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Laboratory Exercises

An Experimental, Hands-on Approach to Epithelial Ion Transport

A SIMPLE TECHNIQUE FOR INTRODUCING STUDENTS TO ION TRANSPORT IN EPITHELIA*

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Andrea Bagdadi, Nadia Orona, Eugenio Fernández, Anibal Altamirano, and Carlos Amorena‡

From the Centro de Estudios en Salud y Medio Ambiente (CESyMA), Escuela de Ciencia y Tecnología (ECyT), Universidad Nacional de Gral. San Martín (UNSAM), San Martín, Argentina

We have realized that our Biology undergraduate students learn biological concepts as established truths without awareness of the body of experimental evidence supporting the emerging models as usually presented in handbooks and texts in general. Therefore, we have implemented a laboratory practice in our course of Physiology and Biophysics, aimed to introduce the students in the way the scientific models and theories are built, through the measurement of Na^+ transport in frog skin. Transepithelial Na^+ transport was assessed in the frog skin, with measurements of short circuit currents. The mucosal Na^+ and serosal K^+ concentrations were modified and the effects were recorded. These effects were reversible. Addition of a drug that blocks epithelial Na^+ channels (amiloride) to the mucosal side solution abolished the short circuit current. Sodium fluxes were calculated, and the results were adjusted to Michaelis-Menten kinetics. The impact of the proposed practice on the students is discussed.

Keywords: Laboratory exercises, methods of science education research, postgraduate education, using modeling as a research tool for investigating teaching.

In this article, we describe a straightforward experiment which is regularly performed by our undergraduate students during the third course on Biology (Physiology and Biophysics). The students have an occasion to apply their theoretical knowledge and to learn the techniques used to measure ion transport in amphibian epithelia. The experiment may be appropriate also for undergraduate and graduate students of biology, medicine, and related fields. It suits particularly well for courses of Physiology and Biophysics, and it may be useful in laboratories where membrane transport mechanisms are studied. In addition, this kind of procedure is of interest in Pharmacology courses. This experiment may also be of interest in college practices, because it provides the students with a general knowledge of the membrane transport processes, may be helpful to identify specific transport systems, and among other aspects, it could be used to discuss the role of membrane potential in Na^+ transport across the epithelia, and to exemplify the kinetics of Na^+ transport.

BIOLOGICAL AND PHYSIOLOGICAL BACKGROUND

Frog skin is formed by several strata of cells: from the serosa to the mucosa, we find the basal or germinative layer, followed by a spiny stratum; then, the stratum granulosum, with several layers of cells with numerous cytosolic granules, and many intercellular unions like tight junctions, desmosomes, and gap junctions. This array of intercellular unions is what confers the frog skin its high electrical resistance (around 1000–3000 ohms), and makes it a tight epithelium [1]. Finally, a cornified stratum of cells completes the skin. Vectorial transfers of dissolved gasses and ions take place through this membrane, and usually, there is osmotic flow of water. The structural units for transport are the apical membrane, the cytosolic compartment, the basolateral membrane, and the paracellular shunt. The apical membrane and the basolateral membrane are generally bathed with extracellular media of different ionic compositions, and have different transport properties. The absorption of Na^+ by the frog skin is accomplished by the outer stratum granulosum. Its basolateral membrane contains the Na-K pump that transfers Na^+ ions from the cytosolic compartment to the interstitium, in exchange for K^+. As a result, the intracellular Na^+ activity is lowered, creating a chemical Na^+ gradient at the apical membrane, which is predominantly permeable to Na^+. The basolateral membrane has also a very large K^+ conductance, the main responsible for the basolateral membrane potential.

Amphibian skin is a well known preparation, cheap and easy to manipulate, and one that gives reproducible results. Measurements of short circuit currents in toad skin have been used extensively by a great number of laboratories [2–4]. The model of Koefoed-Johnsen and Ussing for so-

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‡To whom correspondence should be addressed. Tel.: 5411-45807296 #105. E-mail: camorena@unsam.edu.ar.

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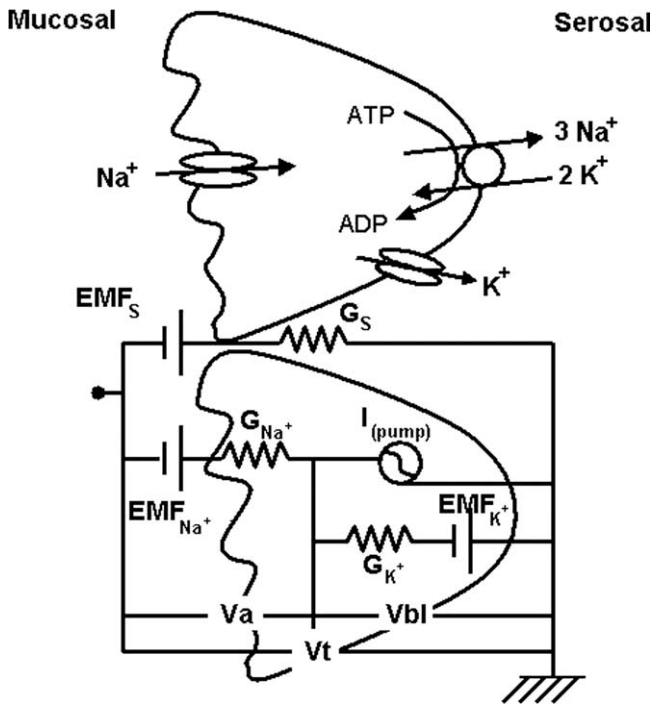

FIG. 1. **Schematic representation of the Koefoed-Johnsen and Ussing model**. Upper cell: location of the transport proteins in the different domains of the cell. In the apical membrane, the Na channel (E_{NaC}). In the basolateral membrane the Na-K pump and the K channel. Lower cell: equivalent circuit. EMF for Na^+ (chemical gradient plus membrane potential) associated to a Na^+ conductance (GNa^+) corresponding to the Na^+ channel. The same for K^+ at the basolateral membrane. In parallel with this a source of current, I, corresponding to the Na-K pump. Also a shunt conductance G_s with its EMFs is depicted on the intercellular junction.

dium uptake by frog skin can be described as follows [2]: on the mucosal side, a barrier contains a resistive pathway for Na^+. On the serosal side, the arrangement is completed with the Na-K pump, connected in series with the resistive component (Fig. 1). Sodium ions are thus transported from the mucosal to the serosal side, in turn, K^+ ions cycle through the Na-K pump, and through the K^+ channel of the basolateral membrane.

Under open circuit conditions, a small flux of anions from the mucosal to the serosal side short-circuits the sodium flux. However, for experiments, the epithelium can be short-circuited to any chosen value with a suitable amplifier. In addition, the correspondence between Na^+ flux and short circuit current has been clearly demonstrated in frog skin, toad bladder, and toad skin. This experimental approach has shown that the whole current measured under short circuit conditions is a good estimation of Na^+ flux across the epithelia when symmetrical solutions are bathing both, the apical and the basolateral sides [3]. The assumptions implicit in this model are:

1) the intracellular K^+ activity is 100 mM; 2) the intracellular Na^+ activity is 26 mM; 3) the basolateral membrane behaves like a K^+ electrode; 4) the apical membrane behaves like a Na^+ electrode.

The first two assumptions are reasonable; the activities of intracellular K^+ and Na^+ in epithelial cells have been estimated to be between 80 and 113 mM and 18–46 mM for K^+ and Na^+, respectively [5]. The last two assumptions are also reasonable because the apical membrane has a Na^+ conductance, and the basolateral membrane has a K^+ conductance.

Students must be made aware that the Koefoed-Johnsen and Ussing model imply an oversimplification, and that biological systems are never as ideal as this. However, this is a good approximation and has been a successful model for research in bioelectrical phenomena in epithelia.

METHODOLOGY

Animals

Frogs (*Rana Catesbeiana*) were purchased from a local animal supplier, and kept in a terrarium. The animals were sacrificed by double-pithing, and a section of the abdominal skin, around 1 inch in diameter, was cut and mounted in an Ussing chamber.

The treatment of the animals in the present work followed the APS's Guiding Principles in the Care and Use of Animals, and the protocol was supervised by the ethical board for the use of animal for experimentation.

Solutions

Sodium chloride, potassium chloride, calcium chloride, magnesium chloride, glucose, and HEPES were purchased from Merck (Darmstadt, Germany). Stock solutions were prepared using glass-distilled water. Amiloride was purchased from Sigma (Sigma Chemical Co, St. Louis, MO). The composition of the solutions needed for this experiment is given in Table I. The concentrations are given in millimoles per liter of solution, with the exception of Solution D which is in Moles per liter. The pH of the basolateral solution was adjusted to 7.4 at room temperature with 1 N NaOH. The osmolality was adjusted to 270 mOsm/Kg with a Wescor osmometer (model Vapro 5520).

Solution A: Ionic composition of the serosal bath, Solution B: Composition of the solution used for increase serosal potassium concentration from 5 mM to 75 mM. Solution C: Starting solution used for the apical side, and Solution D: Solution used for increasing apical sodium by steps.

When needed, amiloride was added from a 1 mM stock solution. This stock solution was prepared in glass-distilled water warmed up to 40°C to improve the low solubility of amiloride. We used a 1:100 dilution for experiments, for a final concentration of 10 μM.

Agar Bridges and Ag/AgCl Electrodes

Agar bridges were prepared dissolving agar (3% w/v) in boiling Solution A. Ag/AgCl electrodes were prepared cleaning silver wire with fine sand paper, followed by immersion in chlorine bleach overnight.

TABLE I
Composition of the solutions (in mM)[a]

Solutions	A	B	C	D
NaCl	140	–	26	2,400
KCl	5	140		
$CaCl_2$	1	1		
$MgSO_4$	1	1		
Glucose	10	10		
HEPES	10	10		

Solution A: Ionic composition of the serosal bath, Solution B: Composition of the solution used for increase serosal potassium concentration from 5mM to 75 mM. Solution C: Starting solution used for the apical side and solution D: Solution used for increasing apical sodium by steps.

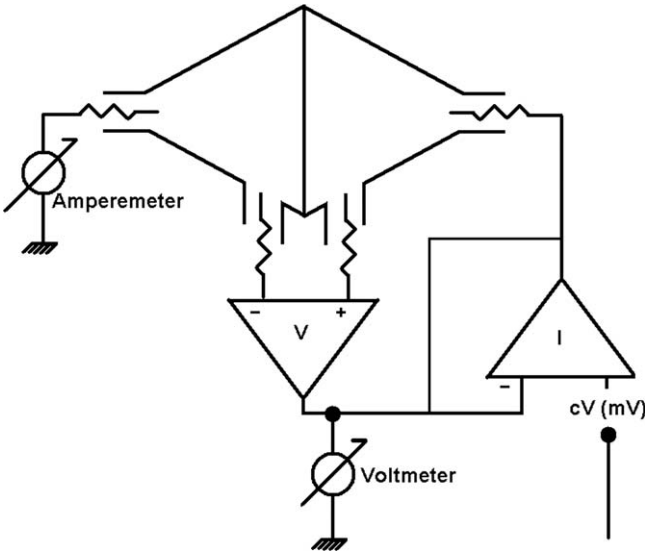

FIG. 2. **Schematic representation of the whole system, including the basic electronics**. The voltage follower (*V*) measures the transepithelial membrane potential close to the skin. The clamping amplifier I compares the measured value with the command potential c*V* and passes current trough the whole preparation, measured by the amperemeter, to make $V = cV$.

List of Elements Needed

The following equipment is required: a Ussing chamber, polyethylene tubing for agar bridges, silver wire for Ag//AgCl electrodes, latex tubing, air compressor, osmometer, voltage-current clamp amplifier: we used a WPI amplifier, model DVC-1000 (World Precision Instruments, Sarasota, Florida). Although very good devices are commercially available, reliable equipment can be built even in a modest electronic workshop [6]. For data acquisition, we used a model MP100WSW (Biopac Systems, Goleta, CA). A pen recorder can be used instead of the acquisition system.

Figure 2 shows a scheme of the equivalent circuit of the whole preparation. The transepithelial membrane potential is measured by the voltage follower with very high input impedance, *V*. The clamping amplifier I compares the measured value with the command potential c*V* and passes current trough the whole preparation, measured by the amperemeter.

Mounting the Frog Skin Into the Ussing Chamber

The experiments were performed on sections of abdominal frog skin, mounted between the two halves of the Ussing chamber. The final volume on each half-chamber was 10 mL. Each half-chamber was connected to a glass perfusion/circulation reservoir, and the skin was bathed on each side by a different solution. Four Ag/AgCl electrodes, two on each half-chamber, were connected to the amplifier through the agar bridges. Two electrodes were placed close to the epithelium to measure the transepitelial voltage, and the other two, placed away from the skin, were used to inject current. The reference electrode was placed on the serosal side. The solutions were bubbled with compressed air connected to the chamber as shown in Fig. 3. This allowed the fluid to circulate as shown in the figure, minimizing the unstirred layer. Because the perfusion-circulation reservoirs are surrounded by a water jacket, the experiments may be conducted at different temperatures if desired. The experiments described here were performed at room temperature.

Currents and Transepithelial Potential Measurements and Data Acquisition

Frog skins were first exposed to Solution A on the serosal side, and to Solution C on the mucosal side until the recorded current stabilized, usually 15 min after the start of the experiment. We then increased the sodium concentration in the mucosal side stepwise by addition of 10 μL of Solution D until a final concentration of 88.4 mM was reached. For each Na^+ addition to the apical solution, short circuit was briefly interrupted and the transepithelial membrane potential recorded. After some time, we replaced 5 mL of the Solution A with 5 mL of Solution B, reaching a final sodium concentration of 70 mM, and a potassium concentration 75 mM on the serosal side. Afterwards we replaced fresh Solution A on the serosal side. Finally amiloride, an inhibitor of Na^+ channels, was first added to the serosal side and then to the mucosal side. Transepithelial

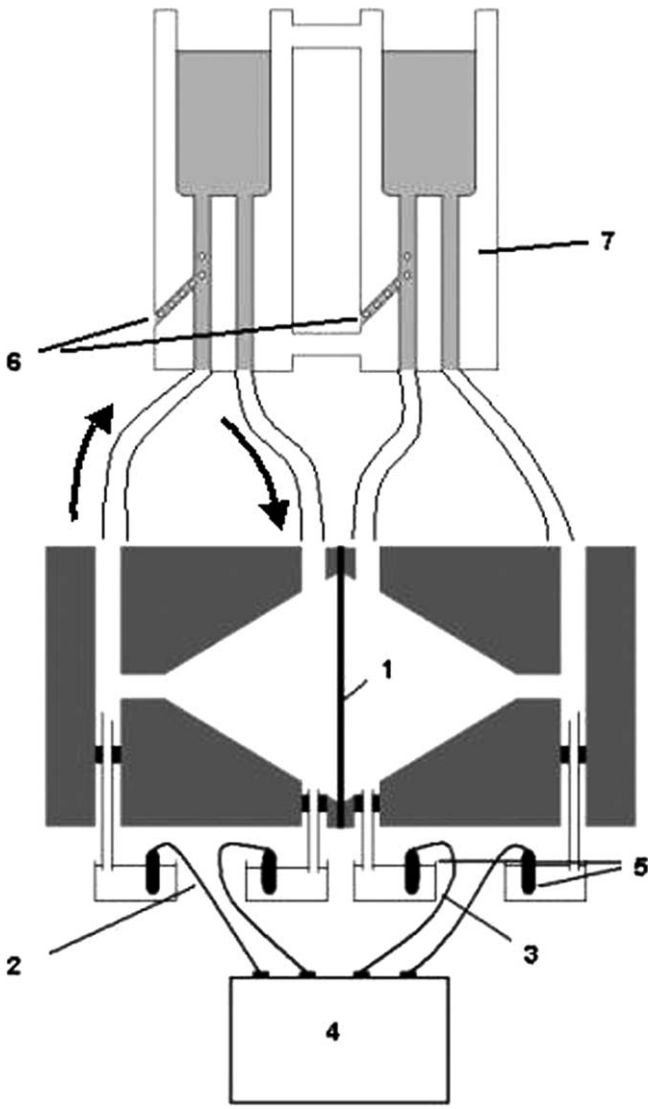

FIG. 3. **Scheme of the setup used for measuring short circuit currents in frog skin**. 1: skin separating the two halves of the Ussing chamber. Each half chamber is filled with 10 mL of solution and is connected to the short circuit clamping device (4) through agar bridges (5). $Ag/AgCl_2$ electrodes for measuring transepithelial membrane potential (3) and for injecting current (2) complete the circuit. Compressed air injected trough (6) makes the solution circulate as indicated by arrows. Finally, a water jacket allows for keeping the solution at the desired temperature.

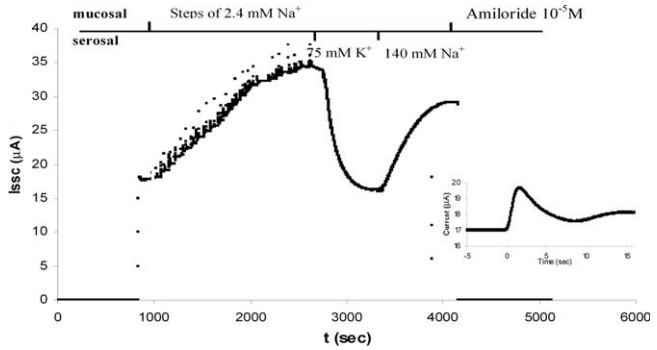

FIG. 4. **A typical experiment showing the effect of increasing the Na^+ concentration at the mucosal side on short circuit current**. The effect of increasing the basolateral K^+ concentration in the serosal side and repolarizing the basolateral membrane with normal K^+ are depicted. The effect of amiloride 10 μM on the apical side is also shown. The insert shows the time curse of short circuit currents after each apical Na^+ increment.

voltages and currents were monitored and recorded. The transepithelial membrane potential was fixed to 0 mV by the voltage follower and the resultant short circuit current was measured. On the other hand, the transepithelial membrane potential can be fixed to any other desired value.

Calculation of Na^+ Fluxes

Currents can be easily transformed to Na^+ fluxes just by dividing the current, usually microamperes, by the Faraday constant. Thus, short circuit current values were converted to fluxes according to: $J_{Na}{}^+ = I_{Na}{}^+/F$, where $I_{Na}{}^+$ is the short circuit current in Amp and F is Faraday's constant, 96,500 Coulomb/Mole. Sodium flux, $J_{Na}{}^+$, was a saturable function of the Na^+ concentration on the mucosal side. The data fitted well with to Michaelis-Menten model.

RESULTS

Figure 4 shows a typical experiment. Increasing the Na^+ concentration in the mucosal side from 26 mM to 88.4 mM in 2.4 mM steps, resulted in a progressive increase of the short circuit current. The insert shows the typical current transient after each addition of sodium to the mucosal chamber. The shape of the transient is due to several causes, one of them being the change in the properties of the Na^+ permeability in the external barrier when Na^+ is increased in the chamber. In addition, the time course of Na^+ concentration in the chamber until the mixing of the high Na^+ added (2.4 M) is complete should be considered. When the K^+ concentration on the serosal side was increased from 5 mM to 75 mM, the magnitude of the short circuit current was reduced by one half, from 34 μA to 17 μA. Then, restoration of Solution A (140 mM of Na^+) on the serosal side increased the short circuit current from 17 μA to 29 μA. At the end of the experiment, addition of 10 μM amiloride to the mucosal side solution abolished the short circuit current. Previous addition to the serosal side of the same concentration did not elicit any response on short circuit current (not shown).

Figure 5 shows a plot of the data, and the inset shows the double-reciprocal plot. The value retrieved for V_{max} was equal to 1.12 nmoles $\times$ cm^{-2} $\times$ sec^{-1}, with a K_m of 84 mM.

DISCUSSION

The simplicity of the setup needed, of the protocol, and the reliable information that can be gathered, make this model an excellent tool to introduce the students to membrane transport in the various aspects it involves: preparation of solutions, dissection, assembly of the experimental setup, collection of data, kinetical analysis, and discussion of the assumptions underlying the effects of several maneuvers that affect Na^+ transport. We have focused these maneuvers to essentially three: 1) changes in apical Na^+ concentration; 2) changes in basolateral membrane potential induced by the increase in basolateral K^+ concentration, and 3) effects of 10 μM amiloride. One additional test that could be performed consists in the addition of amiloride to the basolateral solution, to confirm that this drug has no effect on the transepithelial current (data not shown).

Applications for Teaching

This model gives the students the possibility to experiment and to make a quantitative validation of Na^+ transport in frog skin. It also allows them to interpret the kinetics of Na^+ transport as a process in which there is a strict correspondence between short circuit current and Na^+ transport, thus making possible an appreciation of the electrical nature of biological systems. In addition, the analysis of the data allows to recognize the Michaelian properties of the Na^+ transport behind many physiological and biochemical phenomena. The experiment presented here could be varied in many interesting ways, for example, using agents that affect Na^+ transport, like ouabain, norepinephrine, EGTA for chelating Ca^{2+} at the basolateral side, dopamine and vasopressin, to name just a few. Moreover, the effects of varying the pH of the apical solution can be observed. The temperature of the Ussing chamber and of the solutions reservoirs can be adjusted using a water circulator with a thermostat. A series of experiments such as the one presented here, performed at different temperatures would allow for the estimation of the activation energy of the transepithelial Na^+ absorption.

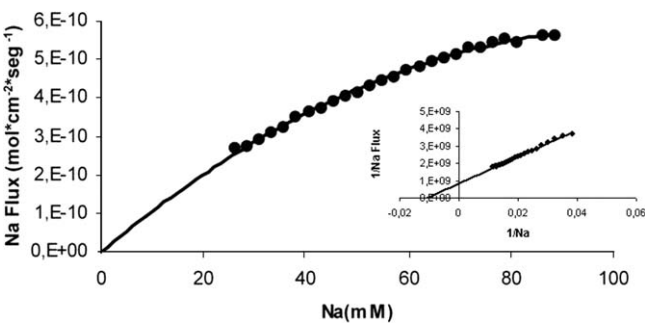

FIG. 5. **Fit of Na^+ flux as function of mucosal Na^+ concentration to a Michaelis-Menten equation**. The insert shows the double-reciprocal plot of the same data.

One of the obvious limitations of this model is its simplification. It does not contemplate other Na^+ pathways, and no attempt was made to account for other ion transport pathways. The transepithelial Cl^- transport in amphibian skin was first supposed to be largely paracellular; however, there is solid evidence that this anion moves also through transcellular pathways [7]. In addition, in some epithelial preparations a current of different sign appears after amiloride inhibition of the Na^+ current. For instance, in the skin of *Rana esculenta*, a VH^+-ATPase moves H^+ from the intracellular compartment through the apical membrane of mitochondria-rich cells [8]. Also, in some preparations chloride is electrogenically transported from the apical to the basolateral side [9]. In spite of the limitations mentioned, it must be pointed out that they do not constitute an obstacle to the use of this model for didactical purposes. Rather, it offers a subject for discussion and analysis, and constitutes a good starting point to build more complex transport models.

Discussion of the Results Obtained by the Students

After the completion of the experiment, the students are to debate their findings. The following questions provide a theoretical frame to lead the discussion:

- What happens with transepithelial Na^+ transport under open circuit conditions?
- What kind of error is introduced at low apical Na^+ concentrations? (Consider junction potentials)
- Why does the magnitude of the short circuit current decrease upon transepithelial depolarization?
- Is the assumed intracellular Na^+ concentration the correct one? (Consider other possibilities)
- Is it valid to assume that the short circuit current is carried only by Na^+?
- How do these data fit other models for epithelial transport?
- Limitations of the model.

Remarks on the teaching implications of the proposed laboratory practice

We used this experiment in a course of Physiology and Biophysics for Biotechnology students with a solid background in mathematics, physics, and chemistry. In general, students of Biology are, with extremely few exceptions, unconcerned with the approaches originated in fields of knowledge which they perceive as unrelated to Biology. It seems odd to them that biological descriptions are basically derived from tools from those other disciplines, and they reject the physical-mathematical approach, as well as electric circuit analogies, because they consider them alien to Biology. Furthermore, there is a strong tendency in Biology handbooks, especially the ones used for introductory courses, to condense the state of the knowledge in the field into cartoons that denaturalize the essence of the biological models, forgetting that they are just extreme reductionist ways to describe experimental results. The oversimplification leave students with notions too simple or fragmentary of how knowledge of biological systems is obtained. Attention to the experimental output underlying the description of the phenomena is often neglected, and as a consequence, the experimental background in which biological phenomena rely is missed. The active involvement of the students in experiments like this, where they actively participate in the gathering of the experimental data, and the subsequent analysis, allows them to become aware of the real nature of the observed phenomena and to understand that the pictures usually found in manuals are constructions that would be better if they were constructed by them after the experiment. Unfortunately, it is not always possible to extend this approach to all the courses of Biology, but we believe this are reasons that make the present experiment a valuable tool.

It is most desirable that the students have previous knowledge on electricity and also on enzyme kinetics. It would be convenient that the students had previously taken Electricity and Biological Chemistry courses. However, if this was not the case, the practice is still valid because the concept of short circuit current can be taken axiomatically and translated with small clarifications into the concept of ion flux. Furthermore, the students find interesting that they can observe the phenomena in real time, and the immediate effects of the treatment with different solutions on the resulting currents. It is not infrequent that some students propose experimental modifications for affecting short circuit currents. It would be convenient, although we have not yet implemented it, to provide the students with a written description of the exercise, supplying the theoretical and experimental background. On the other hand, there is a body of scientific literature, easily available online, that could be used for further clarification of the technique and discussed in the classroom [10]. A suitable implementation of the proposed exercise could be to perform it in two steps. In the first step, the activities would be similar to those described in the present article. In further experiments, the students could prepare the skin as described, and the instructor could introduce different experimental maneuvers affecting the measured short circuit currents in several ways. For instance, changing apical pH, adding inhibitors of the Na-K ATPase, and/or cellular metabolism, and so on. The results of these experiments could be presented as a report with the students' interpretation. This material could be used as evaluation test.

It is important for the student to incorporate the notion that a transport system is a biochemical one with its kinetics properties. Further advantages of this laboratory exercise rely on the use of electric concepts which are deeply mingled with the very nature of ion transport and with the membrane as a barrier controlling the traffic across it, and consequently the construction of an environment of negative entropy. Through the use of this experimental setup, the students should be able to translate the outcome of electric current measurements to mass transport across the skin. This fact not only, as mentioned above, allows visualizing the complete correlation between both phenomena but also will familiarize the students with electronic hardware. The use of the proposed experiment will allow the instructors to lead

students to realize that all biological knowledge relays on experimental tools and that they are just provisory models susceptible to be modified. In conclusion, we feel that this easy experiment will strongly help the students to grasp the way biology knowledge in general and physiology and biophysics in particular are constructed.

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