Chapter 7

Nitrogen Excretion in Insects

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Introduction

The principal goal of this laboratory exercise is to gain insight into the process of excretion in insects from the standpoint of elimination of nitrogenous waste products. Using a combination of spectrophotometric and dissection procedures, students (1) quantify the uric acid content of the hemolymph and feces in one or more insect species, (2) measure the rate of waste removal from the hemolymph, and (3) study the anatomical layout of the excretory system. The technique employed for measuring excretion rate further permits the insect's hemolymph volume to be computed. This exercise is used in a third-year undergraduate course in entomology at Memorial University and is one of several that I employ to introduce students to the field of insect physiology.

From a purely practical standpoint, it is often extremely difficult to accommodate physiological experiments within 3-hour time slots that university timetables frequently prescribe, and where this is possible, the thrust of the laboratory, by necessity, is very often narrow in scope. I have found that upper-level undergraduate students are generally capable of completing this particular exercise within the constraints imposed by a 3-hour laboratory period. Thus, it has the advantage of affording a large amount of information about excretion in insects within a relatively short time-frame.

Much of the laboratory revolves around the use of spectrophotometry as a quantitative assay procedure. While I assume that students in my particular course have received a grounding in the principles underlying the use of spectrophotometry, the exercise could, with some modification, be used in a lower-level undergraduate course (e.g., comparative animal physiology) to illustrate such principles of methodology. Moreover, the precise thrust of the exercise can be readily adjusted to suit the needs of the instructor and/or course. Rather than compare excretion between two insect species, students could compare excretory patterns among phyla (i.e., urico cf. ureo cf. ammoniotelism) or treat an insect species in some way to alter its rate and/or pattern of excretion (e.g., by starving them, injecting a hormone into their hemocoel(s), etc.).

While the techniques involved in this exercise are relatively straightforward and present no barrier to its completion, some students experience occasional difficulty in obtaining clear samples of insect hemolymph, free of fat body and other extraneous tissues. To alleviate this potential problem, it is imperative that the instructor demonstrate the hemolymph extraction technique to the students, stressing that the pressure by the thumb and forefinger on the insect should be applied in a discontinuous fashion (allowing periods of relaxation and recovery) and lightly, not as hard as possible. Also, the use of a large insect with a relatively large hemolymph volume (e.g., locust or

large cockroach) is recommended. In the case of locust, hemolymph volume can be increased by withholding food on the day of the laboratory until approximately 2 hours prior to its commencement.

As a physiological exercise, primarily designed for upper-level undergraduates, it is relatively inexpensive and easy to prepare. The most expensive items of apparatus are a spectrophotometer with UV-range capacity and a high-speed refrigerated centrifuge. Glassware and reagents are of a routine nature, all of the reagents for the uric acid assay being obtainable "ready made" as a kit. The preparation time (for one instructor and 24 students) for the described exercise is 4 hours.

The laboratory outline given to the students should contain an introduction, detailed instructions for carrying out the experiments and writing up the laboratory report, written explanations of how uric acid levels and hemolymph volumes are calculated, and a list of references to enable them to conduct the necessary literature review. It is further essential that students be provided with detailed information regarding the manner in which the fecal extracts had been prepared prior to the laboratory session (see Notes for the Instructor). Students in my entomology course will already have dissected an insect in lower-level undergraduate courses, so I do not find it necessary to supply detailed dissecting instructions and anatomical diagrams. There are several such dissecting aids available should the instructor wish to supply this information; for example, see Harris (1980) and Boolootian and Heyneman (1980) in Appendix A.

Notes for the Instructor

Materials, Supplies, and Equipment

This exercise is best done by students working in pairs, so requirements are expressed on a per pair basis. "Class" requirements are adequate for 24 students.

Per pair of students:

Adult locusts (3)
Adult cockroaches (6)
Safety pipet filler (e.g., bulb type) (1)
0–1 ml graduated pipets (6)
10-ml graduated pipets (6)
15-ml conical centrifuge tubes (4)
Rack for centrifuge tubes and test tubes (1)
Compound microscope (1)
Dissecting microscope (1)
Petri dish (100 mm × 20 mm) containing melted wax (for use as an insect dissecting dish) (1)
Waterproof marking pencil (1)
5-ml fecal extract in a small glass bottle or test tube
25-µl Hamilton microsyringe (1)
5-ml 0.8% amaranth (w/v)
Watchmaker's (No. 5) forceps (2)
Dissecting scissors, small (1)
50-ml bottle of distilled water
Pasteur pipets (6)

For a class of 24 students:

Uric Acid Kit (1)
1000 50-µl disposable micropipets (1 case)
1000 10-µl disposable micropipets (1 case)
Insect mounting pins, fine (size 1)
UV range spectrophotometer (e.g., Spec 710) (1)
Visible-light range spectrophotometer (e.g., Spec 100) (2)
Benchtop centrifuges (e.g., Clay Adams Dynac®) with 15-ml sleeves (3)
0.4-ml glass microcells for use in the spectrophotometer(s) employed in amaranth experiment (4)
Vortex mixers (3)
Quartz spectrophotometer cuvettes for UV range spectrophotometer (2)
1 liter of locust saline
Kimwipes
Parafilm
Glass slides
Cover slides

For the instructor/technician:

In advance of the laboratory session, the instructor and/or laboratory technician will require the usual glassware (beakers, volumetric flasks, pipets, etc.) and equipment (magnetic stirrer, analytical chemical balance, etc.) required for preparing solutions. In addition, the following supplies will be needed:

NaCl
KCl
$MgCl_2 \cdot 6H_20$
$NaH_2PO_4 \cdot 2\ H_20$
$NaHCO_3$
Li_2CO_3
NaOH
Ground glass homogenizer (tissue grinder), manual type, 7-ml capacity (1)
Controlled-temperature (25°C) water bath or incubator (1)
High-speed refrigerated centrifuge (e.g., Sorvall RC-5B) (1)

Sources of Materials and Supplies

The Uric Acid Kit (No. 292) and amaranth are purchased from Sigma Chemical Co. Sources of other chemicals and most of the glassware used in this exercise are not critical. We obtain them from Fisher Scientific or Canlab. All chemicals should be reagent or certified ACS grades. The micropipets are of the disposable microcapillary type, obtainable from Fisher Scientific (Cat. #21-164-2) or Canlab (Cat. #P4518-10; P4518-50C). Watchmakers forceps and small dissecting scissors can be purchased from a surgical supply company (e.g., German Surgicals/Irex, P.O. Box 788, Adelaide St. P.O., Toronto, Ont. M5C 2K1). Insect mounting pins are available from Hamilton Bell Co. (Montvale, NJ).

Reagent Preparation

0.8% amaranth: Dissolve 0.4 g of amaranth in 50 ml of distilled water by use of a magnetic stirrer and teflon-coated stir bar. Store the solution in a reagent bottle prior to apportioning 5-ml aliquots among the student pairs. This solution is stable indefinitely.

Locust saline: Dissolve the following in 1 liter of distilled water using a magnetic stirrer and a teflon-coated stir bar: NaCl, 9.82 g; KCl, 0.48 g; $MgCl_2 \cdot 6H_2O$, 0.73 g; $CaCl_2 \cdot 6H_20$, 0.47 g; $NaH_2PO_4 \cdot 2H_2O$, 0.95 g; NaHCO3, 0.18 g. Store the solution in a reagent bottle. This solution is stable indefinitely.

Fecal extract: First prepare a 5% (w/v) lithium carbonate solution by dissolving 5 g of lithium carbonate in 100 ml distilled water, using a magnetic stirrer and a teflon-coated stir bar. A 5% (w/v) sodium hydroxide solution is prepared in a similar manner. Using a ground glass homogenizer, homogenize 0.1 g of insect feces with 5 ml of 5% (w/v) lithium carbonate plus 5 ml distilled water, then incubate the sample for 24 hours at 25°C in a controlled-temperature water bath or incubator. The extract is then centrifuged for 10 minutes at 4°C at a speed of 12,000 g and the supernatant added to 10.1 ml of 5% (w/v) sodium hydroxide solution. This extract should be stored refrigerated and should be prepared no more than 1 day or so in advance of the laboratory session.

Student Outline

Introduction

When nitrogen-containing organic compounds (principally amino acids, nitrogen-containing bases to a lesser degree) are catabolized by animal cells, ammonia is generated as a byproduct. Free ammonia is extremely toxic, however, and can only be excreted as such when there is ample water for its rapid removal in solution. This would apply, for example, in the case of freshwater insect forms, which inhabit an environment hypotonic to the hemolymph and have a mechanism of osmotic regulation that involves a flux of freshwater passing through them.

Terrestrial insects, however, generally have insufficient environmental water at their disposal to permit ammoniotelism (i.e., excretion of waste nitrogen as ammonia). Like other terrestrial organisms, they deploy a number of strategies to conserve water and this necessitates that the ammonia be detoxified. While mammals and adult amphibians detoxify ammonia by converting it to the soluble substance, urea (i.e., they are *ureotelic*), birds, reptiles, and insects are *uricotelic* (i.e., they convert the ammonia into uric acid, a highly insoluble compound). Most species of insects produce other nitrogenous wastes alongside uric acid, especially the related compounds allantoin and allantoic acid, and there are a few insects which excrete such substances as their principal nitrogenous waste. However, the vast majority of terrestrial insects are uricotelic.

One of the insects that you will be studying today, the migratory locust, *Locusta migratoria* (Orthoptera: Acrididae), is typically uricotelic. The other insect, the American cockroach, *Periplaneta americana* (Dictyoptera: s.o. Blattaria), however, belongs to a group that are by no means typical, or even uniform, with respect to their pattern of nitrogen excretion. While some cockroaches are uricotelic, the vast majority are not—uric acid is stored in the fat body and accessory glands (males) of such insects. The capacity of cockroaches to store, rather than eliminate, uric acid may be correlated to their feeding behavior and phylogeny.

In today's laboratory, you will use an ultraviolet procedure to compare the uric acid concentration in the hemolymph and feces of locusts and cockroaches. The procedure for assaying uric acid depends on the fact that uric acid absorbs ultraviolet light strongly, with maximum absorption at a wavelength

of 292 nm. When the enzyme uricase is applied to the uric acid, the latter is oxidized to allantoin, which does not specifically absorb ultraviolet light of this wavelength. Hence, the decrease in absorbance, measured on a UV spectrophotometer, is proportional to uric acid concentration:

$$\text{Uric acid} + 2H_20 + 0_2 \xrightarrow{\text{uricase}} \text{allantoin} + C0_2 + H_20_2$$

Excretion in most insects (and certainly locusts) is performed by the Malpighian tubules and rectum, functioning as a unit. Wastes (e.g., uric acid) and other metabolites are transported from the hemolymph into the lumen of the Malpighian tubules, along the hindgut to the rectum. Then, selective reabsorption back into the hemolymph of water, salts, and metabolites occurs in the rectum. Today, you will verify this for yourselves, by injecting a known volume of the dye amaranth (not metabolized by the insect) into the locust's hemocoel and following its movement into the Malpighian tubules. As the dye is removed by the insect's Malpighian tubules from the hemolymph, its concentration in the blood progressively diminishes and this can be followed spectrophotometrically. The technique can be used to measure the hemolymph volume of the insect.

Experiment 1: Comparison of Hemolymph and Fecal Uric Acid Concentration in Locusts and Cockroaches

1. Using a 50-µl disposable micropipet and following the procedure which will be demonstrated to you, extract 50 µl of hemolymph from a cockroach and a locust. The easiest way to obtain the hemolymph is to puncture the arthrodial membranae at the base of one of the walking appendages with a fine-pointed insect mounting pin, holding the insect with its ventral surface uppermost between your forefinger and thumb. Your thumb can be used to hold one of the metathoracic appendages apposed next to the sides of the thorax and pointing toward the head, thereby exposing the arthrodial membrane. By alternately squeezing, gently, the sides of the insect's thorax with your thumb and forefinger then relaxing the pressure applied, a droplet of hemolymph will exude from the punctured arthrodial membrane and this is drawn by capillary action to the calibration mark on the micropipet. In the case of the cockroach, several insects may be needed to collectively provide the required 50 µl of hemolymph.

2. Blow the hemolymph aliquots into two test tubes (labelled “L” locust, “C” cockroach) containing 1.0 ml glycine buffer (pH 9.4) and 6.15 ml distilled water. Pipet 0.2 ml of the fecal extract from cockroaches and locusts into each of two further test tubes. To these samples, add 1.0 ml glycine buffer and 6.0 ml distilled water. Label these tubes FECES L and FECES C. Vortex all tubes (10–15 seconds) to insure mixing.

3. Subdivide equally each of the four samples prepared in step 2 into two further tubes, each containing 3.6 ml. In each case, label one BLANK and the other TEST. *Make sure that the* L TEST, L BLANK, C TEST, *and* C BLANK *samples are pipetted into 15-ml conical centrifuge tubes.* You should now have eight (8) tubes, labelled L BLANK, L TEST, C BLANK, C TEST, L FECES BLANK, L FECES TEST, C FECES BLANK, and C FECES TEST. Try and invent your own labelling system.

4. Pipet into each BLANK 0.05 ml distilled water, using a micropipet to measure out the required volume. Vortex for 10–15 seconds. Pipet into each TEST 0.05 ml uricase enzyme. Vortex for 10–15 seconds.

5. Allow all eight (8) tubes to stand at room temperature for 15 minutes.

6. Centrifuge all four (4) samples containing hemolymph (L TEST/BLANK and C TEST/BLANK) for 5 minutes at medium speed, using one of the benchtop centrifuges.
7. Transfer the solution from one of the blanks into a quartz spectrophotometer cuvette and the corresponding test sample into another cuvette (i.e., L BLANK–L TEST, or C BLANK–C TEST).
8. Set the spectrophotometer to a wavelength of 292 nm.
9. With the BLANK in the light path, adjust the instrument to read 0.400 Absorbance. Place the TEST in the light path and record its Absorbance.
10. Wait approximately 5 minutes, then repeat step 8. If absorbance of the TEST has decreased, repeat readings at intervals until a constant reading is obtained. Record the reading as "Final Absorbance."
11. Conduct steps 6 to 9 for the other BLANK/TEST samples thus far not read.

Experiment 2:
Rate of Excretion of Amaranth Dye from the Blood of the Locust

1. Using a 25-µl Hamilton microsyringe, inject a locust (not the same one that you used for Experiment 1) with 10 µl of a 0.8% solution of amaranth into the hemolymph. The best place to inject the locust is through the arthrodial membrane at the base of one of the walking appendages.
2. After 5 minutes, withdraw 5 µl of hemolymph from the locust, as described in Experiment 1, step 1 (10-µl micropipets are easiest to use here; hemolymph is drawn up by capillary action until the calibrated portion of the micropipet is exactly half-filled).
3. Blow the hemolymph sample into 0.4 ml of locust saline in a glass microcuvette. Read the absorbance of this diluted blood/amaranth sample against a saline blank (i.e., locust saline only) at 522 nm using the spectrophotometer.
4. Repeat steps 2 and 3 at 15 and 30 minutes after injection of the dye, respectively.
5. Blow 5 µl of the amaranth dye (i.e., dye which has not been injected into the insect) into 0.5 ml of locust saline and read the absorbance of this sample against the saline blank. This gives the absorbance of the amaranth when undiluted by the locust's hemolymph.
6. Inject a locust with 10 µl of amaranth as in step 1. After 10 minutes, dissect the locust in locust saline under a stereomicroscope (the locust can be killed quickly by decapitation), using insect mounting pins to pin back the flaps of body wall to the wax dish. Separate out the gut, then observe and record the distribution of the amaranth dye along the gut and associated structures. Remove a few Malphigian tubules, make a temporary wet mount of them and examine them under the compound microscope. Record the structural appearance of the tubules, and whether or not the dye is visible within them.

Laboratory Report

Using your data and those obtained by the other students in the laboratory, write a report (see format below) on the process of excretion in the locust, *Locusta migratoria*, and the cockroach, *Periplaneta americana*, adhering to the following format:

Objective: To examine the process of excretion in the locust, *Locusta migratoria*, and in the cockroach, *Periplaneta americana*, in particular, the structures involved in the process, the rate at which excretion occurs, and the capacity to produce and eliminate uric acid.

Materials and Methods: Describe all procedures used, including the theory underlying them, and comment upon any deviations made from the instructions given in the laboratory outline.

Results: (1) State your results clearly and concisely. (2) Present your data in easily read and clearly understood tables, graphs, and/or figures (histograms, etc.).

Discussion: (1) Discuss the results and observations in terms of what they imply or mean and how they compare with other findings. (2) Point out things that do not make sense or cannot be explained. (3) State what you have concluded on the basis of the data and what you would do next if you were going to continue this investigation.

In this particular laboratory, your **Results** section should contain a comparison of the uric acid content of the hemolymph of locusts and cockroaches expressed in mg uric acid/100 ml hemolymph and of the uric acid content of the feces of the two groups on insects expressed in mg/g feces. Also, your data from the amaranth excretion experiment must be expressed in both tabular and graphical form. The graph of absorbance versus time must be a semi-logarithmic plot, with a log scale used for the ordinate. Calculate the percentage of the injected amaranth that had been removed, by excretion, from the hemolymph after 30 minutes. The amaranth clearance experiment also provides a convenient way of calculating the insect's blood volume. Calculate the blood volume of the locust which you injected.

In your **Discussion**, you should discuss your results in terms of what they illustrate about the process of excretion relative to phylogeny, feeding behavior, and protein metabolism. How does the insect regulate the process of excretion?

Calculation of Hemolymph Uric Acid Levels

The test follows a linear relationship, that is, the decrease in absorbance is proportional to the uric acid concentration. Normally, it would be necessary to run a series of standards, uric acid solutions of known and varying concentrations, to construct a standard curve. This would then enable one to calculate the "constant" in the proportional relationship. The uric acid solutions used by you today, however, are supplied in the form of a "kit" by the supplier and the calibration curve has been done by them.

Calculations:

Change in absorbance $\Delta A = (0.400 - \text{Final Absorbance})$

For 50 µl samples:

Hemolymph uric acid (mg/100 ml) = $(\Delta A \times 50)$

For 200 µl samples (as in fecal samples):

Uric acid concentration (mg/100 ml) = $\Delta A \times 50$. But this gives you the uric acid concentration, expressed in mg/100 ml of the solution from which you took the 0.2 ml sample for assay. Since you know how this solution was prepared, you can trace this back to calculate your data in mg uric acid/g feces.

APPENDIX A
References

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Harris, C. L. 1980. Digestive enzymes of the cockroach. Pages 111–123, in Tested studies for laboratory teaching (J. C. Glase, Editor). Kendall/Hunt, Dubuque, Iowa, 272 pages.

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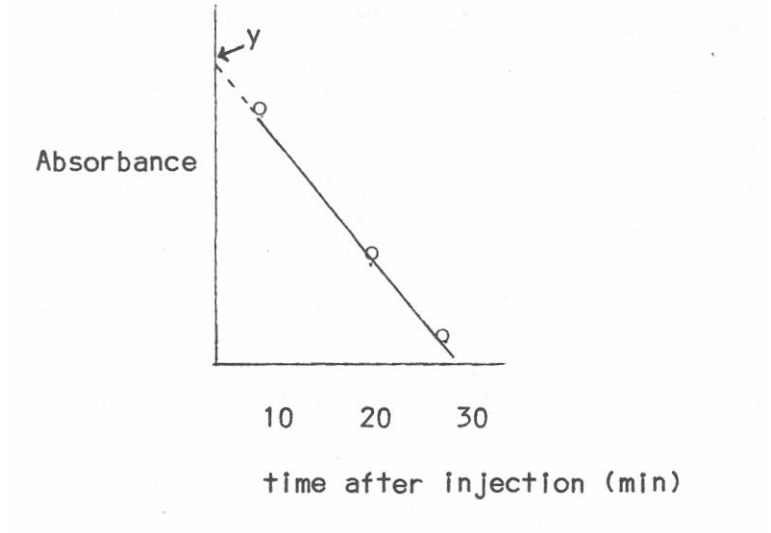
APPENDIX B
Excretion of Amaranth

Since the amaranth is removed from the blood exponentially with respect to time, a semi-logarithmic plot of absorbance against time after injection gives a straight line.



The slope of the graph is an expression of the rate of excretion and can be used for comparisons of excretory rates. Also, the amaranth will be diluted by the hemolymph in accordance with the hemolymph volume and the dilution factor at Time = 0 (i.e., before excretion can occur) can be used to calculate hemolymph volume. Absorbance at Time = 0 cannot be measured, however. It is practically and theoretically impossible. The dye must be given time to evenly mix with the hemolymph before readings can be taken. The graph can be extrapolated to Time = 0, however, and the y-intercept used to calculate blood volume.

If absorbance of amaranth undiluted by hemolymph = X, and absorbance at Time Zero = Y ($Y < X$), then dilution factor = X/Y. This means the 10 µl of amaranth was diluted by the hemolymph X/Y times. Therefore, hemolymph volume = 10X/Y.

APPENDIX C
Sample Data Obtained by Students (in 1983)

Table 7.1. Hemolymph and fecal uric acid concentration.

Student Initials	Spectrophotometer absorbance values (Blank = 0.400)*			
	Hemolymph (50 µl)		Feces extract	
	Roach	Locust	Roach	Locust
J.B./V.A.	0.305	0.274	0.419	0.048
C.H./J.K.	0.427**	0.378	0.444	0.174
I.B./D.B.	0.333	0.382	0.393	0.182
I.B./C.K./W.M.	0.374	0.392	0.412	0.137
C.H./T.H.	0.372	0.370	0.401	0.143
A.J.H./S.N.	0.399	0.354	0.392	0.128
J.P./F.S.	0.367	0.362	0.419	0.152

* Absorbance value ≥ 400 indicates no detectable uric acid.
** This student pair used 25 µl instead of 50 µl of cockroach hemolymph.

Table 7.2. Rate of excretion of amaranth.

Undiluted reading (step 5)	Spectrophotometer absorbance values			
	5 minutes*	15 minutes	30 minutes	Student initials
2.074	0.055	0.030	0.006	T.H/C.H.
2.056	0.192	0.044	0.034	C.H./J.K
1.941	0.043	0.021	0.001	J.B./V.A.
2.010	0.148	0.026	0.031	I.B./D.B.
2.082	0.102	0.040	0.008	J.P./F.S.A.J.H./S.N
2.062	0.056	0.039	0.007	.
2.021	0.063	0.027	0.019	D.S./C.K./W.M.

* After injection.