for the rate to return to steady state.
The unbuffered solutions used in pH stat experiments are typically saline or saline-type solutions that are similar in composition to KBR. The replacement of NaHCO₃ in these solutions is a troublesome aspect in that the ideal replacement anion should not demonstrate appreciable buffering properties nor alter transport function. Unfortunately, these characteristics are not demonstrated by most physiologically nontoxic Na⁺ salts. One method typically used is the replacement of NaHCO₃ and K₂HPO₄/KH₂PO₄ with NaCl and KCl, respectively. Although this establishes a mucosal-serosal Cl⁻ concentration gradient (opposite the HCO₃⁻ gradient), the magnitude of the Cl⁻ gradient is unlikely to significantly affect Cl⁻ transport. Another alternative is to replace NaHCO₃ with a poorly permeable anion such as isethionate⁻ or gluconate⁻. However, gluconate has a low pKa (3.86), resulting in significant buffering capacity at pH 7.0 or 7.4. In contrast, Na⁺ isethionate is an alkali salt, which, at this concentration, yields a pH of ~8 and therefore exhibits much less buffering capacity at pH 7.4. The use of an essentially unbuffered solution in the pH stat method requires two additional considerations. First, rapid titration to the target pH may result in transient exposure of the epithelium to an adverse pH, which may elicit a physiological response. In pH stat studies of the duodenum, we have found that titration with NaOH to rapidly attain pH 7.4 will result in a prolonged cessation of HCO₃⁻ secretion. Since most preparations seem to initially acidify the mucosal bath (likely due to PCO₂ from the dissection bath that has penetrated the mucus layer), we allow HCO₃⁻ secretion of the preparation sufficient time to alkalize the medium to pH 7.4 before a flux period, which improves reproducibility. Second, the addition of agonists-antagonists, even in small quantities, to the unbuffered mucosal bath may have drastic effects on the bath pH; if possible, the drug solution should be neutralized before application.

**Electrical field stimulation.** One of the more novel uses of the Ussing chamber technique has been electrical field stimulation, a technique largely pioneered by Helen J. Cooke and colleagues, which is used to investigate regulation of ion transport by intramural neurons. The method involves the placement of opposing foil electrodes on the serosal half-chamber, which enable the passage of low intensity currents in the plane of the submucosa of the intestinal preparation. Without directly altering the Iₑ, of the preparation, en masse activation of ganglionic cell bodies is possible. Stimulation using 2.5 to 4.0 mA at frequencies in the 5–10 Hz range causes maximal increases in the Iₑ (primarily attributable to anion secretion), which returns to basal levels after cessation of the stimulus. Studies of acetylcholine release by enteric neurons have shown that stimulation at low frequency (0.1 Hz) induces maximal release from the myenteric plexus, whereas high frequencies (5–10 Hz) induce maximal release from the submucosal plexus (21, 60). The technique of electrical field stimulation combined with specific receptor blockade has been instrumental in elucidating not only cholinergic regulation but also other neurotransmitters (e.g., VIP, tachykinin, histamine) involved in the regulation of intestinal ion transport. A description of the technique and an excellent review of the role that electric field stimulation has played in dissecting the various neurotransmitters controlling ion and water transport in the intestine has been provided by HJ Cooke and RA Reddix (10).

Application of this method to intestine from genetically manipulated mouse models (e.g., receptor KO) should provide new routes of discovery in understanding neural regulation of ion transport in the intestine.

**Intestinal restitution and barrier function.** The phenomenon of epithelial restitution involves the rapid dedifferentiation of columnar epithelial cells near a site of superficial injury for the purpose of covering the submucosa and reestablishing barrier function. Early work on epithelial restitution was performed using in vivo intestinal preparations, which seemed necessary given the physiological complexity of the process (25). However, the Ussing chamber provides an organ culture system that sustains viability of the intestinal preparation for hours and therefore has been adapted to studies of barrier damage and restitution. Superficial damage to intestinal epithelium has been induced both in vitro in the Ussing chamber (17) and in vivo followed by intestinal resection for Ussing chamber studies (29). There are clear advantages afforded by the Ussing chamber method including the ability to continuously monitor the Gₑ or its inverse, transepithelial electrical resistance, as barrier integrity is restituted (although caution must be applied to interpretation of this measurement; see TRANSEPITHELIAL CONDUCTANCE). Furthermore, the Ussing chamber provides the opportunities to measure changes in transport properties and manipulate the restitution process through various reagent treatments. The application of this technique to the intestine from genetically manipulated mouse models provides a powerful means of elucidating the key molecular constituents in this physiological process (29).

**Strengths and Weaknesses of the Method**

The principle strength of the Ussing chamber technique is that it provides a short-term organ culture method that enables precise measurement of electrical and transport parameters of intact, polarized intestinal epithelium. Although the technique has been in existence for over 50 years, its utility continues to be relevant in the postgenomic era. The discoveries made
possible by amplification of gene expression in heterologous systems require justification in a physiological context. The Ussing chamber method provides this transition whereby newly discovered reagents or genetic manipulations in mice can be evaluated in native intestinal epithelium.

The principle weakness of the Ussing chamber technique lies in the interpretation of a relatively small number of measurements to describe the complex physiological system of the intestinal mucosa. The intestinal mucosa contains many cell types communicating through a variety of systems, e.g., cell-to-cell contact and paracrine humoral agents, which may not be discerned or even considered during Ussing chamber measurements. The native epithelial cell expresses its full complement of ion transporters available to compensate for or otherwise alter the responses of a transporter in an ostensibly focused investigation. For example, the assignment of an ionic current to either the apical or basolateral membrane of the epithelial cell often requires extensive conceptual and experimental considerations. Another concern often raised with the Ussing chamber method relates to the limited viability and optimal function of an ex vivo intestinal preparation. Despite the number of limitations, progress in Ussing chamber studies is possible through the application of specific inhibitors, transgenic mice, and, as past successes have taught us, deductive reasoning. Many of the technical difficulties with Ussing chamber methodology have been overcome by the introduction of modular systems (for example, Fig. 3) that make the technique more widely accessible. The cautionary note here is that the positive result (e.g., Fig. 7B) or the exciting discovery may be misleading and, on closer inspection, can often be explained by well-documented phenomena. Thus experience and strict adherence to the basic principles of the Ussing chamber method are valuable in the proper application of the technique.

Future Uses

In essence, the Ussing chamber technique has provided life sciences with an important conceptual paradigm. Far beyond Ussing’s landmark discovery that the I_{Na} of frog skin is a Na\(^+\) absorptive current, our present understanding of how to handle intestinal mucosa, preserve epithelial polarity, and sustain viability in ex vivo studies can largely be traced to discoveries made using the Ussing chamber technique. This knowledge serves as the basis for designing chambers that enable the study of individual epithelial cells within the native intestinal mucosa. Although the measurement of cell membrane potential and resistances using intracellular microelectrode and modified patch-clamp techniques were early cell-based assays, the application of fluorometry and confocal microscopy are furthering the study of transport and other functions of individual epithelial cells in situ. Horizontal Ussing-type chambers allowing superfusion of both mucosal and serosal surfaces of intestinal preparations have been developed to enable imaging of cell function. For example, as illustrated by the method shown in Fig. 11, microfluorimetry of intracellular pH using cell-permeant fluorescent dyes combined with KO mice allows mapping of acid-base transporter function in epithelial cells of the lower and upper regions of individual villi (1, 45, 54). Similar studies, but employing confocal microscopy, have described the pH regions established by acid-base transporters at the surface of the apical and basolateral membranes of colonic surface epithelium (30). The application of fluorescently labeled transporter proteins can provide in situ localization to specific cell types, although limits even in the best of optical systems prevent adequate resolution of important events occurring over short distances such as trafficking of transporters between the cell surface and submembrane vesicles. Nonetheless, together with the advantages of genetically manipulated mouse models, new advances in the imaging of cell functions applied with the Ussing chamber method will further our progress in the quest to understand the integration of cellular components in performing the complex functions of intestinal epithelium.

ACKNOWLEDGMENTS

The author acknowledges helpful discussions with Nancy M. Walker (University of Missouri, Dalton Cardiovascular Research Center) and Dr. Lara R. Gawenis (Department of Physiology, University of Utah Medical Center).

GRANTS

Our studies mentioned in this review were funded by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK48816, to L. Clarke) and the Cystic Fibrosis Foundation Therapeutics (CLAR06XX0).

REFERENCES

G1166 USING CHAMBER STUDIES OF MOUSE INTESTINE


