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Chapter 17

Alternative Strategies to the Use of Vertebrates in Undergraduate Physiology Laboratories

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Contents

General Introduction

The use of vertebrate animals has been a tradition in physiology labs. Yet, there are good and current reasons to find alternatives to the use of vertebrates when possible. One reason is the cost of the specimens; another is the concerns dealing with procedures on vertebrates. This major workshop explores alternatives in **four** separate experiments. One explores thermoregulation without using animals at all! Another investigates actomyosin function and membrane excitation using giant algal cells. Finally, insects are used in the investigation of taste receptors and digestion. This workshop will explore the importance of providing good background information to the vertebrate processes and connecting the alternative experimental system to homologous physiological processes in vertebrates. Students can then appreciate the commonality in biological processes and the importance of underlying physiological principles. The four laboratory experiments included in the major work-shop are: A) Effects of Insulation and Antifreeze/Glycerol on Thermoregulation of

Simulated Animals Living in Cold Conditions, B) Giant Algal Cells as a Model System for Investigating Actomyosin Function and Membrane Excitation, C) Tarsal Taste Receptors of Flies, and D) Distribution of Enzymes in Cockroach Gut.

A. Effects of Insulation and Antifreeze/Glycerol on Thermoregulation of Simulated Animals Living in Cold Conditions

Introduction

Ambient and internal body temperatures play very important roles in an animal's survival. All living organisms have limits to the temperature range in which they can survive, whether they maintain a relatively constant internal temperature or conform to their environmental temperature. The degree of environmental temperature tolerance varies with the duration of exposure, seasons and/or their stages of development. The endothermic animals can tolerate only a narrow range of body temperature, but they are able to live in a wide range of environmental temperatures. The ambient temperature range within which the metabolic heat production of an animal is unaffected is considered as the animal's ***thermoneutral zone*** (Withers, 1992). It is advantageous for an animal to modify its behavior and physiological adaptations, such as thicker insulation, to remain in the thermoneutral zone. While in the thermoneutral range, the animal's resting metabolic rate remains fairly constant, and thermoregulatory effort is reduced to a minimum. Thermoregulatory heat production is enormously expensive and makes up the single largest component of the energy budget in endotherms. When ambient temperature is below the thermoneutral zone, the resting metabolic rate of an animal must be increased by shivering to replenish heat that is lost to the environment. The ambient temperature that initiates changes in the animal's resting metabolic rate is called the ***lower critical temperature***. Most tropical mammals have critical temperatures between +20^{o}C and +30^{o}C, and Arctic animals have much lower critical temperatures. A well-insulated animal such as an Arctic fox does not increase its metabolic rate significantly until the air temperature is below –40^{o}C, a point at which it starts shivering (Schmidt-Nielsen, 1997).

In situations where ambient temperature (Ta) is below the body temperature (Tb), heat is transferred from the animal to the environment by conduction and radiation. Endothermic animals adjust their rate of metabolic heat production (H) to equal the rate of heat loss (Q). The heat balance of an animal can be expressed by the following equation:

$$H \alpha Q \alpha C (Tb-Ta)$$

The loss of heat is mediated by the gradient between (Ta) and (Tb). The greater the difference between the ambient and body temperatures, the faster and greater is the heat loss. For example, camels increase their Tb to reduce the gradient between the Ta and Tb, thus reducing heat loss to their environment.

Conductance (C) represents heat flow from the body to the environment or vice versa. To reduce their thermal conductance, animals rely on behavioral adaptations and the efficiency of their insulation such as fat, blubber, and fur. As insulation is increased, conductance decreases, heat loss is minimized, and the animals remain in their thermoneutral zones with minimal expenditure of energy to regulate their Tb. In aquatic environments, fur is not as advantageous as it loses its

insulation value when wet. This is one reason most aquatic mammals have a thick layer of blubber instead of fur.

Arctic animals that live in ambient temperatures below freezing have to avoid the fatal formation of ice in their body tissue. These animals depend on physiological as well as biochemical adaptations in order to live. *Trematomus borchgrevinki,* a fish living in the ocean near the Antarctic, has a high concentration of glycerol in the blood (Schmidt-Nielsen, 1997). Glycerol increases the cold tolerance and lowers the freezing point of the tissue fluid, preventing the deadly formation of ice crystals in the blood and tissues, thus enabling the fish to live in seawater at a temperature of –1.8°C (Schmidt-Nielsen, 1997). Another chemical that is present in animals living in cold environments is a glycoprotein compound, an "antifreeze" complex that resists ice formation by preventing the addition of water molecules to the crystal lattice of ice. For example, Rock cods (*Pagothenia borchgrevinki*), active and thriving in the ocean near the Antarctica, have their body temperatures near –1.9^{o}C (Hill, et al., 2004). These animals metabolically synthesize antifreeze compounds, lowering the freezing point of their tissue fluid (Hill, et al., 2004). Yet another method of coping with subzero temperature for most freeze tolerant insects is the presence of nucleating agents, large hydrophilic protein molecules, promoting ice formation in the hemolymph (Schmidt-Nielsen, 1997). Formation of ice crystal in the hemolymph increases the osmotic pressure of the tissue fluid, creating a hyperosmotic environment for the cell. Water exits the cell, leaving the internal environment with higher osmolality, which in turn lowers the freezing point, and reducing the chance of ice formation within the cell.

Objective:

The purpose of this investigation is to use simulated conditions to study non-metabolic temperature regulation mechanisms for both endo- and ectothermic animals in cold environments. Part one of the experiment studies the effectiveness of fat and/or fur to reduce conductance of heat at Ta of 20^{o}C and 10^{o}C. Part two of the experiment determines the effectiveness of glycerol, antifreeze and various concentrations of physiologic solutions (glucose and NaCl) on freezing point depression.

Materials

- (5) 162-ml aluminum can (Treetop apple juice cans)
- (10) thermometers
- (3) Ziploc bags
- 3 cups lard
- (2) rabbit fur pelts
- Funnels
- Balance
- (5) 1L beakers
- (5) ice buckets
- Rubber bands
- Wax papers
- (4) Styrofoam containers
- Cotton balls to cushion the small test tubes
- Glycerol
- 0.9%, 1.8% sodium chloride solution
- 5 %, 10% dextrose solution
- Commercial antifreeze solution
- Rock Salt
- (4) large test tubes
- (4) small test tubes (fit inside the large ones)
- (4) stirrers
- Crushed ice

Student Outline

Preparation of cans with various insulations

- Prepare fat by weighing two 8-oz portions of lard shortening on wax papers. Place the shortening in two different Ziploc bags. Spread the shortening to an even thin layer, and seal the bags. Label 4 aluminum cans, 1-4. Punch a small hole on the top and insert a thermometer in each can. Wrap can #1 with an 8-oz fat-filled bag, can #2 with a rabbit fur pelt, can #3 with both 8-oz fat filled bag and fur. The last can, #4, is the control; it has neither fat nor fur.
- Heat 1 L of 0.9% saline to 38-39^{o}C. Use a small funnel to fill the cans with warmed saline. By the time the experiment starts, the temperature will drop to 37^{o}C. Attach the wrapped fat and/or fur linings to the can with a rubber band. Make sure the entire can is covered with fat and/or fur, or both. Place all four cans at room temperature (22^{o}C). Record the core temperature of the cans every 5 minutes for an hour, starting at 37^{o}C. Repeat the experiment at 8^{o}C ambient temperature.
- If a cold room is not available, place the can with fat/fur in a large beaker, and then place the entire assembly in an ice bucket with ice and water. Placing the fat/fur wrapped can in a large beaker is to ensure even distribution of temperature around the aluminum can. Use a thermometer to monitor the temperature inside the beaker. Adjust the amount of ice and water until the temperature in the beaker registers around 8^{o}C. The apparatus for measuring internal temperature of the cans is shown in Appendix A.

Preparation of ice-salt baths

Prepare an ice-salt bath using layers of crushed ice and rock salt. Alternate the layers of ice and salt until the Styrofoam container is full. Stir the ice-salt mixture. Clean and dry the smaller test tube and add to it 10 ml of glycerol. Insert the stirrer and thermometer into the tube. Make sure the thermometer is immersed in the solution, and the stirrer moves up and down easily. Immerse the tube into the ice-salt bath, stir the solution with the stirrer, and allow the solution to cool to about 5^{o}C. Remove the smaller test tube from the bath and place it inside the larger test tube. Immerse the entire test tube assembly into the ice-salt bath.

Placing the smaller tube in a larger one ensures even distribution of temperature around the small tube and prevent cold spots outside the small tube. Cover the opening of the tubes with cotton balls. Stir the solution in the test tube continuously at a uniform rate with the wire stirrer. Stirring the solution avoids development of cold spots and ensures the temperature of the solution is the same throughout the small test tube. Continue recording temperature measurements every two minutes until six successive readings are equal. Stir the solution with the stirrer and measure the temperature of the solution. Subsequent freezing point of tap water, deionized (DI) water, 1:1 ratio of saline and antifreeze, 0.9% saline, and 2.7% saline are obtained using the same procedure. The apparatus for measuring freezing point of solutions is shown in Appendix B.

Results:

Part 1

1. The time versus temperature data is arranged in tabular form for each of the three trials, at 22^{o}C, at 8^{o}C, and with twice the shortening as insulation. See Table 1 below.
2. Prepare the graphs from the data tables.

3. Determine the greatest and the least heat loss relative to insulation. Determine the greatest and the least heat loss relative to ambient temperature.

Table 1. Temperature of fluid in cans with various insulations

Time	Temp -^{o}C	Temp -^{o}C	Temp -^{o}C	Temp -^{o}C
Min.	Can – no insulation	Can with fat	Can with fur	Can with fat and fur
0	37	37	37	37
5				
10				
15				
20				
25				
30				
35				
40				
45				
50				
55				
60				

Part 2

1. The time versus temperature data should be arranged in tabular form for each of the six trials: water, glycerol, saline and antifreeze, 0.9% and 2.7% concentrations of saline. See Table 2 below.
2. Prepare cooling curve for water and the other solutions from the data tables.
3. Calculate the freezing point of water—both tap and DI water.
4. Calculate the freezing point depression for each of the solutions

Table 2: Freezing point of fluids in test tube with antifreeze, glycerol and different concentrations of NaCl

Time	Temp -^{o}C	Temp -^{o}C	Temp -^{o}C	Temp -^{o}C	Temp -^{o}C	Temp -^{o}C
Min.	Glycerol	Saline and antifreeze 1:1 ratio	0.9% Saline	1.8% saline	Tap water	DI water
0	5	5	5	5		5
2						
4						
6						
8						
10						
12						
14						
16						
18						
20						
22						
24						
26						
28						
30						

Discussion Questions:

1. In the simulated conditions for endotherms, which aluminum can has the greatest conductance of heat? Which has the lowest? What is the relationship between conductance and insulation?
2. What are the differences in rate of heat loss amongst the four solutions between 22°C and 8°C? Explain the result using the heat loss and heat gain equation.
3. What are the two variables that an animal can adjust, without any changes in the metabolic rate, in order to reduce heat loss to the environment?
4. Why is thicker insulation advantageous to the survival of Arctic animals?
5. How do these terms "thermoneutral zone" and "lower critical temperature" associate with the results of this experiment?
6. How do glycerol, antifreeze, and various concentrations of NaCl affect freezing point?

Notes for the instructor

This lab was presented as mini-workshop in 2003 ABLE conference at UNLV (Watson, 2003). This lab is very time consuming. It is more efficient to divide the class into two main groups, endotherms and ectotherms. At the end of the experiments, share and tabulate the results.

For the endothermic group, I start with the heat gain/loss equation, $H \alpha Q \alpha C$ (Tb-Ta). The students identify the variables and make their hypothesis. There are two variables the students readily noted: 1) Increase insulation, decrease conductance, or 2) Decrease the difference between ambient temperature and body temperature (Tb-Ta), either by a) Changing their habitat (e.g., animals living in burrows where ambient temperature is lower than the surface environment), or b) Changing core body temperature, reducing the difference between Ta and Tb. Hence, variations of this experiment depend on the hypothesis. Heat gain/loss depends on increased insulation (increase thickness of fat and/or fur); some may want to lower the temperature in the can when placed in cold room, or increase temperature of water when placed in warmer environment.

These hypotheses may be tested by 1) Altering the layer of fat: instead of using 8 oz of shortening, use 16 oz or more; or using thicker fur, and 2) Altering ambient temperatures, in one of two ways: a) The ambient temperature may be changed to 15°C from 8°C; b) The temperature of the saline in the cans may be lowered to 34°C or increase to 39°C instead of 37°C.

The freezing point of deionized water is below zero (the deionized water is supercooled). Tap water freezes at 0°C; it has more nucleating agents and is not as pure as the deionized water. Part 2 of the Procedure can be repeated using different solutions such as:

1. 1:2 concentrations of saline and glycerol
2. Increase ratio of antifreeze to saline
3. 5%, 10%, and/or 15% dextrose

The protocol in this lab can also be used in a similar fashion to determine the effects of insulation on heat gain, or the effects of surface area on heat gain/loss, or the effects of different types of insulation, such as paper or different types of wool. For example, the cans can be similarly placed in warm environments, such as outside the lab in the sun, to measure the temperature change in the fluid in the can relative to fur, fat or combination of both. To increase surface area of the can, taller and narrower containers may be used to determine the rate of heat gain/loss. A graph in Appendix C demonstrates the insulation values of fur, fat, fat and fur, and control.

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B. Giant Algal Cells as a model system for investigating actomyosin function and membrane excitation

Introduction

The coordinated action of the muscle proteins, actin and myosin, is necessary for animal movement. In the skeletal muscle of animals, these proteins are organized in regular arrays called sarcomeres. The actin "thin" filaments and myosin "thick" filaments are interposed in these sarcomeres, as shown on the following page. Contraction is produced by the relative sliding of actin filaments induced by myosin ATPase. Even in non-muscle cells of animals, actomyosin is responsible for movement and producing tension. For example, cytokinesis, the division of two daughter cells after mitosis, requires the function of actin and myosin. Actin and myosin are involved in movement of various other cellular cargo, such as membrane vesicles, RNA, and proteins.

The proteins actin and myosin, together called actomyosin, work together in muscle cells to produce tension. They are found in many non-muscle cells and play important roles in intracellular movement. The identification of actin microfilaments in non-muscle cells has taken advantage of the fact that actin and myosin are highly conserved over a wide range of organisms. Thus, subfragments of myosin from skeletal muscle can be used as a label for actin. Specifically, the subfragment of skeletal myosin can be added *in vitro* to actin filament containing cells. The added myosin subfragments bind to actin filaments and form characteristic arrowhead decoration on the actin filaments, thus identifying them as actin filaments.

Early studies on actomyosin function used skeletal muscle to measure macroscopic physical properties of force and contraction velocities. More recently, the studies of actomyosin have focused on microscopic measures of force and velocity. Two classic cell models for microscopic studies of actomyosin movement are the squid giant axon and the giant algal cells in the Characeae family. Since squid giant axons are a little hard to come by, we will use the giant algal cells such as *Chara* and *Nitella*. The internodal cells of these algae are unusually large, almost 1 mm in diameter, and can reach several centimeters long. Rows of chloroplasts are arrayed around the periphery of the cells and actin microfilaments are located on the cytoplasmic side of the rows of chloroplasts. Note

that there are white regions that do not have rows of chloroplasts. These regions, sometimes called the indifferent zones also do not have actin filaments. Microfilaments are not visible by standard light microscope techniques. However, they can be seen by immunofluorescence if the filaments are labeled with fluorescent anti-actin antibodies, or by fluorescently labeled phalloidin, a plant product that binds to actin filaments. These plant actin microfilaments, like other actin microfilaments can be "decorated" with subfragment of skeletal muscle myosin to form "arrowhead" structures. Cytoplasmic streaming in the giant internodal cells involves myosin located on membrane vesicle and other cytoplasmic particles that move these particles along these rows of actin filaments. Even today, *Chara* remains an important model for studying actomyosin function. (Sugi & Chaen, 2003).

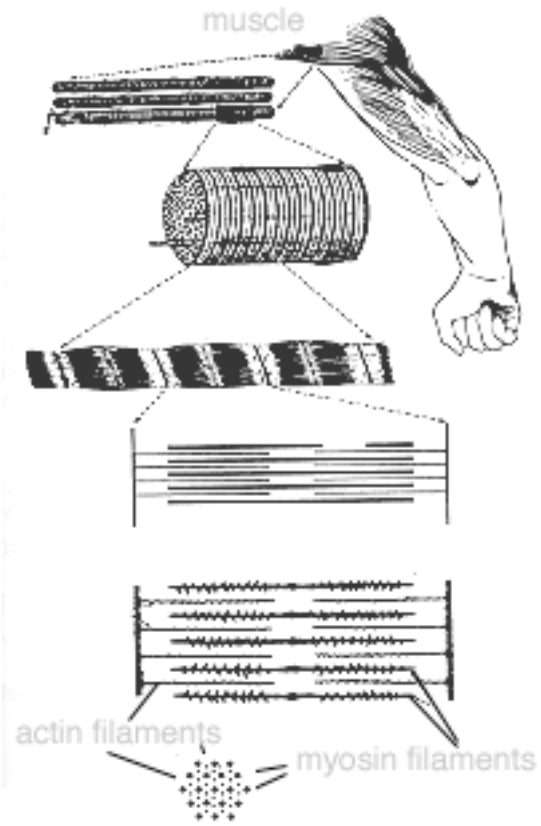

Figure 1. Diagram of levels of organization of muscle from the actomyosin filaments to the whole organism.

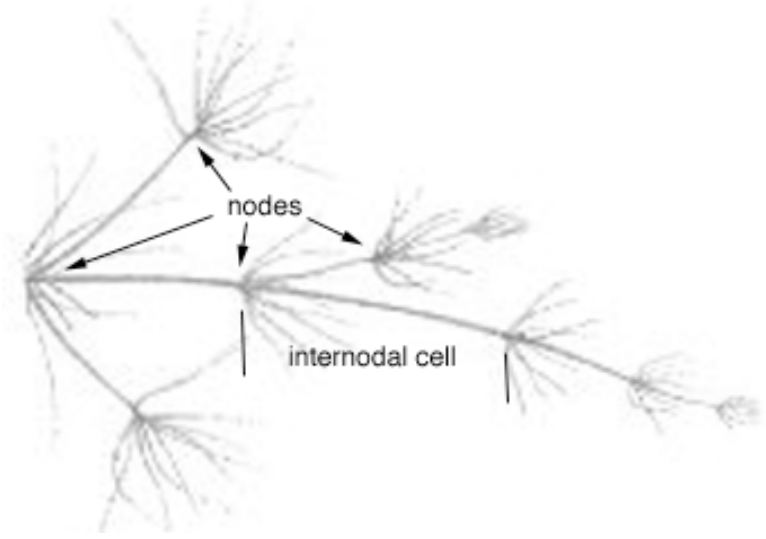

Figure 2. Drawing of a typical characean algal cell. Giant internodal cells span the nodes.

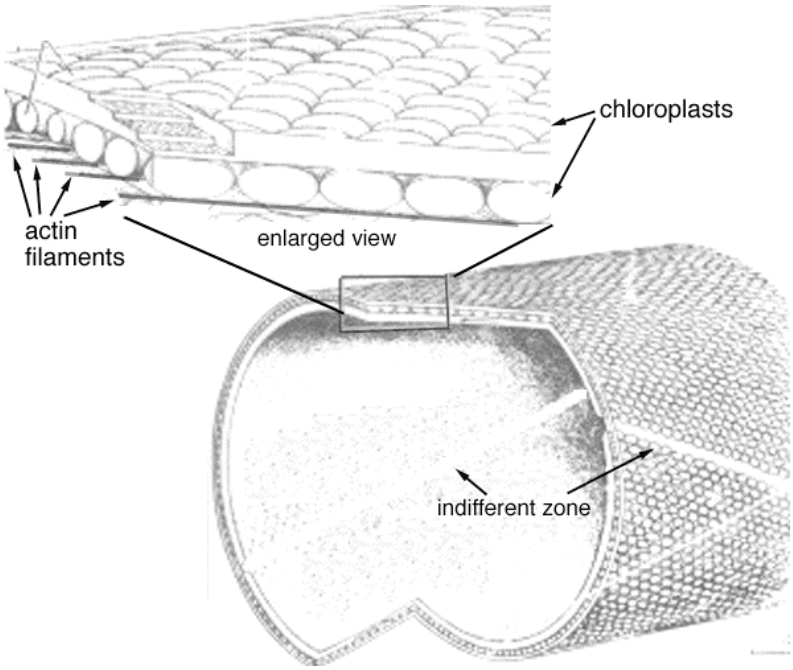

Figure 3. Enlarged view of a Characean cell. A cross-sectional view of an internodal cell is shown below and an enlarged view of the surface showing the relative location of chloroplasts and the actin filaments.

Materials

Characean alga, *Chara* or *Nitella* both work in these experiments and are available from Carolina Biologicals or Wards or can be collected from ponds. They are easy to maintain in a fish aquarium with clay soil at the bottom. In my experience, they do not need aeration or special lighting or special solution. Place them in an area where they get light but not direct sunlight. It is best not to have them in an area that gets too warm or too cold.

Rectangular clear plastic lids from thumb tack or pin containers work great as chambers for viewing these cells alive because it allows the cells to be immersed in liquid and the containers to be held in place on a microscope stage. I ask the departmental office to save for them.

Rings for the perfusion experiments are made by cutting rings from 15-mL conical centrifuge tube and then putting in a small notch suitable for letting the *Chara* cell sit in it.

Solutions for the cells are not crucial as long as there are some monovalent ions as well as calcium ions.

The following solution works well for observation of live cells.

stock solution A	200 mL
100 mM KCl	1.49 g
10 mM NaCl	0.117 g
10 mM $CaCl_2$	0.294 g
10 mM $MgCl_2$	0.407 g

stock solution B	200 mL
10 mM $KHCO_3$	0.2 g

add 1 mL solution A + 1 mL solution B
bring to ~90 mL
adjust pH to 6 with 1 mM HCl
bring to 100 mL

Solutions for internal perfusion experiment

perfusion solution	100 mL
5 mM EGTA	5 mL of 0.1 M (neutralize with 1mM KOH)
6 mM $MgCl_2$	3 mL of 0.2 M $MgCl_2$
290 mM sorbitol	5.28 g
5 mM Tris-maleate buffer pH 7	5 mL of 0.1 M Tris-maleate buffer pH 7

Tris-maleate buffer

80 mL 0.1 M Tris base (1.21 g / 100 mL)
~33 mL 0.1 M maleic acid (1.16 g / 100 mL)

Calcium-containing perfusion solution

has in addition to the above 4.9 mM $CaCl_2$ approx 10^{-5} to 10^{-4} M Ca^{++}
2.45 mL 0.1M $CaCl_2$ reneutralize

Stock ATP solution (0.2 M ATP) to add to the perfusion solution:

Add 0.25 g $Na_2ATP.3H_2O$ to
800 mL 1N NaOH + 1.2 mL water

Micro rulers made by making sub-millimeter markings using Adobe Photoshop and printed on transparency sheet are cheap and handy way to measure distance traveled by the particles

Student Outline

Study cytoplasmic streaming in intact cells.

Place a "frond" of *Chara* in a petri dish with some water to take to your bench. To observe cytoplasmic streaming, cut out a *Chara* cell and place in a rectangular dish. Make sure you have an intact internodal cell, that is, your cell includes the adjoining nodes (note diagram above). It is important to keep the cell moist. Observe them intact under high power of the dissection microscope or low power compound microscope. Do you notice any movement of particles? If not, you may need to let the cell "rest" a bit. Note the movement of endoplasmic particles and vesicles. In what direction are they moving? Is the direction constant? How do the particles move with respect to the whole cell? Figure out a way to estimate the rate of movement of the particles. Make several measurements of the velocity to determine the variation in the rate. Are there differences in rates for different parts of the cell or for different sizes of particles or different times after you begin observation?

Study the effect of electrical stimuli on cytoplasmic streaming

Recall that we let the cells "rest a bit" before we observed movement so we might hypothesize that mechanical stimulus stopped the movement. Try to deliberately stop the movement by mechanical stimulus. Can you get the cytoplasmic streaming to stop? You may need to touch the cell quite vigorously to see an effect. Since it is difficult for us to quantify physical stimulus, we will try electrical stimulation. Place an internodal cell between the two wire electrodes in the electrical stimulus chamber, or place the electrode wires on each side of the internodal cell in the viewing chamber. Focus the microscope on a region where you can observe active cytoplasmic streaming. Do

not begin until you see active cytoplasmic streaming. Begin by first setting the stimulus to 1 volt. Firmly press and immediately release the stimulus button. Do not hold the button down nor give multiple stimuli at this time. Do you see any effect? If you do not see an effect on cytoplasmic streaming, increase the voltage and try again. Continue until you see an effect on movement. Note the voltage that produced a complete cessation of movement. Continue to observe the cell without further stimulus, noting the recovery of movement. Measure the velocity of movement as a function of the time since the stimulus. Consider what the electrical or physical stimuli are doing to the cell. How might this stimulus be coupled to the effect on cytoplasmic streaming? If you wish, try different modes of electrical stimulus, either multiple stimulus or longer stimulus. If you do this, be sure to quantify the stimulus as well as the response. What would be your measure of the stimulus, and what might you measure in terms of the response?

Notes for Instructors

Other experiments using Characean alga

Internally perfuse the cell: Though technically tricky, students can internally perfuse the *Chara* cells to investigate the role of the energy molecule, ATP, and the role of the signal transduction molecule, Ca^{++} in cytoplasmic streaming. A perfusion ring (made from cutting rings from 15-mL conical centrifuge tubes and notched) can be used in the system diagrammed below (Fig. 4). The procedure involves cutting the branches from the nodes and placing the piece on a clean microscope slide. The cell is gently blotted with Kimwipe so that a watertight seal can be formed around the two ends of the cells.

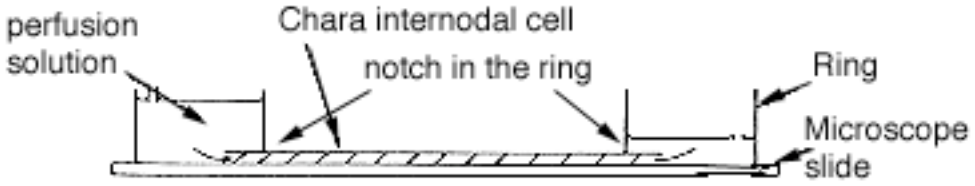

Figure 4. Side view of the setup for internal perfusion experiment. The internodal cell is shown as hatched. The difference in the level of perfusion solutions in the two rings are exaggerated.

Place a layer of grease on the bottom end of the plastic ring (the end with the notch), making sure to have grease *all* around the ring, including the notch. You may want to gently lift the ends of the cell and place a bit of grease under the plant. Then gently place the greased ring on the cell, matching the notch to the cell, and push to ensure a good seal. At this time, seal with additional grease on a toothpick. After about a minute, place a drop or two of perfusion solution into the two rings. If they leak, reconstruct the perfusion apparatus using a fresh sample, clean microscope slide and cleaned and freshly regreased rings. If the rings do not leak, add more fluid into the ring until the cells are covered with fluid. Carefully insert the tip of fine dissecting scissors into the ring to cleanly cut open the internodal cell at both ends.

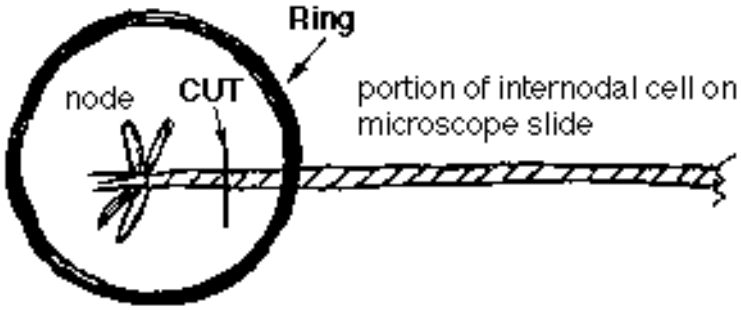

Figure 5. Enlarged view looking down upon one of the rings of the internal perfusion set-up. Only one side is shown.

Place the whole perfusion apparatus with your cell under the microscope. Adding a bit more perfusion solution into one of the rings will cause a pressure difference between the two cut ends of the internodal cell that will cause a flow. Because the initial perfusion solution does not have any ATP, we hope that particles with myosin are tightly attached to the microfilaments, in what is termed "rigor." When there is no pressure difference to cause additional flow, there should be no active movement of the particles since all the ATP should have been washed out. Then the solution in the rings can be replaced with perfusion solution with additions such as ATP and ATP solution with calcium.

Measurement of membrane action potential (Kikuyama et al. 1993): If electrodes and amplifiers for making electrical measurements are available, *Chara* cells are much easier cell to measure action potential than with animal cells. This is because the action potential in *Chara* lasts on the order of seconds rather than milliseconds, so relatively inexpensive amplifiers are sufficient for measurement. The cells are large, and extracellular electrodes can be employed. Intracellular electrodes will also work, but because of the large vacuole, the measurement is the sum of electrical difference across the vacuolar membrane and the plasma membrane.

The physiology of Chara: cytoplasmic streaming, action potential and Ca^{++} regulation

After the students have done their experiment, I explain the physiological basis of their observations. The cytoplasmic streaming in Characean cells is driven by actomyosin ATPase, just as in our muscles. Myosin filaments are attached to all sorts of particles and generally the velocity is fairly constant under given conditions regardless of the size of the particles. The actin filaments are arrayed in parallel with a low helical pitch, thus the movement is unidirectional between the indifferent zones and in opposite direction on each side of the indifferent zone. The role of calcium ions may be confusing at first to students who have been taught that calcium activates actomyosin movement. That is the case with most of our muscles, but Ca^{++} stops the movement in *Chara*. This can be understood as follows. In the case of our muscles, actomyosin is inactive in the unstimulated condition. However. as we saw, cytoplasmic streaming is active in the unstimulated algal cell. In both cases, stimulation, whether by nerves, or mechanical, is mediated by action potential that results in increase in intracellular Ca^{++}. The stimulation results in activation of actomyosin in the case of our muscles, but results in inactivation of actomyosin in *Chara*. As the intracellular Ca^{++} decreases, the actomyosin in *Chara* resumes activity. This can be monitored by the increase in velocity of cytoplasmic streaming after recovery from stimuli.

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C. Tarsal Taste Receptors of Flies

Introduction

Sensory organs associated with feeding include a multitude of organs. The compound eyes are often involved in locating food and they are particularly important in predators. Sensilla of the mouthparts are important in food selection. Antennae are involved in the perception of odor from close-by or from a distance. The tarsi of insects also bear contact chemoreceptors. These receptors play an important role in feeding behavior because they allow different food types and concentration to be distinguished (Chapman, 1982).

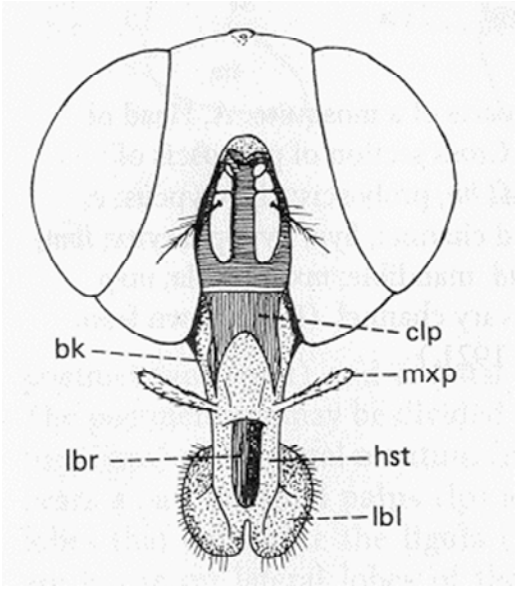

Figure 6. Anterior view of mouthparts of the housefly, *Musca domestica.* (clp—clypeus, bk--rostrum, mxp--maxillary palp, lbr--labrum, hst—hastellum, lbl—labellum with hair.

The tarsal taste receptors in flies play a major role in fluid-feeding regulation. Proboscis extension after stimulation of tarsi brings the labellar hairs into contact with food. The labellar hairs are more sensitive than the tarsal hairs and so are able to detect substances that are present in concentrations too low for stimulation of the tarsal hairs (Wigglesworth, 1953). If the solution presented proves to be suitable, the labellar lobes are spread out and the insect starts to suck up the sugar water. The entry of sugar into the pseudotracheal system immediately stimulates the interpseudotracheal pegs, a final check on the suitability of the food. The month parts of the housefly are shown in Figure 6 (Borree et al., 2004).

Contact chemoreceptors can be stimulated by chemicals in aqueous solution or by chemicals distributed over solid surfaces. The neurons of these receptors are sensitive to different spectra of stimulating molecules. In the tarsi receptors of *Phormia*, one neuron responds maximally to salts, one to sugars, and one to water. Within these classes of receptors, sensitivity also varies with concentration.

Materials

Diptera- housefly, blowfly or flesh fly (10 or more).
Beeswax or paraffin
Slides
Sucrose, lactose solutions: 0.001 to 1 molar concentrations
Glucose, fructose solutions: 0.001 to 1 molar concentrations
Salt solutions: 0.001 to 1 molar concentrations
Pins and ring-stands
Tissue paper- Kimwipe

Student Outline

Objectives:
To stimulate the fly's tarsal chemoreceptors with sugar/salt solutions, and determine the sensitivity of the receptors to various sugar/salt concentrations by the Acceptance-Threshold Method.

Preparation of paraffin blocks

To prepare the apparatus to observe the flies, cut a small block of paraffin or wax about twice the size of length and width of the fly. Clamp the block of wax and attach the clamp to a ring-stand.

Make dilutions of the monosaccharides, disaccharides, and salt solutions from 1 to 0.001 M concentrations. Using CO_2, anesthetize 10 or more flies that have been on a *water diet* for several hours. Five flies serve as control.

Preparation of flies

To prepare the fly for the experiment, push a very fine entomology pin through the abdomen, starting from the ventral side, slightly off center to avoid damaging the centrally located abdominal ganglia. Be sure the pin goes through the abdomen with the head of the pin next to the body. Suspend the fly by pushing the pointed end of the pin into a block of wax, which is held by a clamp on a ring-stand. *The fly should be in a horizontal position.*

Testing procedure

Place a drop of test solution on a glass slide and touch the solution to the tarsi of the flies, starting with the lowest molar concentration of sugar/salt. Observe the labellum. If it is lowered into the solution for one second, then score the test as a (+); if the labellum is lowered for less than a second, then score it as (+/-). If the labellum is not lowered into the solution at all, the score is (-).

Determine the threshold of response to the sugar (glucose, fructose and sucrose) and salt solutions by testing from the lowest to the highest concentration. Between each concentration of solution, cleanse the tarsi with a wet Kimwipe soaked with distilled water. Allow one minute

between testing with another solution. Repeat, going in a reverse direction of concentration to bracket the threshold of response. If necessary, further dilute the sugar/salt solutions to pinpoint the threshold more accurately. Distilled water is used on control flies.

Observe the labellum. If it is lowered into the solution for one second, score the test as a (+); if the labellum is lowered for less than a second, score it as (+/-). If the labellum is not lowered into the solution at all, the score is (-).

Record the data for each sugar/salt dilution. Present the results using a table and summarize your conclusions as to the relative sensitivity of the tarsal receptors to the various dilutions of sugar and salt.

Discussion questions:

Using the scientific method, make your hypothesis, and graph or tabulate your results. Be sure to include the following questions in your discussion.

1. What is the significance of contact chemoreception?
2. What is the lowest acceptance threshold for sucrose, fructose, and glucose?
3. Are there any differences between acceptance threshold between sucrose, fructose and glucose?
4. With graphs, demonstrate differences between control and experimental flies.

Notes for the instructor

In flies, hollow hair structures contain five neurons. One neuron responds specifically to movement (mechanoreceptor). Two neurons respond to salt, and one each responds to sugar and water. The lowest acceptance threshold for sucrose is 0.0098M, for glucose- 0.132 M, and for fructose the threshold level is 0.0058 M. This varies slightly from fly to fly, depending if they are hungry or not.

It is easier to divide the class into three or four groups and each group test for one solution, such as salt, glucose, fructose or sucrose, using 10 flies—five control (test with water only) and five experimental. At the end of the lab, compile the results in a table and compare the sensitivity of the tarsal receptors to various concentrations of solutions.

Other solutions may be used by students to test the sensitivity of the tarsal receptors. Orange juice, Pepsi and diluted Pepsi, vinegar or quinine may be used in additional to sugar and salt.

Literature Cited

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Chapman, R. F. 1982. The Insects- Structure and Function. Harvard University Press, Cambridge, Massachusetts, 919 pages.

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Distribution of Enzymes in Cockroach Gut

Introduction

Animal food consists of organic material, most of which belongs to three major groups: proteins, fats and carbohydrates. Whether the food is used for fuel or for building and maintenance, the large molecules of food are first broken down into simpler units (monomers). These are then absorbed and either incorporated into the body or metabolized to provide energy. The breakdown is achieved in the digestive tract with the aid of enzymes.

Digestion can take place either within or outside of cells. In a unicellular animal, digestion is usually inside the cell. For example, a protozoan takes food into the digestive vacuole, and enzymes that aid in the digestion of carbohydrates, fats and proteins are secreted into the vacuole. Extracellular digestion is usually associated with a well-developed digestive tract that allows secreted enzymes to act on the food material.

The digestive tract may have one opening, as in coelenterates, brittle stars, and flatworms. In these animals, any undigested food is expelled through the same opening that served as a mouth. In more complex animals, the digestive tract has two openings, a mouth and an anus. This permits an assembly-line type of digestion. Food is ingested in the mouth and is passed on and acted upon by a series of digestive enzymes. The soluble products of digestion are absorbed. Undigested material is expelled through the anus without interference with food intake.

The digestive tract of a cockroach is a tube modified into subdivisions, which serve specialized digestive functions: food reception, conduction and storage, internal digestion, absorption, conduction and formation of feces. The three divisions of the cockroach digestive tract are the **foregut**, which includes the crop and proventriculus; the **midgut,** which includes the section below the proventriculus up to the caeca; and the **hindgut,** which includes the Malpighian tubules and the rectum. The products of digestion are absorbed in the midgut, especially in the more anterior parts including the caeca. Absorption also occurs in the hindgut, especially in the rectum, but there is no evidence of absorption from the foregut, which has an impermeable cuticular lining (Chapman, 1982, Slansky et al., 1987).

The enzyme reaction in the digestive tract can be determined two ways. 1) Determine the amount of substrates (starch and proteins) in an enzyme-reaction mixture. 2) Measure the presence of product present, maltose for starch digestion and amino acids in protein digestion. For example, in starch digestion, the easier of the two ways is to measure the amount of starch present. Iodine is a good indicator of presence of starch. In the presence of starch, a blue-black stain will appear. As starch is digested, the blue-black color will disappear.

Some of the factors that may influence the enzymatic process are pH, temperature, time, and concentration of substrate and enzyme. In this experiment, all variables are held constant except the concentration of enzymes (amylase and protease) and pH of substrates. Thus, the presence and the concentration of enzymes such as amylase and proteolytic enzymes can be studied in the gut of cockroaches.

An enzyme extraction procedure usually requires a method that destroys the integrity of the cell. The broken cells then release their molecular constituents including enzymes. One method that can be used to lyse (break open) cells involves treating tissue with a detergent that breaks or dissolves cell membranes. In the procedures described below, the tissue extracts will be mixed with an enzyme extraction buffer, which contains a few drops of detergent.

Objective

The objective of this experiment is to determine the optimum pH for pepsin and chymotrypsin, both proteolytic enzymes, and the presence and activity of these enzymes and amylase, a starch digesting enzyme, in the different divisions of the cockroach digestive system.

Materials

- Agar powder- 10 gm
  Agar gel buffer- 0.1M NaCl, 10 mM Tris, pH 7.4 - 500 mL
- Enzyme extraction buffer- 20 mM NaCl, 0.02%, 3 drops of 'Dawn" detergent, 10 mM Tris, pH 8– 100 mL
- Starch solution- 1% - 50 mL
- Skim milk- 20 mL or 12% casein (pH=8, titrate with 6N NaOH)
- 3 large glass test tubes
- 10 1.5 mL tubes
- 3 petri dishes per group (30)
- 18 glass Pasteur pipettes
- 10 small transfer pipettes: one pipette per student group, one for the starch solution and one for each of the amylase and chymotrypsin solutions.
- Amylase- 4 mg/mL - 10 mL
- Chymotrypsin-10mg/mL- dilutions needed for various standards - 10 mL
- Pepsin- 2mg/mL- 10 mL
- IKI solution (Lugol's iodine solution)
- Large beaker-2 to boil water
- Cockroaches, CO_2 tank
- 20 scissors and 20 forceps
- Insect saline (500 mL), and 20 petri dishes for cockroach dissection
- 3 glass tissue grinders
- Hot plates
- Acetic acid – 5% 500 mL
- Microcentrifuge tubes
- Centrifuge
- Rulers

Student Outline

Removal and processing of the digestive tract of the cockroach.

Anesthetize 20-25 cockroaches with carbon dioxide (2-3 cockroaches per students for 10 groups of students). Frozen cockroaches may be used. Cut the anus free from the body wall with scissors. Remove the complete digestive tract of the cockroach by holding the cockroach in insect saline and slowly pulling off the head and attached gut with forceps. Should the gut break between the proventriculus and midgut, open the abdomen centrally and dissect out the posterior gut portion. Refer to the diagram and *identify (do not isolate the three regions until they are rinsed)* the foregut, midgut, and hindgut.

Slit open the gut and, holding it with a pair of forceps, vigorously rinse in a dish of insect saline to remove any contained food. Using a scalpel, isolate the three regions of the gut to be studied (foregut, midgut, and hindgut). Obtain four test tubes and label them 1-4. Collectively, weigh the *isolated sections* of cockroaches' gut; be sure not to mix up the different sections of the intestine. Place the isolated sections of gut in separate *labeled* glass tissue grinders containing enzyme extraction buffer (1 to 1.5 mL of extracting buffer per gram of tissue). After grinding, allow the tissue to settle. Transfer the supernatant by Pasteur pipette into separate test tubes (1- foregut, 2- midgut, and 3- hindgut). Bring the volume of each extract to 2 mL with enzyme extraction buffer. Pipette 1 mL of each of the extracts (1-3) into the 4th tube. Heat this combined extract for 5 minutes

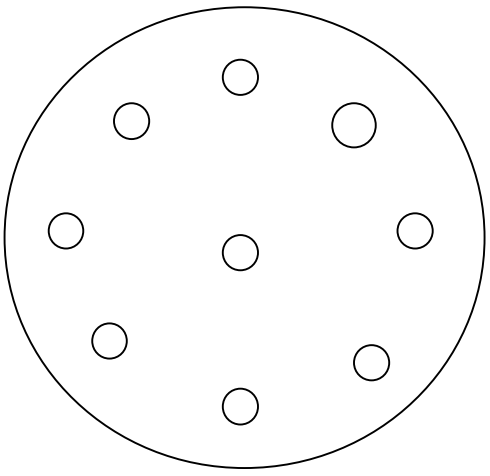
in a beaker of boiling water to denature the enzymes present. Cool and bring to about 3 mL with enzyme extraction buffer. This will serve as a suitable control for the enzyme tests. Why?

Shake the remaining 3 tissue extracts, and divide the homogenized gut extract into 10 portions (for 10 groups of students) by placing 1 ml of each extract in a labeled 1.5-mL microcentrifuge tube. Cap and shake the tubes vigorously for 3 minutes. Centrifuge the tubes for 5 minutes and remove the supernatant fraction (the liquid) with a pipette. Place these solutions in clean labeled 1.5-mL microcentrifuge tubes. The extracted enzyme is now in these tubes.

Preparation of the agar gels:

1. Label three large tubes, A, B and C. Dispense 18 mL of agar gel buffer into tubes A and B, and 19.5 mL to tube C. Add 0.32 gm of agar to each tube.
2. Place the tubes into a boiling water bath and allow the agar suspensions to come to a vigorous boil. After boiling for 4 minutes, remove the test tubes from the bath and cool at room temperature for about 3 minutes. The solution should be clear (Anderson, 1987).
3. Add 2 mL of skim milk or 1 mL of 12% casein to tubes A and B. Add four drops of concentrated NaOH to the skim milk so that the pH is around 8. Add 0.5 mL of 1% starch solution to tube C and swirl the tube until the agar forms a suspension. Skim milk (casein) and starch will serve as the substrates for the enzymes used in this experiment. Each tube should have 20 mL of fluid.
4. Pour the melted agar into three petri dishes. One tube per dish. Label the dishes: A, B and C; A and B for milk (casein), and C for starch substrate.
5. Let the agar cool at least 15 minutes. The gels can be used or stored in the refrigerator for up to a week.
6. Make 9 wells in each of the three agar plates using the large end of a Pasteur pipette.
7. Add about 5 mL of vinegar to agar plate B. After 15 minutes, discard the acid. The acid will denature and precipitate the casein in the milk, which will cause the protein to turn white.
8. Prepare the appropriate dilutions of chymotrypsin and amylase solutions

Pattern for arrangement of wells in the petri dish



*Sample Application***:**

Using the transfer pipettes, carefully fill the wells (about 0.1 ml) of the plates as indicated below. Separate pipettes should be used to disperse the chymotrypsin and pepsin solutions. (Plate B was treated with 5% acetic acid before extracts were placed in the wells)

Well number	**Proteolytic enzymes (A and B)**
1	Agar gel buffer
2	Chymotrypsin (20 ug/mL)
3	Chymotrypsin (200 ug/mL)
4	Chymotrypsin (2000 ug/mL)
5	Pepsin (2 mg/mL)
6	Standard foregut extract (not boiled)
7	Standard midgut extract (not boiled)
8	Standard hindgut extract (not boiled)
9	Boiled intestinal extract

Well number	**Amylase C**
1	Agar gel buffer
2	Amylase- 4 ug/mL
3	Amylase- 20 ug/mL
4	Amylase- 100 ug/mL
5	Amylase- 500 ug/mL
6	Standard foregut extract (not boiled)
7	Standard midgut extract (not boiled)
8	Standard hindgut extract (not boiled)
9	Boiled intestinal extract

After all samples (about 0.1 mL) have been loaded in the wells, place the lids on the dishes. Do not overfill the wells. The dishes should not be moved at this time. The plates should remain at room temperature for 12-24 hours.

Analysis of agar plates:

A. Detection of proteolytic enzyme activity (A and B):

1. Place about 5 mL of vinegar or 4% acetic acid onto agar in plate **A**. The transparent rings around the sample wells where the casein has been degraded by the enzyme indicate protease activity on both plates A and B.
2. After 20 minutes, discard the acid on plate A, and measure the diameter of the clear rings around each well.
3. The amount of chymotrypsin in the tissue extracts can be compared to the diameters of the rings around the sample wells containing known concentrations of the enzymes.

B. Detection of amylase activity (C):

1. Place about 5 mL of the Lugol's iodine solution onto the agar in the plate.
2. After 10-20 minutes, discard the iodine and fill the petri dish with water.
3. After 10-20 minutes, measure the diameter in mm of the clear rings around each well and record your results below (Table 1). The amount of amylase in the tissue extracts can be compared to

the diameters of the clear rings around the sample wells containing known concentrations of the enzymes.

Table 1. Diameter of rings, measuring protease and amylase activities

Well number	Diameter of clear rings- mm Protease activity		Amylase activity
	Plate A	**Plate B**	**Plate C**
1			
2			
3			
4			
5			
6			
7			
8			
9			

Suggestions for Data Analysis and Discussion:

1. Determine the amount (if any) of amylase and chymotrypsin activity in the cockroach gut extracts, using Excel [diameter of ring (cm) on Y axis, and concentration of enzyme on (log) X axis].
2. Explain the basis for the formation of rings in the agar-starch gels.
3. What is the function of the enzyme extraction buffer?
4. Describe the effects of the heat treatment on the activity of the enzymes in the cockroach gut extract.
5. Relate the findings from the cockroach foregut, midgut, and hindgut to the site of action of proteolytic and carbohydrate enzymes in the digestive tract.

Notes for the instructor

This lab was presented as a mini-workshop in 2002 ABLE conference (Watson, 2002). Frozen cockroaches may be used in this lab. Presence of amylase is well demonstrated in the agar plates from all three regions of the gut from previously frozen cockroaches. However, proteases do not show up as well. Freshly dissected cockroach guts have more definite rings, showing presence of proteases. In addition, when using skim milk as protein substrate, the agar gel has to have a pH of 8. This can be achieved by adding 7-8 drops of 1N NaOH to the agar solution.

Preparation of agar gels in the lab is very time consuming since it takes time for the agar gel to cool. To save time, agar gels with starch or milk substrates may be poured into petri dishes a day or two ahead of time. Be sure to refrigerate the plates after the agar is poured. To prevent confusion with the labels (1 through 9) on the top half of the petri dishes and the actual wells at the bottom part of dishes, use a permanent maker draw a short line that extends from the top to the bottom half of the dish. The top and bottom lines must be matched up in order to identify the wells and their contents properly. In addition, mark *top and bottom* halves of petri dishes as follows: "A" = gels with milk

substrate, "B" = gels with milk substrate that will be washed with acetic acid, and "C" = gels that have starch substrate.

Examples of gel plates are given in the appendices. Appendix D demonstrates the presence of amylase with starch substrate in the agar gel, and Appendix E demonstrates the presence of protease in agar gel with skim milk as substrate.

The following are recipes for Enzyme extraction buffer and gel buffer (50X):

Enzyme extraction buffer:
NaCl—0.116 g (20 mM), and Trizma—0.121 g (10mM) in 100 mL deioniozed water
Three drops of "Dawn" detergent
Final pH = 8

Gel buffer (50X)
0.06g NaCl (0.1 M) and 0.03g Tris- HCl (20mM) in10 mL deionized water
Final pH = 7.4

2% solution gel buffer = 10 mL of 50X gel buffer and 490 mL deionized water

Literature Cited

Anderson, J. N. 1987. Teaching Modern Biology in the Laboratory. Modern Biology Inc., Dayton, Indiana, 9 pages

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Slansky Jr, F., and J. G. Rodriguez. 1987. Nutritional Ecology of Insects, Mites, Spiders, and Related Invertebrates. A Wiley-Interscience Publicaion, John Wiley & Sons, New York, 1016 pages.

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Appendix A. Apparatus for measuring temperature of fluid in a can with insulation

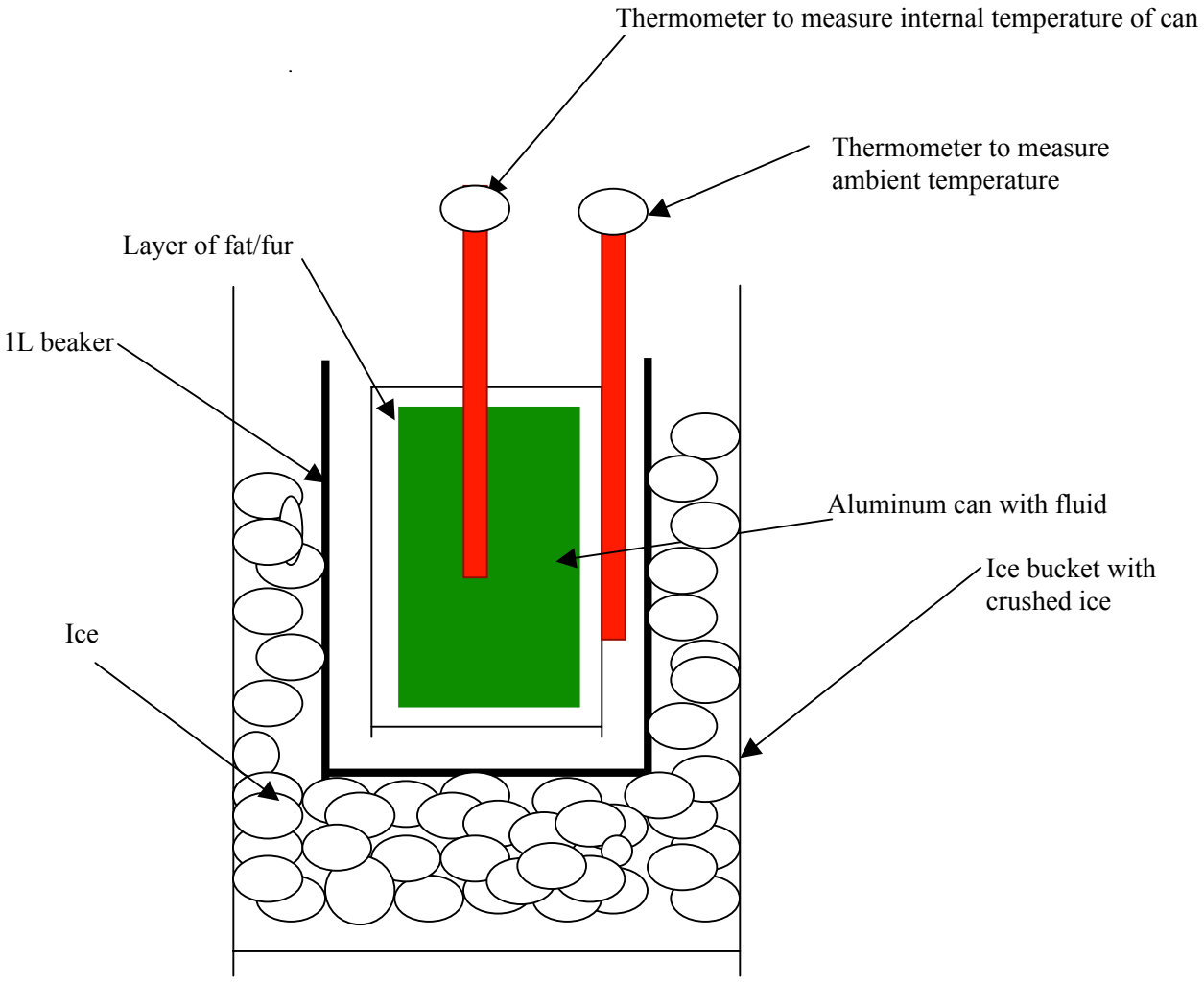

Figure A. Apparatus for measuring internal temperature of fluid in the can at ambient temperature of 8° C

Appendix B: Apparatus in measuring freezing point of fluid in a tube

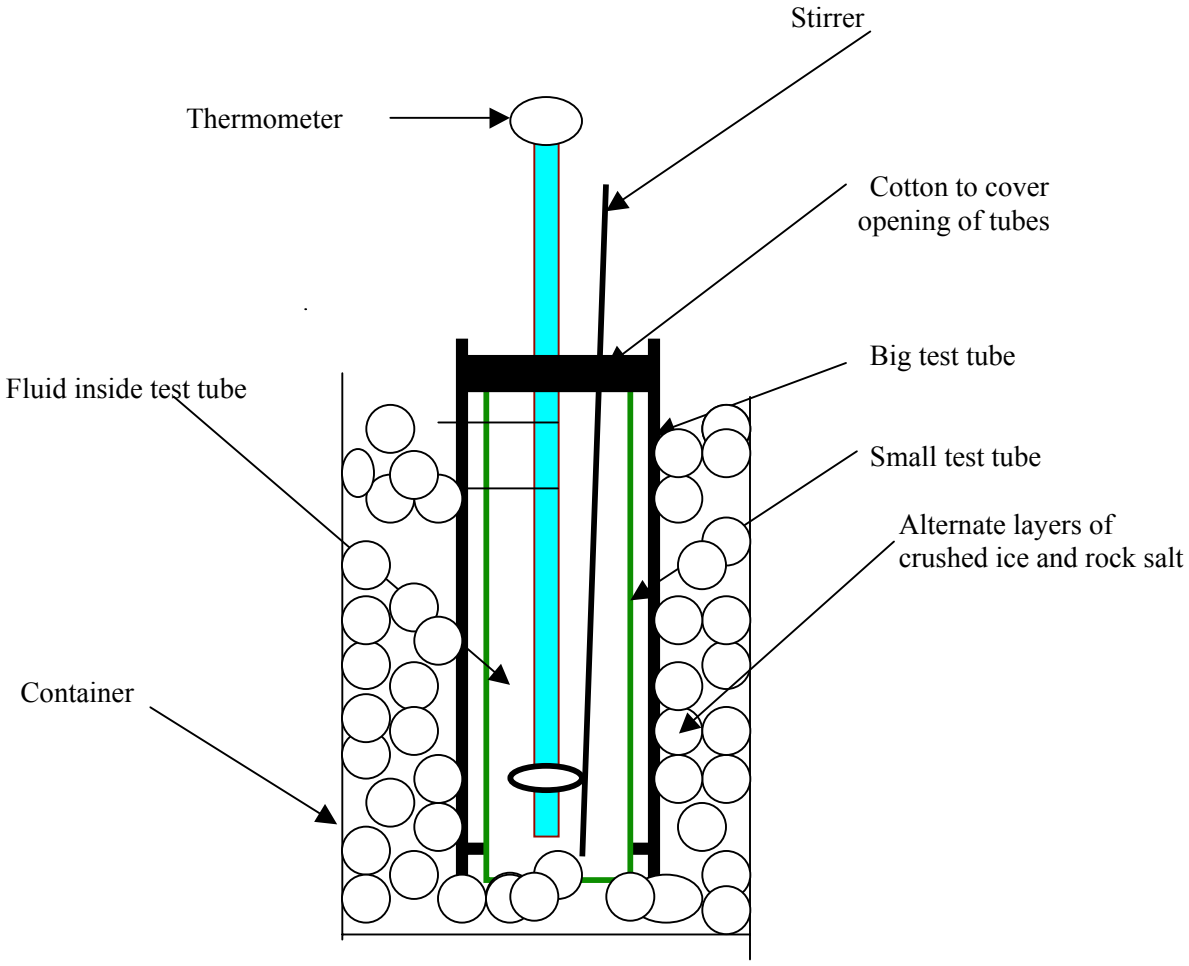

Figure B. Apparatus for measuring freezing point of fluid in the small test tube

Appendix C: Typical result showing temperature of fluid in cans with different insulations at ambient temperature of 8° C

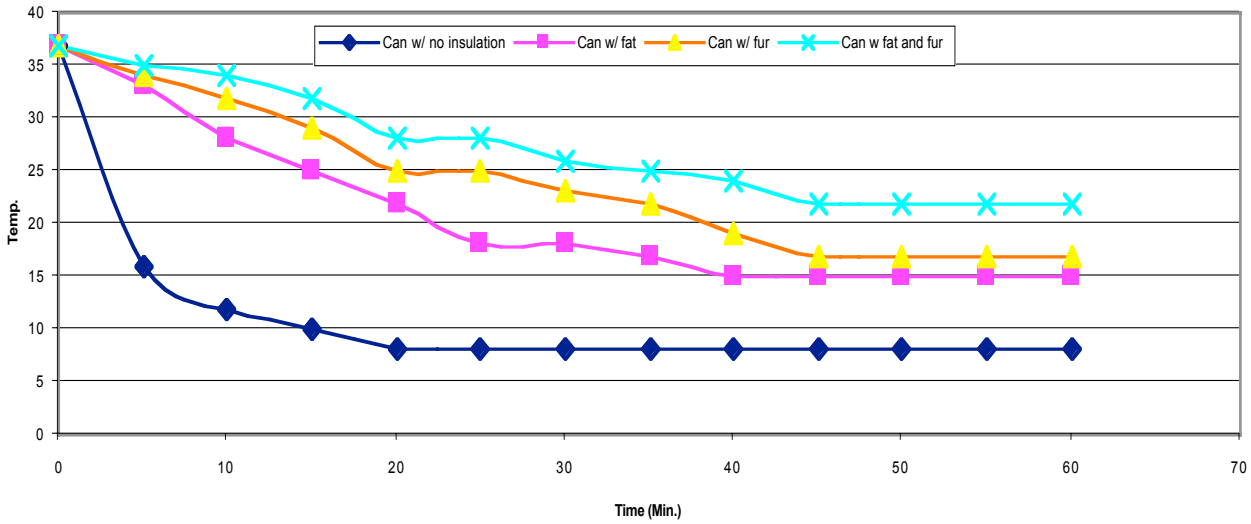

Figure C. Core temperature of control can (with no insulation), and cans with fur, fat and fur insulations in ambient temperature of 8°C

Appendix D: Rings in agar gel with starch as substrate

Clear rings in agar gel with starch substrate demonstrated the presence of amylase in different regions of cockroach gut. Three of the seven clear rings were amylase with various known concentrations. Wells without rings had gel buffer and boiled gut in them. Rings in agar gel with starch as substrate

Figure D. Typical results showing clear rings in agar gel with starch substrate demonstrated the presence of amylase in different regions of cockroach gut. Three of the seven clear rings were amylase with known concentrations. Wells without rings had gel buffer, and boiled gut in them.

Appendix E: Rings in agar gel with milk as substrate

Clear rings in agar gel (pH =7) with skim milk substrate demonstrated the presence of protease in different regions of cockroach gut. Three of seven rings were proteases with known concentrations. Wells without rings had gel buffer, pepsin and boiled gut in them.Rings in agar gel with protein as substrate

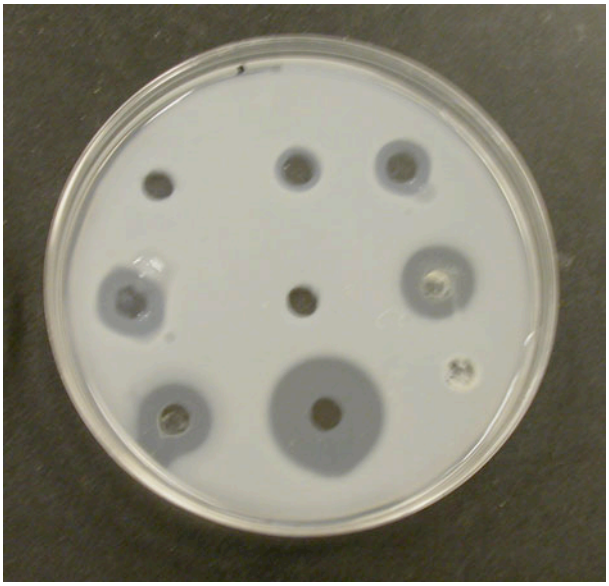

Figure E. Typical results showing clear rings in agar gel (pH =7) with skim milk substrate demonstrated the presence of protease in different regions of cockroach gut. Three of seven rings were proteases with known concentrations. Wells without rings had gel buffer, pepsin and boiled gut in them.