Chapter 3

Control of Surface Exudation by Slugs

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Introduction

Objectives: This exercise is directed at one subdivision of the analysis of water regulation in molluscs. Terrestrial pulmonates are the immediate subjects, and this exercise investigates how slugs react to excess of water. Much more work has been directed to resistance of slugs to desiccation, but this important topic suffers, in the student laboratory, by requiring excessive time for completion of significant procedures. The analysis to be described here has the great advantage of yielding easily measurable, quantitative results in the course of one laboratory period. The several aspects of regulation can be assessed by different groups, and the results compared in class discussion at the end of the laboratory period.

Level: This exercise can be directed at any one of several levels. It can illustrate neuron-effector relationships, and so may be a part of a study of neurobiology; or the emphasis can be placed on neurotransmitters and neurohoromones. Expensive chemicals can be employed, and the phyletic implications pursued; or the analysis can be kept to a very elementary (and inexpensive) level.

Time: The period should extend for at least 2 hours; better results will be obtained with a period of 3 hours, of which the last 20 minutes can be devoted to comparisons and discussion.

Preparation: The preparation of equipment is simple; each group of students should have two set-ups. For a group of 20 students working in pairs, this means preparation of 20 set-ups, which should be accomplished at least one day in advance of the class period. The instructor is advised to practice making the preparation in advance, and to save the time of the students by giving a 5 minute demonstration before students begin individual work.

Some reagents worth testing can be made up a day in advance of the exercise. Others, such as acetylcholine, should be made up just before the laboratory period.

Suitable animals include the common, introduced European species *(Limax maximus, Arion ater)* and large native American slugs (such as *Ariolimax californicus* and *Ariolimax columbianus*). *Arion* species are annuals, available in spring through fall, whereas *Limax* and *Ariolimax* may be kept throughout the year. They are collected in the field, where they are found on plants or under rocks or boards. Collection is easiest in the early morning or late evening. We keep our slugs at room temperature *(Arion)* or at 10°C *(Ariolimax, Limax)*. The latter animals are brought into the laboratory for 24 to 48 hours at room temperature before use. Slugs should be kept in cages (e.g., plastic shoe boxes) with free access to lettuce and carrots for at least a week before the exercise. However, students can also bring in freshly collected slugs in season and expect to obtain valid results in a majority of cases.

Instructors' Materials

In brief, the empty posterior end of a slug is mounted on a glass tube filled with Roach pulmonate Ringer solution (Appendix A), a stimulus is delivered, and the resulting fluid flow is measured. Each of these steps requires detailed explanation.

The body wall of the slug is made up of a complex organization of smooth muscles which can effect contraction or elongation in complex patterns, as well as coordinated waves of movement along the sole of the foot. The epidermis, a single cell layer, has characteristic bands of ciliated cells. The slug's effectors are controlled by nerves which secrete neurotransmitters also characteristic of vertebrates (e.g., acetylcholine, serotonin, noradrenaline, dopamine).

Make a cut around the body wall behind the mantle and press and draw out the viscera, both digestive and reproductive, leaving a tight sac for the experiment. (Appendix B) In working with small slugs the entire posterior part of the body is used; in large slugs the resulting sac is too large and only 2 to 4 cm of the posterior end is fastened to the tubing. Unless care is taken during dissection, the crop will be ruptured and digestive fluid will pour over the preparation. This must be washed away soon, or the preparation will prove unsatisfactory.

The tubing system can be set up to follow either flow at pressures set by contraction of the slug body, or flow at constant pressure. The first system is shown in *A*, the second in *B*, in Fig. 3.1.

Procedure

A folded paper towel provides a disposable dissection area. Just along its right border a 6-inch piece of soft cotton cord is laid, and, just to the right of the cord, the flared T-tube (already connected to the long glass tube) is placed on the table. The body wall is cut through, the viscera are withdrawn with mouse-tooth forceps, and the posterior end is picked up and pushed smoothly over the flare. The tubing is then laid down so that the cotton thread is just above the flare and the slug body is resting on a clean piece of paper towel. The cord is knotted quite tightly about the body just above the flare in order to prevent leaks. (If the cord used is too thin it may cut clear through the body wall, and the body chamber will have to be moved further up the T-tube. Linen thread will do, but care must be taken with the knots; ordinary parcel cord has been found to do nicely, and is easily available). The glass tube is mounted along the groove of the plastic rule, with the slug body not quite touching the base of the rule. The tube is fastened in a vertical position, and the long piece of vinyl tubing slipped down the glass tube. The experimenter should watch the tip of the vinyl tube as it enters the slug's body, making an effort to position the tip of the tube about two-thirds down the length of the chamber, that is,

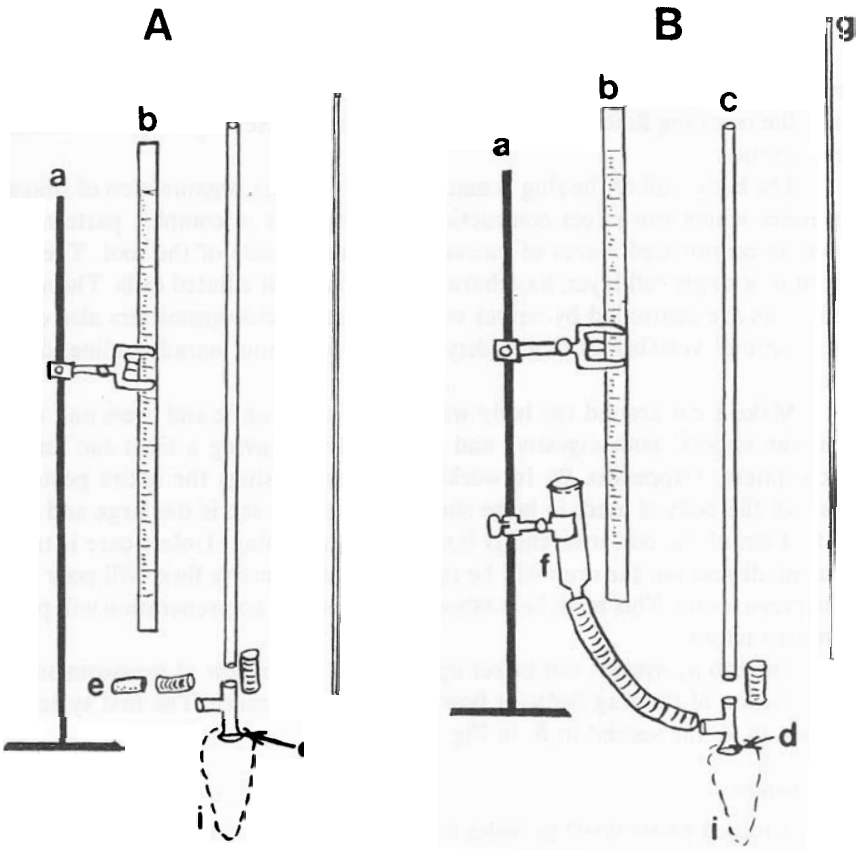

Figure 3.1.

A. Arrangement to measure changes in flow and pressure.

B. Arrangement to measure flow at constant pressure. a. ring stand; b. plastic rule; c. 5 to 8 mm glass tubing, about 30 cm long; d. glass T-tube, size chosen to fit the slug's body (we use 4 to 8 mm diameter tubing) of which one expanded end has been cut off and a small flare blown on the end in a Bunsen burner, to hold the slug body from slipping off; e. a glass plug chosen to fit the rubber tubing connecting it to the glass T-tube; f. a 50 ml plastic syringe body, adjustable with a clamp to set the system pressure to a constant level; g. pieces of polyethylene or vinyl tubing small enough to pass through all of the glass tubing, and long enough to reach from the bottom of the slug sac to extend through the top of the glass tubing, so that injected material may reach the slug body lumen directly; h. syringe needle, sharp point cut off, size 27 to 16, to fit tightly into the plastic tubing *g;* i. position of slug sac when attached to the apparatus.

almost to the bottom. Slug Ringer solution is then injected, allowing the excess to flow out through the open T-tube. The body is also washed off with slug Ringer, and at least 10 ml is forced through the system to get rid of any biologically active agents released during the dissection. The time is noted, and the preparation is allowed to rest for 30 minutes. The pressure may be set to any desired level, but we have found that a pressure of 10 cm of Ringer is optimal. During the period when this preparation rests, the second preparation may be set up according to the same procedure; in this case, the arrangement provides for constant pressure throughout the experiment (see Fig. 3.1B).

Measurements

Experimental procedures will now cause the production of drops of fluid from the slug body. The responses are often time-limited, so that the recovery period, and subsequent stimuli, are of interest. We use stop watches to measure time, and collect the drops in tared petri dish bottoms for periods of 2 to 15 minutes. At this point judgment must be made to fit the experiments to the means at hand. In 5 minutes a good preparation may put out 500 mg of fluid, with the actual amount determined by weighing the covered petri dish on an analytical balance to the nearest mg. If sensitive balances are not available, a less accurate but adequate method of measurement would be to count the drops formed per unit time (as measured by a watch with a second hand or any sort of bench top timer). Pressure measurements are recorded as well (in set-up A). In our laboratory we follow as many as 4 experiments simultaneously, and recommend that even the beginners make 2 preparations, and treat and follow them both. Even if only a single period is used for this study, one can estimate that one, at least, of the 2 preparations will yield satisfactory results.

The results of each experiment can be plotted as a block diagram, with the mg of fluid or the number of drops per unit time plotted as the ordinate against time as the abscissa. Pressure changes can also be plotted. If a means of projection is available to the class, these graphs will make for easy comparison of the extent and time course of responses.

Suggested Experiments

I. Chemical stimuli

A variety of transmitter and blocking agents may be investigated: acetylcholine, 5-hydroxytryptamine (serotonin), histamine, dopamine, noradrenaline, atropine, propanolol, etc. (1 to 2 mM concentrations are appropriate for test solutions).

Within a range of about pH 4 to 10, hydrogen ion internally appears to be without significant effect. Potassium ions would be expected to act either

on neurons or effectors, and this proves to be the case. Other ions and osmotic changes may be investigated.

II. Mechanical stimuli

Students are usually familiar with the generalization of vertebrate physiology that stimulation of a nervous element must act on an effector through a spinal reflex. (At this point the instructor can comment that the vertebrate gut is an exception to this rule.) It is easy to use the posterior chamber of the slug, obviously free of any central nervous system, to see whether this is true of molluscs. For external stimulation, the slug may be stroked with a glass stirring rod at a rate of about 2 strokes per second, proceeding regularly around the entire body. A sac preparation which has not been producing fluid will now show one of two typical responses. In system A, which allows the pressure to change with contractions of the slug body, the pressure will rise steadily to from 20 to 30 cm Ringer (assuming a starting point of 10 cm); at the same time, drops of fluid will form and drip from the tip of the body. The responsiveness to stroking will decrease with time. Students can ask several questions, and try a number of experiments in attempting to answer them. For example, is the decrease in fluid formation due to reduction in the amount of fluid available inside the sac? (The constant pressure set-up B, with its considerable reserve of fluid can be used to advantage in attacking this question).

Another immediate question that can be addressed is the nature of the neurotransmitter at this peripheral synapse. Known blocking agents (e.g., atropine for acetylcholine; propanolol for norepinephrine; and others) can be used (e.g. with 2 mM or higher concentrations of agents injected and allowed to act for 30 minutes prior to testing).

A further question may occur to students. Is it possible to use mechanical stimulation inside the body? The polyethylene tube makes it easy to provide such stimulation: the tube is simply raised and lowered while, at the same time, it is turned about within the sac. This provides a stroking action to the inside of the body wall which can be compared in effectiveness to the external stroking, although a new slug preparation must be prepared if one agrees that fatigue at synapses is likely to take place.

III. Extraction of active substances from the head ganglia

This experiment demands more preparation on the part of the instructor, and the provision of extra equipment: a good centrifuge and a glass homogenizer. The pleural, pedal and parietal ganglia are easily freed from the cerebral ganglia and surrounding tissue. The entire contents of one pair of such tissues will be needed for each sac tested. The ganglionic tissue is homogenized on ice for 2 to 5 min. with a ml or 2 of water, then diluted with an equal volume of 2x slug Ringer. The resulting homogenate is centrifuged (e.g., for 10 min

at 5000 rpm), the entire supernatant volume is injected into the sac, and the resulting outflow assessed.

Small peptides have long been known to be important hormones of vertebrates, but only recently has evidence been found for similar activity in molluscs. The slug sac preparation makes it easy to assess the effects of some of these compounds. Arginine vasopressin (antidiuretic hormone) is easily obtained and may be tested at the level of 1 mM. Arginine vasotocin is only a little more difficult to obtain, and has proven to be far the most effective of agents tested in producing the fluid response. It has been shown that arginine vasotocin (AVT) is closely similar to, though not identical with, the active material extracted from the ganglia of slugs. A number of compounds are available which block the action of AVT in vertebrates, and these prove to be blockers of the action in the slug as well.

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APPENDIX A

Reagents and Ringer Solution for Slug Experiments

A standard Ringer solution appropriate for the slug *Arion ater* was proposed by Roach, D. K. Analysis of the haemolymph of *Arion ater* L.; J. Exp. Biol. 40:612–623; 1963:

Stock 1	NaCl	2.52 gm
	KCl	0.26 gm
	$CaCl_2 \cdot 6H_2O$	0.66 gm
	$MgSO_4$	1.0 gm
Stock 2	Na_2SO_4	0.53 gm
	$NaHCO_3$	0.76 gm
	NaOH	0.064 gm
	Na_2HPO_4	0.03 gm

Dissolve the salts of Stock 1 in 100 ml H_2O; likewise, dissolve the salts of stock 2 in 100 ml H_2O.

Store in refrigerator before use.

At the time of the experiment, mix 10 parts Stock 1 + 10 parts Stock 2 + 80 parts H_2O

Add 1.15 gm glucose

For large classes it would be convenient to make up Stock 1 and Stock 2 in proportionately large amounts, e.g., x 10 or x20; and add glucose to give a final concentration in the Roach Ringer solution of 1.15%.

Neurotransmitter substances and hormones are obtained from biochemical supply companies, such as Sigma Chemical Co., P.O. box 14508, Saint Louis, Mo. 63178. Arginine vasotocin, while closely similar to slug neurohormones, may be substituted with arginine vasopressin; the latter is much less expensive than arginine vasotocin.

APPENDIX B

A Note on the Dissection for Preparation of the Slug Body Wall Sac

A simple way to start the dissection, minimizing unwanted mucus secretion, is to place the slug in a relaxed position on a piece of paper towel. Then, with a *sharp* one-edged razor blade, divide the body with a single swift cut, directed exactly perpendicularly to the horizontal surface, at the posterior edge of the mantle (at 1 in Fig. 3.2). Use a pair of scissors to cut through the mantle and head region, to destroy the head ganglia for humane reasons (at 2 in Fig. 3.2). Then, with a pair of forceps, gently pull the viscera in a single mass from the posterior half of the body (3). Avoid puncturing the crop. Transfer the body wall sac to a clean piece of paper towel, and proceed with the preparation as described in the main section of this experiment.

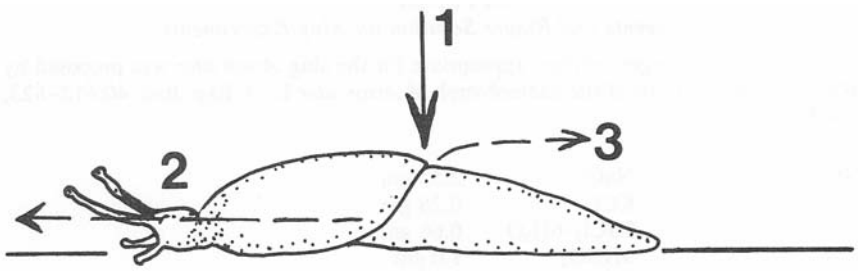

Figure 3.2.

APPENDIX C

Additional References on Various Aspects of Slug (and Snail) Biology

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