Chapter 14

Regulation of Blood Glucose Levels in Normal and Diabetic Rats

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Introduction

This laboratory exercise was designed for inclusion in an Introductory Organismal Biology Laboratory course, which is a freshman-level course. It could also be used in upper-division animal physiology courses. It was specifically designed to provide a valuable laboratory experience involving several mammalian body systems. Since the exercise was implemented in 1991, it has been both reliably successful and very popular with the students.

As designed, this exercise is fairly expensive to run; the major cost is the glucose reagent strips, but the costs for the rats and their maintenance are also significant. This exercise also requires more preparation time for the instructor(s) than most labs, particularly in the induction of chemical diabetes. To save time and money, the exercise could be conducted using only normal rats and omitting the diabetic rats. Students would not have the opportunity to observe diseased organisms' responses and learn about normal physiology from such observations, but it would still be a valuable learning experience.

In order to conduct this exercise, and, in fact, in order simply to purchase the rats, you must receive approval from your Institutional Animal Care and Use Committee. The use of this exercise in our course has been approved by the Institutional Animal Care and Use Committee at the University of Texas–Austin. In preparing a protocol for committee review, you may wish to consult Baker et al. (1980) and Waynforth (1980).

The Animal Care and Use Committee recommended that we not allow students to actually handle the rats, at least until we had conducted the exercise several times and become well-experienced with its operation. There is plenty for the students to do in the lab, even without handling the animals. For example, we have students weigh rats held in the weighing container, fill syringes, administer feedings, operate blood glucose monitors, tabulate data, and observe the animals for any unusual symptoms. Teaching assistants and assistant instructors need to be thoroughly trained for handling the rats and administering the treatments; these are simple procedures and don't require a great deal of time to learn.

The laboratory exercise as written takes about 3.5 hours to complete. It takes most classes about 1.5 hours to get set up and administer the treatments. It then takes 2 hours to monitor the rats' blood glucose levels. This much time is required in order to observe the normal rat administered a standard glucose tolerance test (i.e., the normal rat given a glucose feeding and a saline injection), increase its blood glucose level in response to the glucose feeding, and then decrease its blood glucose level to about the fasting level in response to its body's release of insulin. This portion of the exercise could be shortened somewhat, if necessary, to observe a decrease in this rat's blood glucose level but not a return to the fasting level..

Materials

The following quantities of materials are the numbers required for each group of students. I suggest that you have only one group per laboratory room, unless your classes are very large and you have two teaching assistants or assistant instructors. You should order about 30% more rats than are needed for the exercise, as some of the rats injected with Alloxan will likely die and some will likely not become diabetic.

Rats (*Rattus rattus*), weighing about 150 g each, fasted for 12–18 hours, housed together in one or two cages (6; these will include 3 normal rats and 3 chemically diabetic rats)
Balance, 500 g capacity (1)
Plastic container with lid for weighing rats (1)
Cloth gloves (2 pairs)
Restraint devices of two different sizes, depending on the sizes of the rats (2)
Nembutal (sodium pentobarbital) solution (10 mg Nembutal per 1 ml solution, in physiological saline), 5 ml, sterile, in a covered 16 × 100 mm test tube (1)
Dissecting scissors, small (1)
Razor blade (1)
Ethanol (95%), 40 ml, in a 50 ml beaker (1)
Blood glucose monitors (Tracer II, Boehringer Manheim Diagnostics), + operator's manual (3)
Reagent (test) strips for blood glucose monitor (140)
Cotton balls, white (150)
Ethanol (95%), 100 ml, in a 125 ml flask (1)
Ethanol (70%), 40 ml, in a 50 ml flask (1)
Cotton-tipped sticks (10)
Kimwipes, boxed (1)
Glucose solution (1 g glucose per 2 ml solution, in distilled water), 20 ml, in a 50 ml beaker (1)
Distilled water, 20 ml, in a 50 ml beaker (1)
Insulin solution (1.0 Units insulin per 0.25 ml solution, in physiological saline), 5 ml, sterile, in a covered 16 × 100 mm test tube (1)
Physiological saline solution (0.85% NaCl in distilled water), 5 ml, sterile, in a covered 16 × 100 mm test tube (1)
Feeding tubes (6)
Syringes, 1 ml, sterile (12)
Needles, 25 G 5/8, sterile (12)
Syringes, 5 ml, clean (6)
Beakers, 50 ml (2)
Paper towels, folded or rolled (30)
White tape, on a roll (1)
Permanent marker, black (1)
Rat food, about 30 g, in a 100 ml beaker (1)

Student Outline

Introduction

The circulatory system of an animal transports nutrients, oxygen, water, and other substances throughout its body, thus providing every cell with the products it needs for metabolism and growth. Nutrients and water are absorbed by the blood as it travels through the organs of the animal's digestive system. Animals cannot eat constantly yet their cells and the tissues and organs must constantly be supplied with nutrients from which they can obtain energy. Energy stored in the form of fat or protein

can be converted in most tissues to a compound which can enter the Kreb's cycle and from which energy can be derived. The tissues of the vertebrate brain and a few other tissues, however, can use only glucose as their source of energy. Because of this requirement, it is important that the concentration of glucose in the blood remains nearly constant. The maintenance of a nearly constant blood glucose level is achieved by a complex set of interactions which include regulation by a number of hormones of the endocrine system.

The hormone insulin is secreted into the blood when a vertebrate eats and the glucose level in the blood increases. Among the numerous functions of insulin is the stimulation of the uptake, storage, and use of glucose by tissues of the body. These activities result in a decrease in the level of glucose remaining in the blood. Insulin, in turn, is broken down rapidly; this prevents the blood glucose level from continuing to drop. Several other hormones, such as glucagon, promote the release of stored energy reserves into the blood and thus increase blood glucose levels. Vertebrates that have diabetes either produce insufficient quantities of insulin or have insufficient numbers of insulin receptor sites in target cell membranes. In either case, these animals are unable to maintain nearly constant blood glucose levels because cellular uptake is inadequately stimulated.

Objectives

1. To make direct observations on some of the interacting functions of the mammalian circulatory, digestive, and endocrine systems.

2. To observe the effects of fasting, fasting followed by a glucose load, and fasting followed by a glucose load plus an injection of insulin on the level of glucose in the blood of normal and chemically diabetic rats.

3. To gain an understanding of some of the complex processes involved in maintaining a nearly constant blood glucose level in a mammal.

4. To gain an appreciation for the speed of response of the normal (i.e., non-diabetic) mammalian body to changes in blood glucose levels and the very tight control it maintains over these levels.

5. To gain an understanding of the significance of the role of insulin in regulating the level of glucose in the blood of a mammal.

Principles

The Glucose Tolerance Test is a standard test used to determine whether or not a mammal produces sufficient insulin to promote the uptake of glucose from the blood after it is given a high glucose feeding. This test must be administered after a significant period of fasting to ensure that the level of glucose in the blood is low enough that it does not trigger the release of more than trivial amounts of insulin.

A standard Glucose Tolerance Test will be performed on two rats in this exercise: one of these will be a normal rat and the other will be a chemically diabetic rat. (Chemical diabetes is induced by the administration of a substance, Alloxan, in our case, which is cytotoxic specifically for the ß-cells of the Islets of Langerhans in the pancreas.) This test involves administering a glucose dose orally, through a feeding tube. (This is preferable to letting the rats drink the glucose willingly because you will know exactly how much glucose each rat receives.) A second pair of normal and diabetic rats will be given a standard Glucose Tolerance Test plus they will receive a treatment of exogenously supplied insulin (by injection); this treatment will emphasize the effect of this hormone on the level of glucose in the blood. A third pair of normal and diabetic rats, the controls, will continue to fast for the

duration of the exercise. The pair of rats that do not receive a glucose load will be administered a water "load" and the two pairs of rats that do not receive an insulin injection will instead be administered injections of saline. These placebo treatments will enable you to separate the effects of the glucose and insulin treatments from the effects of the actual process of administering a substance. Blood samples will be taken from all of the rats at 20-minute intervals during the 2-hour test period; these will be analyzed for the level of glucose they contain.

Procedures

Part A: Assemble Materials

Before you begin this exercise, make certain that your group has all of the following materials readily available:

Rats (*Rattus rattus*), weighing about 150 g each, fasted for 12–18 hours, housed together in one or two cages (6; these will include 3 normal rats and 3 chemically diabetic rats)
Balance, 500 g capacity (1)
Plastic container with lid for weighing rats (1)
Cloth gloves (2 pairs)
Restraint devices of two different sizes, depending on the sizes of the rats (2)
Nembutal (sodium pentobarbital) solution (10 mg Nembutal per 1 ml solution, in physiological saline), 5 ml, sterile, in a covered 16 × 100 mm test tube (1)
Dissecting scissors, small (1)
Razor blade (1)
Ethanol (95%), 40 ml, in a 50 ml beaker (1)
Blood glucose monitors (Tracer II, Boehringer Manheim Diagnostics), + operator's manual (3)
Reagent (test) strips for blood glucose monitor (140)
Cotton balls, white (150)
Ethanol (95%), 100 ml, in a 125 ml flask (1)
Ethanol (70%), 40 ml, in a 50 ml flask (1)
Cotton-tipped sticks (10)
Kimwipes, boxed (1)
Glucose solution (1 g glucose per 2 ml solution, in distilled water), 20 ml, in a 50 ml beaker (1)
Distilled water, 20 ml, in a 50 ml beaker (1)
Insulin solution (1.0 Units insulin per 0.25 ml solution, in physiological saline), 5 ml, sterile, in a covered 16 × 100 mm test tube (1)
Physiological saline solution (0.85% NaCl in distilled water), 5 ml, sterile, in a covered 16 × 100 mm test tube (1)
Feeding tubes (6)
Syringes, 1 ml, sterile (12)
Needles, 25 G 5/8, sterile (12)
Syringes, 5 ml, clean (6)
Beakers, 50 ml (2)
Paper towels, folded or rolled (30)
White tape, on a roll (1)
Permanent marker, black (1)
Rat food, about 30 g, in a 100 ml beaker (1)

The rats will already have been identified by your instructor with different numbers of bands drawn with permanent marker at the base of the tails. Either rat #1 or rat #2 will be normal and the

other will be diabetic. Either rat #3 or rat #4 will be normal and the other will be diabetic. Either rat #5 or rat #6 will be normal and the other will be diabetic. You will not be told which rat of each pair is normal and which is diabetic.

The rats you will be using in this exercise are very docile animals. This is true even after they have fasted for 12–18 hours. Nevertheless, because we wish to reduce trauma to the animals, your teaching assistant or assistant instructor (TA/AI) will be responsible for handling them. Please do not attempt to handle the animals yourself.

Part B: Determine the Treatment Dosages

1. *Your TA/AI will weigh the rats.* One of the students should first determine the weight (to the nearest half of a gram) of the empty weighing container using the balance; record the weight.

2. The TA/AI will remove one of the rats from the cage, place it in the weighing container, and affix the lid. The total weight of the rat and the container should then be determined. The rat should then be placed back inside the cage and the cover of the cage replaced. Write down any other identifying characteristics of the rat, including its sex, color, coloration pattern, etc. You should then calculate and record the weight of the rat alone.

3. The weighing procedure should be repeated for the remaining five rats.

4. Calculate the amount of the glucose solution that rats #1 and #2 should get for their glucose doses. These rats will each get glucose plus a placebo injection of physiological saline. The glucose dose a rat receives is determined by its weight as follows:

   Glucose dose: 0.5 g glucose per 100 g body weight
   Glucose solution: 1 g glucose per 2 ml solution

5. Calculate the amount of placebo solution that rats #1 and #2 should get in their injections. The dose of placebo that a rat receives is determined by its weight as follows:

   Placebo dose: 0.125 ml saline per 100 g body weight

6. Calculate the amount of glucose solution that rats #3 and #4 should get for their glucose doses. These rats will get both glucose and insulin. Use the same procedure you used for calculating the glucose dose for rats #1 and #2 (in step 4 above).

7. Calculate the amount of insulin solution that rats #3 and #4 should get for their insulin doses. The insulin dose a rat receives is determined by its weight as follows:

   Insulin dose: 0.5 Units insulin per 100 g body weight
   Insulin solution: 1.0 Units insulin per 0.25 ml solution

8. Calculate the amount of water that rats #5 and #6 should get for their placebo feeding. These rats will get a placebo feeding plus a placebo injection. The placebo feeding dose a rat receives is determined by its weight as follows:

   Placebo feeding dose: 1.0 ml water per 100 g body weight

9. Calculate the amount of placebo solution that rats #5 and #6 should get in their injections. Use the same procedure you used for calculating the placebo dose for rats #1 and #2 (in step 5 above).

10. Calculate the amount of Nembutal that each of the rats should get to subdue them initially for the blood samplings and the administration of treatments.

Nembutal dose: 1.0 mg per 100 g body weight
Nembutal solution: 10 mg Nembutal per 1 ml solution

The rats may be given 20% of this amount (0.2 mg per 100 g body weight) in subsequent injections at 30 to 40 minute intervals throughout the exercise to keep them subdued. Calculate this dosage for each of the six rats used in your exercise.

11. Have your TA/AI check your calculated dosages of glucose, insulin, placebo, and Nembutal solutions.

Part C: Designate Groups of Students for Monitoring Different Rats

Your TA/AI will divide your class into six groups of two to three students each. Each of these six groups of students will be monitoring the blood glucose levels of all six rats; however, only half of the groups will do monitoring at each of the 20-minute time intervals. For example, groups #1, 2, and 3 will monitor at the start of the exercise, and groups #4, 5, and 6 will monitor 20 minutes after the start of the exercise. At the first monitoring time, groups #1, 2, and 3 will each receive one blood sample from each of the six rats. At the next monitoring time, groups #4, 5, and 6 will each receive one blood sample from each of the six rats. Thus, there will be three replicates of the data obtained for each rat at each monitoring time. At the end of the exercise, the data from all of the groups will be pooled and analyzed together.

Part D: Prepare Your Data Collection Sheet

You will find a data collection sheet on which you will record the data that your class obtains in this exercise in Table 14.1. Using a red or a blue pen, circle your group number wherever it appears on this data sheet. You will record the data that your own group collects in these rows. You will later collect data from the other groups to fill in the other rows of Table 14.1.

Part E: Subdue the Rats with Nembutal

This part will be performed by your TA/AI.

Part F: Determine Fasting Blood Glucose Levels

1. *Your TA/AI will be responsible for obtaining the blood samples from the rats for these determinations.* You will obtain the blood samples from him/her and determine the blood glucose levels using the blood glucose monitors. The rats' cage should be opened at the top and a restraint device of the appropriate size for rat #1 placed inside the cage. The TA/AI will place rat #1 in the restraint device. The rubber stopper should be securely fitted into the open end of the device, with the rat's tail protruding out through the v-shaped notch in the stopper. The rat in the restraint device should then be removed from the cage and placed on a counter with a piece of paper towel under its tail. The rats' cage should then be closed.

2. One of the students should place the dissecting scissors, opened and pointed-end down, into the second beaker containing 95% EtOH. If preferred, you may use the razor blade, cleaned in alcohol, instead.

3. If you are a member of group #1, 2, or 3, remove a reagent strip from the container in the glucose monitoring kit and place it right side up on a paper towel.

Table 14.1. Blood glucose levels (mg glucose per deciliter blood).

Time (minutes)	Group	Rat #					
		1	2	3	4	5	6
0	1						
	2						
	3						
20	4						
	5						
	6						
40	1						
	2						
	3						
60	4						
	5						
	6						
80	1						
	2						
	3						
100	4						
	5						
	6						
120	1						
	2						
	3						

4. Press the On/Off button on the glucose monitor and wait for the code to be displayed. The code should be the same as that on the container for the reagent strips (use the code for "Tracer II"). If it is not, enter the correct code for the reagent strip by following the steps in the operator's manual.

5. You will see a symbol of a blood drop and of a reagent strip displayed to the right of the code on the monitor. The blood drop symbol will be blinking, indicating that the monitor is properly prepared for a sample.

6. One of the students should remove the scissors from the EtOH and dry them *thoroughly* with a kimwipe.

7. Your TA/AI will provide a blood sample for groups #1, 2, and 3 from the same rat, at the same time. When he/she is finished with rat #1, the scissors should be placed, opened and pointed-end down, back into the beaker containing 95% EtOH.

8. As soon as the blood drop has been applied to the reagent strip, push the start button on the glucose monitor. You will then see the time elapsed, in seconds, displayed on the screen. The symbol of the blood drop will also be displayed.

9. The glucose monitor will emit short beeps at 57, 58, and 59 seconds after the start time. At 60 seconds, it will emit a longer beep. At this time, take a cotton ball and carefully wipe off any liquid blood on the test site (all traces of blood must be removed). Then immediately insert the test strip in the strip slot (in the upper left corner of the monitor); push it in as far as it will go. The strip will protrude vertically out of the monitor and *must* have the side with the test pad site and the letters "bG" facing the words "Tracer II" on the monitor.

10. The monitor will continue to display the time elapsed, along with the symbol of a reagent strip, until it reaches 120 seconds. There will then be a pause with a dash displayed. This will be followed by a display of the blood glucose level of the sample, expressed in mg glucose per deciliter of blood. Record this number on your data sheet in the appropriate place.

11. Remove the used test strip from the strip slot and discard it. Turn the monitor off.

12. The rat in the restraint device should be placed back inside the cage. The rubber stopper should be removed from the restraint device and the rat let out. The restraint device should be removed and the cage closed.

13. Steps 1 through 12 above should now be repeated for rats #2 through #6, using appropriately sized restraint devices. Before your TA/AI snips the tails of these rats, the dissecting scissors should be removed from the EtOH and wiped *thoroughly* dry with a kimwipe. The scissors should be placed in the EtOH between sampling from different rats.

14. Note that if a test result is less than 40 mg/d liter, "LO" will appear on the monitor display screen. If a test result is more than 400 mg/d liter, "HI" will appear on the monitor display screen.

Part G: Administer Treatment Dosages

1. One student should pour about 15 ml of the glucose solution into one of the small, empty beakers. Another student should pour about 15 ml of distilled water into the other small, empty beaker. Label both beakers with white tape and permanent marker.

2. One student should fill one of the 5-ml syringes (without a needle) with the correct amount of glucose solution for rat #1. Insert the tip of the syringe into one of the feeding tubes. Do *not* depress the plunger in the syringe. Set the syringe and feeding tube down on a clean paper towel. Label the syringe "glucose" and the feeding tube "rat #1" with tape and permanent marker.

3. Your TA/AI should attach a sterile needle to a sterile 1 ml syringe. The cap should be removed from the syringe. The syringe and needle should be inserted into the bottle containing the placebo solution and the correct amount of solution obtained for rat #1. The sample should have as few air bubbles as possible. The bubbles will not hurt the rat but they will make it difficult to obtain the exact amount of solution needed. The stopper should be replaced on the placebo solution bottle. The cap should be replaced loosely on the needle and the syringe and needle set down on a clean paper towel. Label the syringe "rat #1" with tape and permanent marker.

4. Your TA/AI should now insert the feeding tube into rat #1. One student should record the time at which the glucose dose was administered.

5. Next, your TA/AI will administer insulin or a placebo.

6. One student should record the time at which the injection was administered. Rat #1 should now be returned to its cage by the TA/AI.

7. One student should fill a 5 ml glucose syringe (without a needle) with the correct amount of glucose solution for rat #2 (see step 2 above). Label the feeding tube "rat #2" and label the syringe "glucose."

8. Your TA/AI should attach a second sterile needle to a second sterile 1 ml syringe. The syringe should be filled with the correct amount of placebo solution for rat #2 (see step 3 above). Label the syringe "rat #2."

9. Step number 4 above should be repeated to administer the glucose dose to rat #2.

10. Step number 5 above should be repeated to administer the placebo dose to rat #2. Record the time at which the placebo dose was administered. Rat #2 should now be returned to its cage.

11. Steps number 2 through 10 above should be repeated for rats #3 and 4, with the following changes:

    (a) Label the feeding tubes "rat #3" and "rat #4."

    (b) Label the 1-ml syringes "rat #3" and "rat #4."

    (c) Rats #3 and 4 will receive an injection of insulin instead of the placebo injection of physiological saline. The sterile 1-ml syringe can be inserted into the bottle of insulin and the appropriate amount of insulin removed. The stopper should then be replaced on the bottle.

12. Steps number 2 through 10 above should be repeated for rats #5 and 6, with the following changes:

    (a) Label the feeding tubes "rat #5" and "rat #6."

    (b) Rats #5 and 6 will receive a placebo water "feeding" instead of glucose. Fill the second 5 ml syringe with water and attach it to the intubation needle. Label the syringe "water."

    (c) Label the 1-ml syringes "rat #5" and "rat #6."

    (d) Rats #5 and 6 will also receive a placebo injection of physiological saline. The sterile 1-ml syringe can be inserted into the bottle of saline and the appropriate amount of saline removed. The stopper should then be replaced on the bottle.

13. Rinse the six feeding tubes thoroughly with tap water and return them to the supply table (on a piece of paper towel).

Part H: Monitor Blood Glucose Levels for 2 Hours

1. If you are a member of group #4, 5, or 6, remove a reagent strip from the container in the glucose monitoring kit and place it right-side up on a paper towel. Group #4 should use the same glucose monitor kit as group #1, group #5 should use the glucose monitor kit used by group #2, and group #6 should use the glucose monitor kit used by group #3.

2. Your TA/AI will place a restraint device appropriate for rat #1 in the cage a few minutes before the end of the 20-minute period immediately following the administration of its glucose dose. Following the procedure outlined in section F above, your TA/AI will obtain blood from rat #1. The rat in the restraint device should be removed from the cage and placed on a counter with a paper towel placed under its tail. The rat cages should be closed.
3. A blood sample will be obtained from the rat by the TA/AI for each of the groups #4, 5, and 6. The students in these groups should then determine the blood glucose level of the samples according to the procedures described previously in Part F, steps 1 through 12, with the following modification: *Only the dried blood at the tip of the rat's tail needs to be removed in order for a fresh drop of blood to be obtained.* Use a cotton ball dipped in the 95% EtOH in the flask to remove the dried blood. Make sure that you use the *second* drop of blood that you obtain from the rat's tail for the blood glucose determination (because the first drop may be diluted with ethanol, which would give an erroneous reading).
4. Steps 1 through 3 above should now be repeated for rats #2 through 6.
5. The blood sampling and glucose determination procedures should be repeated for all six of the rats at 20-minute intervals. Remember that student groups #1, 2, and 3 will alternate with groups #4, 5, and 6 the times at which they will determine blood glucose levels. These procedures should be continued until a period of 2 hours from the time of the glucose administrations has elapsed.
6. Clean the blood glucose monitor periodically during the exercise, as described in the operator's manual. Use the 70% ethanol and the cotton-tipped sticks for cleaning.
7. After 30 to 40 minutes from the time of the initial injection of Nembutal, each of the rats may be given a second injection. *These injections will be administered by your TA/AI, as described in Part E above.* The dosage for this injection should be 20% of the amount given in the initial injection and should have been calculated previously. Record the time at which the second injection was administered. Inform your TA/AI when 30–40 minutes have elapsed from the time of the second injection so that he/she can administer another injection, if necessary. These injections may be repeated for the duration of the exercise.
8. Recall that a blood sample that gets a "LO" result on the blood glucose monitor contains less than 40 mg of glucose per deciliters of blood. If any of the blood samples you obtain gets a "LO" reading, watch the rat carefully. If it has *two* consecutive readings of "LO," you will need to terminate the experiment for this animal. Notify your TA/AI immediately. He/she should fill the 5 ml glucose syringe with about 2 ml of the glucose solution. He/she should hold the rat and insert the tip of the syringe (without a needle, of course) in the rat's mouth. The plunger of the syringe should be gently depressed, allowing the rat to swallow the glucose solution, until it will not take any more. Return the rat to its cage. It should recover from any signs of weakness or lethargy within 10 to 15 minutes.
9. Make sure that all of the rats receive their regular food as soon as possible after the end of the 2-hour monitoring period.

Results and Analysis

1. Make a graph of the results you obtained in this exercise, plotting blood glucose level as a function of time. Consider the time at which a glucose dose was administered as time=zero and plot the fasting blood glucose level at this point.

2. Discuss the results of your experiment in terms of the mammalian circulatory, digestive, and endocrine systems. What were the physiological effects of fasting on the rats and of the different treatments tested? Were the results you obtained similar to those that you had predicted you would observe? If not, can you suggest an explanation for the difference(s)?

3. Explain why the rats' fasting blood glucose levels were in the range that you observed. Can you predict which of the rats are diabetic from the fasting blood glucose levels? What physiological mechanism keeps the blood glucose level in normal, fasted rats from going much lower?

4. Can you predict which of the rats are diabetic from the results of the different treatments tested? Explain your answers.

5. What would you expect to be the effects of administering insulin alone to one of the normal, fasted rats? What about the effects of administering insulin alone to one of the diabetic, fasted rats?

6. Describe the sequence of events relating to insulin and blood glucose that occur under normal conditions when a (non-diabetic) mammal ingests a meal.

Notes for the Instructor

Obtaining Rats

The rats for this exercise can be obtained through the National Cancer Institute, which is a part of the U.S. Department of Health and Human Services (301/846-1151; ask to speak with Ms. Kim Cassidy). You are required to have an approved protocol number from your Institutional Animal Use and Care committee in order to purchase the rats.

Preparations Before the Laboratory Period

To begin at least 4 days before lab:

Obtain seven rats weighing about 150 g for your group of students. Three of these will simply be housed until the day of the laboratory exercise and will be the non-diabetic (normal) rats in the exercise. Four of the rats should be treated to make them chemically diabetic, and three of these four rats will be used in the student exercise. (See Appendix A for the procedures for inducing diabetes; these procedures should be conduced 4–5 days prior to the day of the laboratory exercise.) The rats must be obtained from a supplier authorized by the U.S. Federal Government. Try to get rats all of the same sex and avoid getting any rats that are obviously pregnant. The cage must have a floor area of at least 140 square inches and be at least 7 inches high. Supply the rats with water and rat pellets. Twelve to eighteen hours before the beginning of the laboratory period, remove the rats' food.

Check the glucose monitoring equipment and make sure it is in good operating condition. Clean the equipment, replace old batteries, and obtain additional reagent strips if necessary.

To be completed the day before or on the day of the lab:

1. Prepare the glucose, insulin, and Nembutal solutions as follows:

Glucose: In a sterilized beaker, combine 20 ml of distilled water and 10 g of glucose. Stir the solution while heating it slightly until the sugar is completely dissolved. Pour the solution into a sterilized, stoppered bottle (about 30 ml capacity). Label the bottle and keep it refrigerated.

Insulin: Either bovine, porcine, or a combination of these two types of insulin may be used. We use the "regular" (i.e., fast-acting) form of the insulin. It is supplied in a sealed 10-ml bottle at a concentration of 100 Units of insulin per ml. Obtain a sterilized, stoppered bottle (about 30 ml capacity). Add to this bottle 24 ml of sterile physiological saline (0.85% NaCl in distilled water). Then add 1 ml of the insulin. To do this you will need a sterile syringe and a sterilized, small gauge needle (e.g., 25 g). Retract the plunger of the syringe to the 1 ml mark, insert the needle through the rubber seal on the bottle, and turn the bottle and syringe upside down. Depress the plunger and then fill the syringe with 1 ml of insulin. Be careful to avoid air bubbles as much as possible because they will make it difficult to get the exact amount of insulin that you need. Place the stopper in the bottle and swirl it gently to mix the contents. Label the bottle and keep it refrigerated. Do not autoclave the insulin solution.

Nembutal: Obtained a sterilized, stoppered bottle (about 100 ml capacity). Add to this bottle 4.5 ml of sterilized physiological saline. Then add 50.0 mg of Nembutal (the amount of liquid Nembutal solution required should be determined by the solution concentration stated on the bottle). It is supplied in a sealed bottle similar to the one in which the insulin is supplied; you will need to use a syringe to remove the Nembutal from the bottle and add it to the saline. Place the stopper in the bottle and swirl it gently to mix the contents. Label the bottle and keep it refrigerated.

2. Identify the rats by drawing the appropriate number of lines with a permanent marker across the base of each rat's tail. Either rat #1 *or* #2 should be a diabetic rat; either rat #3 *or* #4 should be a diabetic rat; and either rat #5 *or* #6 should be a diabetic rat. Keep a record of this information, but do not give it to the students. Keep the rats in two cages.

Procedures Performed by the Instructor During the Laboratory Period

General procedures for handling of the rats (in Part A):

1. These rats are docile creatures and can be easily handled. It is important, however, that when they are held it is done with a firm grasp. Rats are extremely agile creatures, and they can squirm out of a person's hands and jump to the floor if they are not held securely.

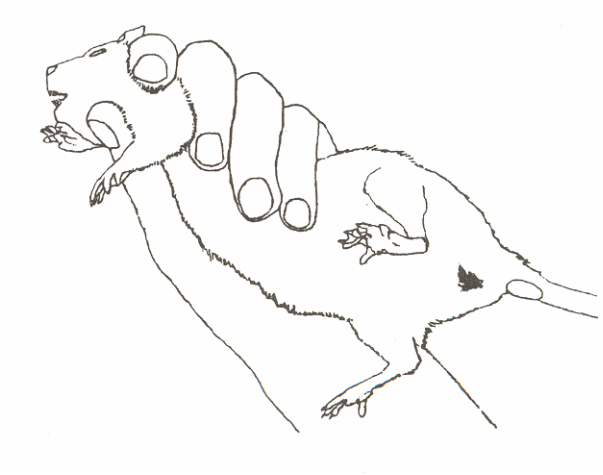
2. When picking up a rat, it is usually easiest to grab hold of some of the loose skin on its back. The handler should then put his/her thumb behind the forelimb on that side of the hand and his/her forefinger in front of the forelimb on the other side; the thumb and forefinger should pass around the animal's neck, with the thumb ending up under the rat's jaw (see Figure 14.1). This will prevent the animal from biting or moving its head. The remaining fingers should be placed around the rat's body, extending as far down the body as possible. The rat must be held firmly until it is to be put back into its cage.

3. If a rat starts to get away, it can be grabbed by its tail. However, it is generally advisable to pick it up by its back with the handler's other hand rather than pick it up by its tail.

4. The cloth gloves may be used when the rats are handled. They will provide good protection in the unlikely event that a rat should bite, and they will not significantly hamper the handler's dexterity

as thick rubber gloves would. If the gloves will give the handler the confidence to hold the rats firmly and securely, then he/she should certainly wear them.

5. The rats may urinate and/or defecate while they are being handled; try not to startle if this happens. When the rat has been put back in its cage, clean up any soiled equipment and thoroughly wash your hands.



Administering Nembutal to the rats (in Part E):

Remove the rats one at a time from the cage and inject them with the previously calculated initial dosages of Nembutal. This injection should be made using a sterile 1 ml syringe with a sterile 25 G needle. It should be administered intraperitoneally in the lower left quadrant of the abdomen (to avoid injection into hollow or solid organs); only the tip of the needle needs to be inserted into the peritoneal cavity. Hold the rat firmly in your other hand, as described previously. Label the syringe used for each of the rats (using tape and permanent marker) with the appropriate number of the rat; place them needle-end down, in one of the beakers of EtOH. Following the injection, each rat should be returned to the cage. Have one of the students record the time at which the injection was made for each of the rats.

Bleeding the rat (in Part F):

Snip off the very tip of the rat's tail. This should involve removing not any more than a few millimeters of the tail and will cause very little discomfort to the subdued rat. If blood does not begin to appear at the tip of the tail, cut it again a little closer to the animal's trunk.

The rat's tail should be gently squeezed, starting at the base of the tail and moving towards the site of the cut, to get a drop of blood to form at the incision site. When a *complete* drop is visible, the drop of blood should be carefully brought in contact with the test pad site on the reagent strip (the yellow rectangle). It must be a large enough drop to nearly cover the entire test portion; you may have to move the tip of the tail as you touch it to the test pad site to get it to cover the area. The test will *not* be accurate if the blood sample does not contain a full drop of blood or if it consists of two separate drops.

Inserting intubation needle and administering oral solutions (in Part G, step 4):

Pick up a rat and hold it firmly with one hand. Pick up the appropriate syringe with feeding tube and insert the tip of the tube into the rat's mouth. The plunger on the syringe should be depressed a little bit so that the rat gets a taste of the glucose solution. Since the rat will be hungry,

this taste will make him eager to get more of the solution. The tip of the intubation tube should be well inside the rat's mouth, but not down its esophagus. The tube should be kept towards the side of the rat's mouth so that he can't bite the tube with his very sharp incisors. The plunger on the syringe should be slowly depressed until the rat has swallowed its entire contents. The feeding tube should be gently removed.

Administering insulin or placebo injection (in Part G, step 5):

Pick up the syringe containing the placebo solution and remove the cap. Position the rat so that the muscles depicted in Figure 14.2 are accessible. The placebo may be injected into any of these muscles. The syringe should be held at a narrow angle to the rat's body (*not* perpendicular to its body). The tip of the needle should be inserted through the rat's skin; the resistance of the skin can be felt as this is done. The needle should not be inserted too deeply or bone may be encountered. The plunger of the syringe should then be depressed to administer the placebo. The needle should then be withdrawn, recapped, and discarded in a container specifically designated for needle disposal.

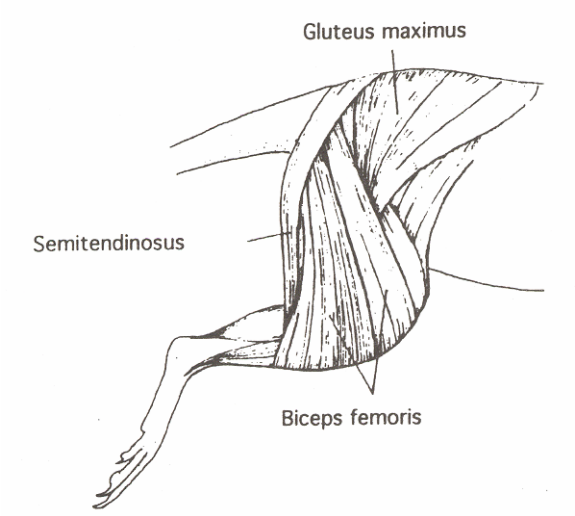


Figure 14.2. Muscles for injection of insulin or saline.

Safety Precautions

This exercise involves working with live animals that have sharp teeth. Even though laboratory rats are docile animals, the possibility of someone being bitten does exist. Because of this, and because many of the students may not be able to handle the rats properly, it is best if the teaching assistant, assistant instructor, staff, or faculty member does all of the handling of the rats. Rats that are held firmly and handled confidently are very unlikely to bite. This exercise also involves working with hypodermic needles. The needles must always be handled with care. They should be capped except when a treatment is being administered. After they have been used, they should be discarded in a container specifically designated for needle disposal. The students must be instructed to *never* walk around the laboratory room carrying an uncapped syringe.

Supplemental Information

Be sure that each group of students checks their calculated dosages of glucose, insulin, placebo, and Nembutal solutions with you before these are administered to the rats. You can use the following calculations to check each student's determinations:

For a rat weighing 200 g:

Glucose dose:	200 g × 0.5 g glucose per 100 g = 1.0 g glucose
	1.0 g glucose × 2 ml glucose solution per 1 g glucose = 2 ml glucose solution
Insulin dose:	200 g × 0.5 U insulin per 100 g = 1.0 U insulin
	1.0 U insulin × 0.25 ml insulin solution per 1.0 U insulin = 0.25 ml insulin solution
Nembutal dose:	200 g × 1.0 mg Nembutal per 100 g = 2 mg Nembutal
	2 mg Nembutal × 1 ml Nembutal solution per 10 mg Nembutal = 0.2 ml Nembutal solution

It is extremely important that the students follow the directions for using the blood glucose monitoring equipment very carefully. Using less than a full drop of blood, failing to remove all of the blood when wiping with the cotton or rayon ball, and/or improperly following the timing instructions can result in erroneous values. You might want to assign one student in each group to thoroughly read the operator's manual for the monitor (say, while you are weighing the rats) and to operate the monitor during the exercise. Have the students clean the monitors periodically during the exercise, as described in the operator's manual. They should use the 70% ethanol and the cotton-tipped sticks for cleaning the monitors.

As the different groups of students in your laboratory work on this exercise, periodically observe the rats that receive an insulin injection. Be alert to unusual behavior on the part of these animals. If any one of them appears particularly sleepy or lethargic, its body seems limp, or it exhibits any other unusual symptoms, check the blood glucose results the students have obtained for the animal. If there is any doubt about the animal's condition, make sure that you immediately administer a dose of glucose solution to the rat as described in the laboratory protocol. This animal will then be eliminated from the remainder of the experiment. This will *not* be cause to terminate the experiment, however. The students will include the effects on this rat, up to the time it was removed from the experiment, as part of their results.

Answers to Results and Analysis Questions

1. Graphs will vary depending on results with individual rats. Check that students have followed all of the instructions for presentation of data in graphic form. We typically find that about 20% of the diabetic rats have normal, or approximately normal, fasting blood glucose levels. An elevated fasting blood glucose level should not be the only piece of evidence that a student uses to determine which rat of each pair is diabetic.

   The first pair of rats (#1 and 2) should show dramatically different responses to their experimental treatment (a glucose load). The non-diabetic rat's blood glucose level should become elevated and return to approximately the fasting level within about 2 hours. The diabetic rat's blood glucose level will likely rise and remain quite high for the duration of the experiment.

The second pair of rats (#3 and 4) may be more difficult to distinguish based on their responses, especially if the diabetic one does not exhibit an elevated fasting blood glucose level. Both will likely demonstrate a drop in their blood glucose levels beginning early in the experiment; the drop is usually more rapid and more dramatic in the non-diabetic rats (typically, some of these rats will have to be removed from the experiment because their blood glucose levels drop below 40 mg glucose per deciliter blood).

The third pair of rats (#5 and 6) can usually be distinguished, even if the diabetic rat does not exhibit an elevated fasting blood glucose level. The non-diabetic rat will likely show some drop in its blood glucose level, while the diabetic rat will likely show little or no drop in its blood glucose level.

2. Student answers should center on the function of insulin at the cellular level (i.e., stimulating cellular uptake of glucose), the fact that the increase in blood glucose level in a fasted rat that is fed glucose will trigger the release of insulin from the animal's pancreas, and the fact that diabetic rats will produce insufficient quantities of insulin to effect a significant reduction in blood glucose level.

   The students should know that endocrine glands secrete their hormone products into the circulatory system which transports these hormones to all of the tissues of the body. The circulatory system also picks up the products of food digestion in the small intestine and transports these substances to all of the tissues of the body. Their answers should also indicate an understanding of negative feedback control in the endocrine system of mammals.

3. Students should discuss the role of the hormone glucagon. Glucagon, which is also produced and secreted by the pancreas, has many effects which are directly opposite to the effects of insulin. Specifically, they should note that glucagon's target organ is the liver, in which it promotes the breakdown of glycogen into glucose and the conversion of amino acids and fatty acids into glucose. The glucose is then transported in the animal's blood.

   The release of glucagon from the pancreas is stimulated by low blood glucose levels, and the result of the breakdown of glycogen is an increase in blood glucose levels. Thus, glucagon's effects prevent the blood glucose level from getting below the level needed to support normal brain function. (Prolonged starvation, of course, would deplete glycogen reserves so drastically that glucagon is ineffective.)

   The fasting blood glucose levels of the normal rats will usually be around 90 mg glucose per deciliter blood. The fasting blood glucose levels of the diabetic rats *may* be close to that of the normal rats; we observe this in about 20% of the diabetic rats. More commonly, however, the fasting blood glucose levels of the diabetic rats is elevated (e.g., about 150–200 mg glucose per deciliter blood) or extremely high (e.g., 300–400 mg glucose per deciliter blood or above). Since all of the rats that were diabetic were tested for the presence of glucose in their urine samples, and all typically show very high urine glucose levels, the most likely explanation for a normal fasting blood glucose level in a diabetic rat is that the fasting has caused a reduction in the level of glucose in the rat's blood.

4. See answers given for question #1. Student explanations should center on the function of insulin at the cellular level (i.e., stimulating cellular uptake of glucose) and the knowledge or prediction of which rats have endogenously produced insulin and/or exogenously supplied insulin.

5. A normal, fasted rat that was administered insulin alone (no glucose) would likely exhibit a rapid, dramatic decrease in its blood glucose level. It may even require rapid administration of glucose to prevent death. A diabetic, fasted rat that was administered insulin alone would likely also exhibit a decrease in its blood glucose level, but it would probably be less rapid and less dramatic. This would possibly be the case even if the diabetic rat's fasting blood glucose level was in the normal range.

6. When a normal (non-diabetic) mammal ingests a meal, digestion of the contents of the meal begins in the mouth, continues in the stomach, and is completed in the small intestine. Monomers of polysaccharides and proteins and subunits of lipids will then be absorbed across the wall of the small intestine and into blood vessels of the circulatory system. As glucose enters the blood from the intestines, the blood glucose level of the mammal begins to rise. This elevation in blood glucose level triggers the release of insulin from the ß-cells of the Islets of Langerhans in the pancreas. The insulin is released into the blood and is transported to all of the tissues of the body. In these tissues, insulin stimulates the cellular uptake of glucose present in the blood. As the body tissues take up glucose, the level of glucose in the blood will decrease. When this occurs, the release of insulin by the pancreas is halted.

Acknowledgements

I thank Dr. Mary Ann Rankin, Chairman of the Division of Biological Sciences, for her encouragement and assistance in developing this exercise, for her assistance in obtaining approval for conducting the exercise, and for obtaining the financial resources that have made it possible for this exercise to become a regular exercise in the organismal biology laboratory course at the University of Texas–Austin.

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Waynforth, H. B. 1980. Experimental and surgical techniques in the rat. Academic Press, San Diego, 269 pages.

APPENDIX A
Procedures for Inducing Chemical Diabetes

Materials

The following quantities of materials are the numbers required for each group of students. I suggest that you have only one group per laboratory room, unless your classes are very large and you have two teaching assistants or assistant instructors. You should order about 30% more rats than are needed for the exercise, as some of the rats injected with Alloxan will likely die and some will likely not become diabetic.

Rats (*Rattus rattus*, Sprague-Dawley), all male, weighing approximately 150 g each (5–6 weeks of age), housed three per cage (4) (We have found approximately a 20% mortality rate among female rats injected with Alloxan, compared with approximately a 5% mortality rate among males.)
Balance, 500 g capacity (1)
Weighing container with lid (large enough to weigh 1 rat) (1)
Permanent marker, black (1)
Gloves, latex (several pairs)
Alloxan (200 mg)
Balance, electronic, accurate to 0.1 mg (1)
Weighing spatula, small (1)
Weighing paper (1)
Test tubes, 16 × 100 (4)
Test tube rack (1)
Physiological saline (0.85% NaCl in distilled water), sterile, in a sterile, stoppered bottle (20 ml)
Syringes, 1 ml, sterile (4)
Needles, 25 G 5/8, sterile (4) (*Note:* When attached to the syringe, the tip of the needle must be able to reach the bottom of the test tubes.)
Urine glucose test strips (Ames Keto-Diastix) (6)

Procedures to be Conducted 4–5 Days Prior to the Laboratory Period

Weigh all of the rats by placing them, one at a time, inside the weighing container with the lid affixed and determining the weight of the rat plus the container (to the nearest 1 g) using the large balance. Number the cages and, using the permanent marker, draw one, two, or three stripes at the base of each rat's tail. This will provide identification of individual rats. Determine the weight of the empty weighing container. Calculate the weight of each of the rats.

Calculate the Alloxan dosage that each rat will receive, based on a dose rate of 20 mg per 100 g body weight. Obtain a test tube rack containing enough test tubes for one per rat. Label the test tubes with each rat's identifying numbers (i.e., cage number and rat number), using the permanent marker. Put on a pair of gloves. Weigh out the Alloxan dosage for each rat separately, placing each aliquot in the appropriate test tube. *Use extreme caution when handling the Alloxan as it is a hazardous chemical.*

Take the test tubes in the rack and the syringes and needles to the room where the rats are being kept. You should have an assistant to hold the rats for you. Set out the materials so that you will be able to work quickly and efficiently. This is of critical importance because of the short half-life of Alloxan in water; Alloxan has a half-life in water at 20°C of 5 minutes. For this reason, you will add saline to one rat's Alloxan aliquot and administer the solution to the rat before mixing the next rat's solution.

Have your assistant remove the first rat from its cage and hold it in preparation for its injection. Put on a pair of gloves. Remove a syringe and a needle from their sterile wrappers and attach the needle to the syringe. Remove the stopper on the saline bottle, insert the needle into the saline, and withdraw 1 ml of saline into the syringe. Replace the stopper on the saline bottle. Add the 1 ml of saline to the test tube containing the first rat's aliquot of Alloxan. Mix the contents of the test tube by drawing the solution up into the syringe and then releasing it into the test tube several times. Then draw the 1 ml of solution up into the syringe. Administer the contents of the syringe into the first rat via an intraperitoneal injection in the lower left quadrant of the abdomen. Discard the syringe and needle into a sharp's disposal container and have your assistant return the first rat to its cage. Repeat these procedures for each of the remaining rats. The injected rats should be kept in an animal care facility until the day of the laboratory exercise.

Procedures to be Conducted 1 Day Prior to the Laboratory Period

Test the urine of each of the rats for the presence of glucose, which indicates that a rat is diabetic. Remove a urine glucose test strip from the container and hold it in one hand. Remove the first rat from its cage, holding it by its tail with your other hand. Set the rat on the floor, keeping hold of its tail, until it urinates. Most of the rats will be diabetic and will be urinating frequently. Place the test pad site of the urine glucose test strip in the stream of urine. Return the rat to its cage. Check the test pad site after 30 seconds by comparing its color to the color chart on the side of the test strip container. Record the rat's urine glucose level. In our experience, most of the rats will have urine glucose levels of greater than or equal to 2%.

Note: When obtaining a urine sample, make sure that you get free-flowing urine from the rat. The litter in the rat cages tends to get wet readily, and we have found that two diabetic rats in a cage can urinate enough to moisten the fur of a non-diabetic rat in the same cage. If the latter animal's fur is touched with the test pad site of the urine glucose test strip, you might get a false positive reading. Repeat the urine glucose testing for the remainder of the rats.

Remove each rat's food from their cages 12–24 hours in advance of the beginning of the laboratory section in which the rats will be used. Make sure that they have access to ample water during the fasting period.

We recommend that you do *not* test each rat's urine for the presence of glucose immediately before the laboratory section, after they have fasted for 12–24 hours. We have found that diabetic rats may not spill glucose into their urine after fasting for this period of time. Thus, you could get a false negative test for diabetes if you test them after they have fasted.

APPENDIX B
Instructions for Assembling Restraint Devices

Materials

For making two restraint devices of different sizes (enough for one lab group):

Pipe, PVC, schedule 40, 5 cm (2 inch) inside diameter (30 cm in length)
Screen, wire, fine gauge (e.g., 1 mm^2 openings), 10 cm diameter circle (2)
Hose clamp, to fit around outside of PVC pipe (2)
Stopper, rubber, No. 11.5 (2)
Saw, wood (1)
Sandpaper, 100 grade (1 piece)
Screwdriver, regular head (1)
Razor blade, single edge (1)
Wire cutters (1 pair)
Permanent marker, black (1)

Procedures

Using the saw, cut the 30 cm piece of PVC pipe into two pieces depending on the weight range of the rats that you have, one 14 cm long (for rats weighing approximately 150 g) and one 13 cm long (for rats weighing approximately 100 g) or 15 cm long (for rats weighing approximately 200 g). Sand both ends of each piece of pipe until they are fairly smooth. Wrap one of the circular pieces of wire screen over one end of one piece of pipe. Secure the screen by placing the hose clamp around it and the pipe and tightening the hose clamp by turning the adjustment screw with the screwdriver. Make sure that the hose clamp completely covers all of the cut edge of the screen (so users cannot be scratched by the screen), or remove the screen and trim it with the wire cutters so that it can be safely secured. Using the razor blade, cut a notch in the perimeter of one of the rubber stoppers (see Figure 14.3). The notch should be large enough so that, when the stopper is placed inside the open end of the piece of pipe, a rat's tail will be able to fit through the space created by the notch. (The base of a 150 g rat's tail will be approximately 1 cm in diameter.) Using the permanent marker, write the rat weight for which the restraint device is suitable on the side of the piece of pipe. Repeat the above procedures to assemble the second restraint device.

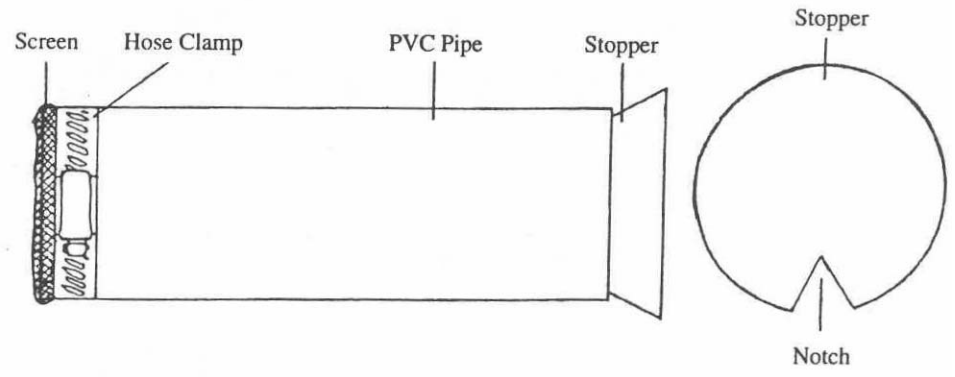

Figure 14.3. Rat restraint device.

APPENDIX C
Instructions for Assembling Feeding Tubes

Materials

For making six feeding tubes (enough for one lab group):

Needle, 18 G, 2.5 cm long (6)
Syringe, 3 ml or 5 ml (6)
Tubing, Tygon, 0.05 ID, 0.09 OD (180 cm in length)
Saw, jewelers (1)
Pliers (1 pair)
File, metal, fine (1)
Super glue, gel (1 tube)
Beaker, 30 ml, about half full of water (1)
Scissors (1 pair)

Procedures

Remove the cap from one of the needles and hold the metal part of the needle securely in the pliers. Using the jewelers saw, cut off the end of the needle so that the remaining part is 1–1.5 cm long. Remove the needle from the pliers. Smooth down the cut end of the needle with the file. (You may want to inspect the needle under a dissecting microscope.) Remove the cap from the syringe. Attach the needle to the syringe by turning it as you push it into the end of the syringe (the inside of the syringe is threaded at this point). With the plunger of the syringe depressed, insert the tip of the needle into the water in the beaker. Pull up on the plunger. If water moves up into the syringe, the needle has been properly cut. If water does not move up into the syringe, you will need to recut the needle or discard it and restart with a new one. Cut a piece of Tygon tubing about 30 cm long, using the scissors. Slide the piece of tubing over the cut end of the needle until it abuts the colored, plastic part of the needle. Slide the tubing back down the needle a couple of millimeters and place a drop of super glue at the junction point of the metal and plastic parts of the needle, a slide the tubing back up the needle until it again abuts the plastic part of the needle. Hold the tubing in place for about 20 seconds to allow the glue to adhere and then release the tubing. Repeat the above procedures to assemble the five remaining feeding tubes. A rat feeding tube is shown in Figure 14.4.

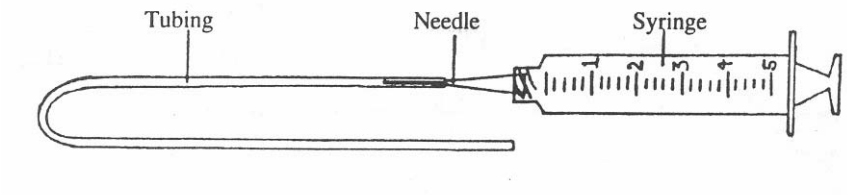

Figure 14.4. Rat feeding tube.