Content

Introduction....120
Notes for the Instructor....121
Materials....123
Student Outline....124
Acknowledgements....130
Appendix A: Further Reading....131
Appendix B: Alternative Methods for Extracting Hemolymph....132
Appendix C: Making Solutions and Measuring Concentrations....133

Introduction

This exercise is designed to be completed in one 3-hour period. At the University of Toronto we have used it in an introductory biology course, but it might also be suitable for an introductory course in animal physiology. We have usually presented it as the second in a series of four related physiology laboratory exercises, although it will stand on its own if students already have the appropriate background.

The first exercise in the series introduces students to various techniques: using pipets, measuring volumes, making dilutions, the serial dilution technique, and using a spectrophotometer for measuring concentration. Equipped with these skills and a standard curve for dye concentration versus optical density, the students would then be ready to measure hemolymph volume in a cockroach in the second exercise. In the third exercise students determine protein concentrations in the cockroach hemolymph. They produce standard curves for the spectrometer using a dilution series of albumen, and its quantitative binding to the dye naphthalene blue-black. They then work with unknown protein samples and samples of cockroach hemolymph. The fourth and final exercise allows the students to apply the techniques learned in the previous three exercise to a physiological investigation of the effects of starvation (including dehydration) on cockroaches.

The exercise as presented in this chapter assumes students know how to use a spectrophotometer, pipets, and an electronic balance. Background readings on blood volume in insects and on cockroaches are provided in Appendix A. Alternative methods for extracting hemolymph from a cockroach are provided in Appendix B. The first exercise in the series which introduces students to various techniques (as described in the above paragraph) is presented in Appendix C. I can provide you with copies of the third and fourth exercises if you are interested.

Why cockroaches?

Why cockroaches are used in this exercise is addressed in a 2-hour lecture which accompanies the four laboratory exercises described above. In practical terms, cockroaches are cheaper than vertebrates, and since they are neither “furry” nor “cuddly” their use does not (yet) anger the animal-rights enthusiasts. In terms of economics and public health, we need to know everything we can about insects because of their enormous impact on humans as crop pests, carriers of disease, pollinators, etc. But they also have a fascination in their own right. For instance, they represent the only other major group of animals that, with the “higher” vertebrates, have successfully conquered the terrestrial habitat. I introduce the exercises which use the cockroach in this context: comparing insects and vertebrates (specifically mammals) to look for similarities and differences. Insects and mammals have a vastly different evolutionary heritage: evolution has had to work with very different basic body plans to produce two highly successful terrestrial organisms. What can we

learn about animal design by looking at a sample system, such as circulation, in the less familiar creature?

First, what is the function of a circulatory system? Here I discuss living cells requiring the constant exchange of materials with their surroundings, and the limited distances over which diffusion is an adequate process. To build a "large" organism, cells must be surrounded by an aqueous fluid which is then stirred around (bulk flow), just like you stir your coffee to distribute the sugar evenly. One can, in this sense, identify two fluid "compartments" within a larger organism: the intracellular fluid (ICF), and the stirred or circulated extracellular fluid (ECF).

Next, I use some "gross" observations that I extract from the students: what are some obvious things about the blood systems of insects and mammals?

Insect	*Mammal*
Colorless blood	Red blood
Little or no blood pressure	High blood pressure
Slow circulation	Rapid circulation

The mention of color leads to hemoglobin, and the role of the blood system in the transport of oxygen in mammals. Oxygen is arguably the most "critical" of the transported substances: cut off the blood supply to the brain, and we go unconscious not through lack of glucose, amino acids, hormones, etc., but from lack of oxygen. This is because of the low solubility of molecular oxygen in water; it is potentially the critical rate-limiting substance in cellular respiration. Hemoglobin then increases the amount of oxygen that can be transported. Insects do not have a respiratory pigment such as hemoglobin in their blood; how do they manage without it? After all, they are highly active creatures, just like mammals (large flying moths maintain high body temperatures, and have metabolic rates similar to that of a humming bird). Answer: They supply oxygen via air-filled tubes, the tracheal system. One can use a good analogy here: insect flight muscle is permeated with the tree-like branches of tracheoles, with no part being more than a few tens of micrometers from a tracheole. Mammalian muscle similarly contains vast numbers of capillaries, with no part being more than a similar distance away.

The lack of needing to supply oxygen via its blood "allows" the insect circulatory system to be slow, have low-pressure, and be "low-tech" — no capillaries, no branching arterial and venous system, and just a simple tubular heart. Blood is not part of the ECF separately circulated under high-pressure and intensely regulated as it is in mammals; it is simply the ECF itself. Given this, the insect can allow its blood (hemolymph) volume to change more than can a mammal.

Insect blood acts as a water storage organ (critical in a small high surface-area-to-volume ratio terrestrial organism). It also acts as a storage organ for metabolites such as amino acids, carbohydrates, and proteins. This gives rise to interesting questions of regulatory physiology that can be approached experimentally: as water is lost in a dehydrating insect, its hemolymph volume declines. This means the osmotic pressure must be adjusted lest water is drawn from the cells, for example, by converting amino acids to proteins, or removing metabolites from the hemolymph.

Notes for the Instructor

Most students are understandably nervous about handling the cockroaches, particularly since the best results are from large, healthy, active ones! We make sure the teaching assistants (TAs) have actually done the lab beforehand, and are comfortable picking up rapidly reviving roaches and recapturing escapees; a nervous TA does not inspire confidence.

We get the students to use CO_2 anaesthesia for the cockroaches prior to injecting and taking samples. It is important that the roaches not be over-anaesthetized though, or their circulation becomes very intermittent and unreliable, causing poor mixing of dye and hemolymph (one

potentially serious source of error). Since CO_2 is dense, it can remain at the bottom of the handling jar, prolonging exposure. I tell the students they need only keep the valve of the CO_2 cylinder open only long enough to displace the air; in reality, if the valve is closed when the cockroach starts to go into spasms, there will be plenty of CO_2. When the cockroach is "out," take the lid off, and blow quickly into the jar, replacing the CO_2 with air again.

Not keeping the roach in CO_2 is particularly important *after injection and between blood samples*; it will be returned to its holding jar to recover and keep its circulation going. If the jar is still full of CO_2, neither recovery nor circulation will be optimal.

The anesthetic does not last long — a few minutes at most, so it is important that everything be ready for injection or sampling *before* the roach is exposed to CO_2, and the students are familiar with what to do (one reason for requiring each student to prepare a written protocol before the lab). I suggest that each pair of students do a "dry run" through the injection procedure, pretending they are holding a cockroach, before using the real insect. This way, they should have sorted out who will be where, what angles to hold things at, where the cockroach holding jar will be, and so on.

A good injection will result in a visible spreading of the red dye under the abdominal cuticle, with no leakage at the point of injection (obviously a source of error). The rest of the cockroach will start to become pinky red, particularly where the cuticle is thin or lightly pigmented, after a minute or two. Sometimes, the injected dye may appear to be trapped around the injected region, hardly spreading. The only thing to do in this case is to try again with another insect.

The trickiest part of the exercise is taking blood samples; again, we make sure the TAs have actually done it, and can demonstrate the technique. The blood clots quickly (typically well less than a minute), so everything must be ready when the cuticle is pierced. Holding the micropipet horizontal, or even tilted downward is very important, as is applying its end to the drop as soon as possible after puncturing the membrane. Ideally, the roach should be squeezed as little as possible — all the blood should not be lost in the first sample — but this really only comes with practice. Nevertheless, we find most students get enough samples to produce a curve of optical density versus time.

Note that it is not necessary to take samples at exactly 5, 15, and 30 minutes; some students may try to do this, and find they were not quite ready, or it took longer than expected to anesthetize the roach. Since they are plotting a curve, all that is necessary is to record the *actual time* when the sample was taken.

Typical results we get in cockroaches from our colony, given free access to food and water, are hemolymph volumes ranging from 80 to 250 µl with body weights from 0.8 to 1.2 g, giving relative volumes of 8% to 25%. We ask students at the end of the lab to enter their results for hemolymph volume, together with the sex and weight of their cockroach, into a table of class data. They are then given a copy of the entire class data when the table is complete so they can calculate means, standard deviations for volumes, and compare males and females. This is part of an ongoing effort to get them to use simple statistical methods in analysis of data.

Handling Cockroaches

Twenty-four cockroaches (*Periplaneta americana*) are transferred from our breeding colony to a large (1 liter) stock jar 24 hours before the laboratory class; both food (dry rat pellets) and water (in a small vial with a cotton plug) are available in the stock jar. This amount is sufficient for two lab groups of 20 students each; students work in pairs. Just prior to the lab, a TA anesthetizes the cockroaches in the stock jar and transfers one roach to each of 10 small (4" diameter) holding jars per class. The holding jars are without food and water. Each pair of students is provided with one roach in a holding jar. Both stock and holding jars have screw tops with several small air holes

drilled in them; a center hole should be large enough to allow for the delivery tube from the CO_2 cylinder.

Only healthy adult specimens are removed from our breeding colony for use in this exercise. Extraction of hemolymph can be tricky if the cockroaches are dehydrated or unhealthy. Thus, plump, active cockroaches from a well-maintained colony are desirable. We do not select specimens whose wings are torn or tattered.

Sources of Error

Excessive anesthesia will slow or abolish the heart rate. This in turn will slow mixing of the dye and give poor readings (they could be high or low, depending on dye concentration in the sample).

In a good preparation, the three data points for optical density (OD) will lie approximately on an exponentially-declining curve (dye excretion rate may be a function of dye concentration). The extrapolation back to zero time should therefore follow this curve. Often, however, the first point will be noticeably high or low compared to the trend of the later points. This is probably due to poor mixing, either because of poor circulation (see above), or poor injection (the dye sometimes appears to be "trapped" close to the point of injection). In this case, the best that can be done is to extrapolate from the later points (which, since there are only two points, will have to be a linear extrapolation).

A factor which should be discussed is the removal of hemolymph from the cockroach during the 30-minute sampling period. This will of course reduce the hemolymph volume. The error becomes progressively larger as the 5 µl samples are removed. If mixing is incomplete, the OD may apparently increase. Another possible source of error is the distribution of the dye, that is, how long does it take for the dye to be distributed uniformly throughout the hemolymph?

Materials

The following materials are required per pair of students:

Cockroach (1) in a holding jar
Hamilton syringe, which uses disposable needles
Syringe needle, 25G 22 mm, disposable
Syringe, disposable, plastic, 1 µl
Test tubes, small (75 mm) (12)
Test tube rack, to hold minimum of 12 test tubes
Calibrated micropipets, 5 µl capacity (1 container of at least 100 per class)
Beaker, 100 ml, to hold micropipets for cooling
Large beaker or dish of ice, into which the container holding micropipets is placed
Crushed ice to cool micropipets
Pipet, 5 ml, for dispensing Ringer solution
Bulb-type or other pipet manipulator (if desired, though Ringer is not toxic!)
Dropper bottle, 30 ml, containing 2% w/v Amaranth Red dye in Ringer solution*, filtered
Spotting plate
Ringer solution* in 250 ml bottle
Plastic squeeze bottle of distilled water
Plexiglas block, approx. 3" × 1" × 0.5" for supporting Hamilton syringe (see Figure 8.1)
Adhesive putty or plasticine, to hold syringe to block and block to bench (see Figure 8.1)

Pins (straight) for pricking cockroach
Parafilm and scissors (approximately 1" squares can be pre-cut, 12 per pair of students)
Black grease pencil or marker
Paper tissues
Plastic dish pan for collecting used test tubes (1 per class)
Disposal container(s) for used hypodermic needles and micropipets (per class)
Graph paper
Spectrophotometers (5–10 per class)
CO_2 cylinder and regulator (1 per class)
Electronic balances, weighing to 1 mg (5–10 per class)

* The Amaranth Red dye should be made up in Ringer solution so that the injected fluid does not cause osmotic or ionic stress to the cockroach. We have used Ringer throughout as a diluent and as the blank for the spectrophotometer; this was for simplicity and consistency since other related labs needed Ringer. We use a simple Ringer solution since it is only injected in a small quantity. For 1 liter of Ringer, dissolve the following in distilled water: 9.8 g of NaCl, 0.77 g of KCl, 0.66 g of $CaCl_2.2H_20$, and 0.18 g of $NaHCO_3$.

Student Outline

Objectives

In this laboratory you will apply the techniques of dilution and measurement of concentrations to investigate a physiological parameter: the hemolymph (or blood) volume of an insect. A parameter is a quantifiable variable of a system you wish to study. Determining values for a parameter such as hemolymph volume allows you to measure the effect of a treatment in an experiment, or to calculate other things. For example, you can determine the absolute amount of a metabolite such as an amino acid if you know both its concentration and the volume of the hemolymph containing it. We have chosen the cockroach because it is readily available and it is large (for an insect!).

Your objectives are:

1. To determine cockroach hemolymph volume by injecting a known amount of Amaranth Red dye, and measuring its dilution by the hemolymph.
2. To think about and correct for possible errors in the method.

Outline

1. Derive a standard curve for dye concentration by a method simulating the dilution by the hemolymph.
2. Inject a cockroach with a known amount of Amaranth Red.
3. Extract hemolymph samples at given times
4. Plot your data and calculate hemolymph volume.

Introduction

Unlike vertebrates, insects (and other arthropods) have an open circulatory system, in which the fluid pumped by the heart freely bathes the tissues rather than being confined to blood vessels. The body cavity of insects which contains the fluid and the internal organs is called the hemocoel (pronounced "heem-o-seal"), and the fluid (the "blood") is called *hemolymph.* Insect hemolymph is circulated by a *heart,* which is simply a long muscular dorsal tube. The heart takes in blood from the abdominal hemocoel and empties into the head and thorax. The hemolymph then flows slowly through the tissues back to the abdomen and is recirculated by the heart. Insect hemolymph contains a wide variety of *metabolites,* including amino acids, proteins, lipids, organic acids, and inorganic ions. It is thus a fluid from which the various organs of the insect body obtain their nutrients. This is the major role of hemolymph — the transport of nutrients between organs. In addition, the hemolymph functions to *store* metabolites, to *transport waste products* to the organs of excretion (the Malpighian tubules), and to *transport regulatory hormones* from the organs of synthesis to their sites of action. The hemolymph is also *well buffered* and so provides a *stable environment* for the tissues in the face of changing external environments. And finally, the hemolymph functions in the protection of the tissues from invading parasites and micro-organisms. The hemolymph can be regarded as a vital organ.

Insects regulate their hemolymph volume by a variety of mechanisms including hormonal and metabolic factors. In an aquatic environment, insects must continuously excrete water to compensate for water uptake, whereas in a hot, dry terrestrial environment the insect must conserve water — in both these instances, the volume of the hemolymph is regulated to prevent excessive water gain or loss. In any given insect species, the volume of the hemolymph at any particular time during the life cycle will tend to be fairly constant. However, stressing the insect, for example by exposure to very high temperatures or by starvation, will result in changes in the hemolymph volume.

In a number of laboratories around the world biologists are investigating factors which control hemolymph volume, particularly in the light of the discovery that some new insecticides exert their toxic effects by causing excessive loss of water from the insect. How is hemolymph volume measured? One common method is *to measure how much the hemolymph dilutes a known quantity of a substance*. In this laboratory, you will use a dye called Amaranth Red. This dye is not toxic to the insect, and it does not penetrate the cells, two conditions necessary for the method (why?). However, the method is limited by the fact that the insect excretes the dye, and so it is necessary to estimate blood volume at several times after injection and extrapolate the dilution to zero time.

Procedures

Part 1: Deriving a Standard Curve

To find the cockroach's hemolymph volume, you will be injecting a small quantity (10 µl) of concentrated dye and finding how much this dye is diluted. You will use a spectrophotometer to find the dye concentration in the cockroach. A spectrophotometer works because dyes and other chemical *absorb* light of characteristic wavelengths, and the *amount of absorption* depends on the *concentration* of the chemical. Knowing the original concentration and the final concentration you could calculate what volume (the hemolymph volume) would have been necessary to dilute the dye that much. An easier way, and one which has other advantages (what are these?), is to inject the same amount of dye into *known volumes of fluid*, then measure absorption for each of these, using the same procedure you will use on the cockroach. If you choose a range of volumes extending

from below to above the probable values for hemolymph volume, you can produce a *standard curve* for optical density of solution on which you can read hemolymph volume directly. One advantage of this is that any errors in the procedure with the cockroach are likely to be similar to those for determining the standard curve, and they should therefore cancel each other out.

1. Set up four test tubes and add 100 µl of Ringer to one tube, 200 µl to the next, 300 µl to the next, and 400 µl to the last tube (use a 1-ml syringe).
2. For injecting small volumes accurately, you will use a Hamilton syringe. *Be careful* — they are precision instruments and expensive. To fill the Hamilton syringe with 2% Amaranth Red, first fill one cavity in the spotting plate with dye solution. Start *without* a needle on the syringe. Push the plunger right in, then fill as full as possible with the dye from the spotting plate. Holding the syringe vertically with the opening upward, place the needle firmly on the end. Slowly push in the plunger; this will displace dye into the needle cavity. When and if dye starts to come out of the end of the needle, stop, and refill the syringe with the needle in place. If the needle does not entirely fill, carefully remove it, refill the syringe, replace the needle with the syringe pointing upwards and repeat. In this way you should be able to fill the syringe with little or no air trapped. Finally, eject sufficient dye to line up the dye meniscus with a convenient mark (e.g., 30 or 40 µl). The drop(s) of dye coming from the needle should be removed with a tissue. Don't worry about the small amount of air between the end of the plunger and the dye meniscus.
3. Add 10 µl of Amaranth Red to each of the tubes using your Hamilton syringe. Do this very carefully as even small errors in dispensing the Amaranth will result in very large errors in your final readings (why?). Mix the solutions in the tubes by covering each with a piece of parafilm and shaking vigorously.
4. Using a 5 µl micropipet, remove 5 µl samples from each tube and add these to a series of four tubes containing 2.5 ml of Ringer solution. The micropipets fill by capillary action; stop the filling when the meniscus reaches the 5 µl mark by placing your finger tip on the end. If you get too much, allow some to be drawn out on an absorbent tissue. If you cannot obtain exactly 5 µl, don't worry — just estimate the volume by reading the meniscus position according to the 1-µl gradations, and use the determined volume for your graph. Empty the micropipet by gently blowing through it. Flush out any remaining contents by filling it with the solution in the tube three times and blowing out.
5. Mix the contents of the tubes and read the optical density (OD) of your samples at 522 nm. Use a tube containing 2.5 ml of Ringer as your "blank." Remember to use the same tube for each reading; dispense the samples into this tube after emptying and rinsing the previous sample.
6. Plot your data on graph paper: OD versus volume of diluent (100, 200, 300 and 400 µl). Note that a plot of OD versus volume will give a curve. Since OD is proportional to concentration, which is proportional to 1/volume, you can fit a straight line if you plot *1/OD* versus *volume of diluent*. Plot both graphs. From these graphs, you can read hemolymph volume directly. Do this from both graphs. Compare these values with each other. Which do you feel is most *accurate*? Using these graphs, you will be able to read off the hemolymph volume of your cockroach directly, once you have determined the OD.

Part 2: Injection of Cockroach

1. Prepare four test tubes and place 2.5 ml of Ringer solution in each ready for the hemolymph samples (do this *before* injecting the cockroach).
2. Read this entire section carefully *before* you inject the cockroach.
3. It is easiest to inject the cockroach if the syringe is mounted parallel to the bench and about 2 cm above it (see Figure 8.1). Use the plastic block and adhesive putty to do this. Two small pieces of putty under the block and two under the syringe will hold everything in position well enough. If the bench surface is dark, slide a white piece of paper under the plastic block to make the volume markings on the syringe stand out more clearly.

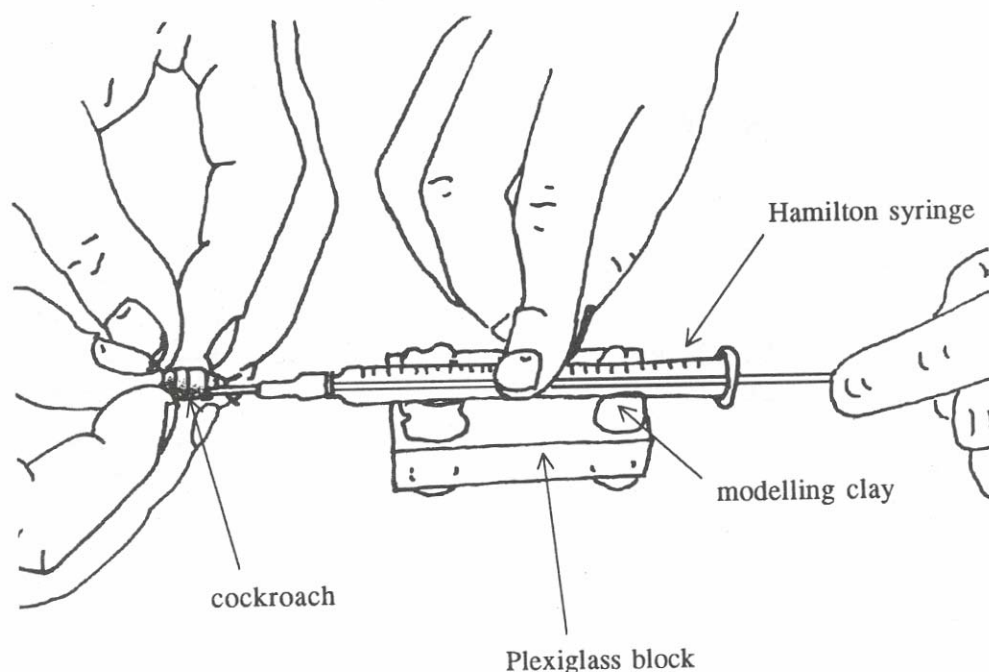

Figure 8.1. Injecting dye into a cockroach.

4. Make a mark with a grease pencil about 1 cm from the tip of the needle for your Hamilton syringe; this will help you see how far you have inserted the needle during the injection.
5. Refill the Hamilton syringe with 2% Amaranth Red. Mount the needle on the syringe with the needle opening *on the same side as the volume markings* to ensure the opening is upward when you inject.
6. Mount the syringe on the plastic block with the needle opening and the volume markings uppermost.
7. Anesthetize the cockroach with CO_2. Insert the CO_2 delivery tube into the holding jar so its tip is near the bottom of the jar. Use a gentle flow of CO_2. Wait until all movement of the cockroach stops (about 5–10 seconds). Turn off the CO_2 and remove the tube and the jar lid.

Tip the cockroach onto the weighing pan of a balance, and record its weight. Do this quickly — the cockroach will not stay anesthetized for long.

8. Inject 10 µl of Amaranth Red into the abdominal hemocoel. One person should hold the insect, and the other steady the syringe and make the injection. Pick up the cockroach and hold it between thumb and fingertips, *ventral side up, posterior end toward the syringe* and needle. (Practice holding the cockroach in this position before injection). Keeping your hand steady by resting it on the bench, gently slide the cockroach onto the needle so that the needle enters between the last two abdominal sternites close to the lateral margin (see Figure 8.2). Use both hands if necessary. Keep the needle more or less parallel to the long axis of the insect, and its tip visible just below the cuticle — this will avoid puncturing internal organs. Insert the needle about 1 cm, until the grease pencil mark is reached.

   When the needle is in place, *slowly* inject the 10 µl; the injection should be done smoothly over about 5 seconds. Read the dye meniscus, not the plunger end, for volume determination.

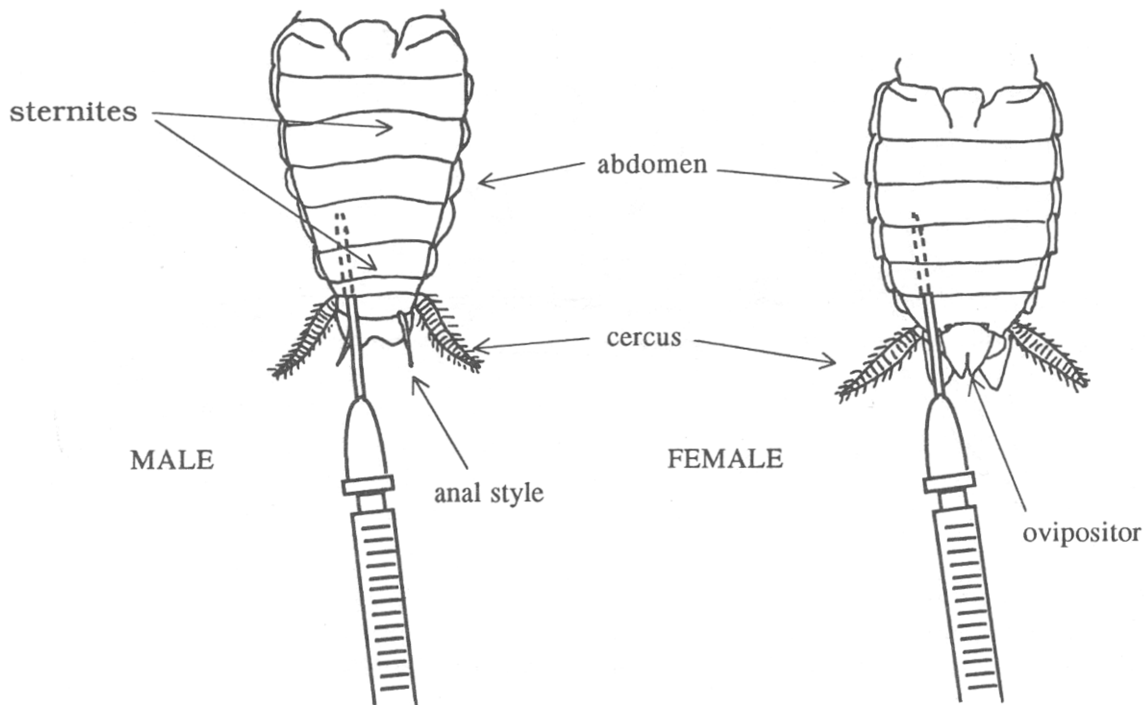

Figure 8.2. Injecting dye between the last two abdominal sternites in a male and female cockroach.

Part 3: Taking Hemolymph Samples

1. Have a pin, your prepared sample tubes containing Ringer, small squares of parafilm, and a chilled micropipet ready. The micropipet is kept cold to delay coagulation (clotting) of the hemolymph.
2. 2.Samples of hemolymph will be taken at 5, 15, and, if possible, 30 minutes after injection. Start anesthetizing the cockroach about 2 minutes before the sampling time is due. Anesthetize only until the cockroach stops moving; *do not over apply the anesthetic*.
3. To take a hemolymph sample, hold the cockroach ventral side up with it's neck between your first and second fingers, and your thumb on the thorax. With the other hand, grasp the end of the hind (metathoracic) leg, fold the leg toward the head and across to the other side, and hold it in place with your thumb; this should expose the membrane at the base of the leg. When you are ready to take the sample, your partner should note the time. (*Practice* holding the roach in this position before extracting hemolymph. You might find it easier if one person holds the roach while steadying their hands on the bench, and the other person draws the hemolymph sample.)
4. Puncture the membrane with a pin, and quickly apply the end of a 5 µl graduated micropipet to the drop of hemolymph. Hold the micropipet horizontally — it should fill by capillary action. Squeeze the cockroach gently, if necessary, to release enough hemolymph. This step must be done rapidly — the hemolymph clots in under 30 seconds. If you cannot get the full 5 µl, don't worry — anything more than 3 µl is probably enough, and the volume can be estimated by reading the meniscus position according to the 1-µl gradations; remember to record the exact amount obtained!
5. *Quickly* return the cockroach to the holding jar, and blow out the sample into one of the prepared test tubes. Place a small square of parafilm over the end of the tube, and shake the tube well until the dye is evenly dispersed.
6. Take further samples at 15 and 30 minutes after injection, adding each to a fresh tube of Ringer. If necessary, make the puncture at the base of another leg. See Appendix B for alternative methods for removing hemolymph. Use a fresh dry micropipet for each sample — used ones are more difficult to fill.

Part 4: Determination of Hemolymph Volume

1. Blank the spectrophotometer with 2.5 ml of Ringer and read the optical density of your hemolymph samples at 522 nm.
2. Plot a graph of *OD of samples* versus *time.* If your samples were not 5 µl, correct for this by multiplying your OD values by 5 and then dividing by the sample volume. The time (the independent variable) should be on the horizontal axis, and OD on the vertical axis. Draw a smooth curve (not a straight line) to fit the points, and extend it back to zero time.

   Why read the value at zero time? The problem is that it takes time for the dye to mix thoroughly in the hemolymph, and it is at the same time being removed by the Malphigian tubes of the insect. So you can never obtain a "true" reading. Extrapolating back to time zero will give you a theoretical value, as if the dye had dispersed throughout the hemolymph instantaneously, and none of it had disappeared. What assumptions do you make in this extrapolation? What possible sources of error are there?
3. Using your estimate of OD at zero time, convert this to hemolymph volume using the standard curve you prepared above.

Part 5: Alternate Method for Determining Hemolymph Volume

If standard curves for OD versus concentration of Amaranth Red have been determined in a previous lab, then you can estimate hemolymph volume by determining how much the injected dye is diluted. To do so, omit Part 1 above and proceed with parts 2 to 4, but omit part 4, step 3. Then do the following:

1. Using the determined value for OD at time zero, find the concentration of Amaranth Red from the standard curves you obtained earlier.

   This concentration refers of course to the solution in the tube after you diluted it. To determine concentration in the cockroach, you must correct for this dilution. If you added 5 µl of sample to 2.5 ml (2500 µl) of Ringer, the dilution is 5:2500 or 1:500. (Actually it is 5:2505, but the difference is well within pipetting error). So the dilution factor is 500 divided by 1, or 500.

   Dilution factor: The dilution factor is the number of times a substance is diluted in the final solution. For example, in a 1:10 dilution, the dilution factor is 10. In a 2:50 dilution, the dilution factor is 25.

   In other words, given a dilution of $x{:}y$ the dilution factor is y divided by x. In order to determine the concentration of the original substance (x) you must multiply the concentration of the diluted substance by the dilution factor.

2. Multiply your dye concentration by the dilution factor to obtain the dye concentration in the cockroach:

   Concentration in cockroach (µg ml^{-1}) = Concentration in tube (µg ml^{-1}) × 500

   ________________ µg ml^{-1} = ________________ µg ml^{-1} × 500

   Dye concentration in the cockroach can now be converted to hemolymph volume by determining how much the hemolymph diluted the original injected solution. For example, if the hemolymph diluted the injected dye 100 times, then its volume must be 99 times greater than the volume of the injected dye. (Remember that a dilution of 1:100 is made with 1 part stock to 99 parts diluent.)

3. Calculate hemolymph volume as follows (all volumes in µl):

$$Hemolymph\ volume = \left(\frac{Conc.\ of\ injected\ dye}{Conc.\ of\ sample\ at\ time\ zero}\right) x\ (\ Volume\ injected\ )$$

Note that the first term is a ratio (the dilution factor) and so any units could be used as long as they are the same for dividend and divisor.

Acknowledgments

Many people have contributed to this exercise and the other exercises in the series. The exercises were first conceived of and written by Stephen S. Tobe. I also thank, in particular, Anne L. Cordon and Corey A. Goldman for valuable modifications and improvements in both procedures and the written text.

APPENDIX A
Further Reading

The following articles pertain to blood and its volume in cockroaches. See also Rockstein's *Physiology of the Insecta*, Wigglesworth's *Principles of Insect Physiology*, or other animal and comparative physiology texts.

Jones, J. C. 1977. The circulatory system of insects. C.C. Thomas, Springfield, 255 pages.

Lee, R. M. 1961. The variation of blood volume with age in the desert locust (*Schistocerca gregaria* Forsk.). Journal of Insect Physiology, 6:36–51.

Miller, T. A. 1985. Structure and physiology of the circulatory system. Pages 289–353, *in* Comparative insect physiology, biochemistry and pharmacology, Volume 3 (Kerkut and Gilbert, Editors). Pergamon Press.

Wall, B. J. 1970. Effects of dehydration and rehydration on *Periplaneta americana*. Journal of Insect Physiology, 16:1027–1042.

Wharton, D. R. A, M. L. Wharton. and J. Lola. 1965. Blood volume and water content of the male American cockroach, *Periplaneta americana* L.: Methods and the influence of age and starvation. Journal of Insect Physiology, 11:391–404.

The following are more general texts on cockroaches:

Bell, W. J., and K.G. Adiyodi. 1981. The American cockroach. Chapman and Hall, London, 550 pages.

Cameron, E. 1961. The cockroach. William Heineman, London, 111 pages.

Cornwell, P. B. 1968. The cockroach. 2 volumes. Hutchinson, London.

Guthrie, D. M., and A. R. Tindall. 1968. The biology of the cockroach. Edward Arnold, London, 408 pages.

APPENDIX B
Alternative Methods for Extracting Hemolymph

If absolutely no hemolymph can be extracted from the base of the legs there are alternative methods. You must remember that these may be fatal, and thus used only as a last resort. Therefore, if necessary, use only for the 30-minute sample. Remember, you want to extract hemolymph (reddish fluid); do not pipet unrecognizable substances. *Consult your TA (for permission) if one of these techniques is to be used.*

1. **Snipping antennae:** The preferred method is snipping the antennae near the base with sharp scissors. Gently compress the thorax of the cockroach, rolling it from posterior to anterior to force hemolymph towards the head. Repeat. If both antennae are cut, about 2 µl should be extracted.

2. **Snipping of tarsi:** Snipping tarsi (terminal segment of limb) should provide additional hemolymph. Begin with hind limb, but any limb should do. Follow procedure as above (compressing in direction as is appropriate).

3. If the cockroach is extremely desiccated cut a “V” in the chitin posterior to the head. Pull the head forward and down to cut. Remove the chitin wedge and slice the exposed membrane with a thin, shallow cut (if too deep the esophagus will be pierced). Extract hemolymph.

APPENDIX C

Making Solutions and Measuring Concentrations

Many of our students have had little or no experience in pipetting, making solutions, etc., let alone in using a spectrophotometer. So we have usually preceded the hemolymph lab with a lab giving them a chance to practice these basic techniques, as well as learn how to use a spectrophotometer. Doing this lab beforehand enables them to complete the hemolymph lab much more easily in the allotted 3 hours. What follows is the essence of this lab. Not included are details of the spectrophotometer, which will depend on the instrument available.

Objectives

Any physiological experiment depends on your ability to make and dispense solutions accurately. In this lab you will (1) learn how to measure volumes and weights accurately using pipets and balances. Then with only a simple balance and a pipet you will (2) learn how to make a wide range of dilutions. Finally, you will (3) learn to use a spectrophotometer to measure the concentration of solutions.

Your objective in this laboratory will be to master these techniques in order to:

1. Prepare a standard concentration curve using the Amaranth Red dye.
2. Identify the possible sources of error in your measurements.
3. Determine the concentrations of unknown solutions using your standard curve.

Outline

1. Examine your pipets and determine their types.
2. Practice pipetting, and investigate the accuracy and reproducibility of pipetting.
3. Make a series of solutions of the Amaranth Red dye using the single dilution technique.
4. Become familiar with the operation of the spectrophotometer.
5. Measure the optical density (OD) of your solutions, and plot the results as a function of concentration.
6. Determine the concentration of a given unknown solution of Amaranth Red dye.

Procedures

Part 1: Pipets and Pipetting

A pipet is a slender tube of glass or polypropylene used for transferring or measuring small quantities of liquids. The following information on pipets is taken in part from the Canlab Laboratory Equipment catalogue (1981:730).

The true measure of a pipet is defined in specifications established by The National Bureau of Standards and various other agencies. In order to meet these standards, certain criteria must be followed in the calibrating process. (1) The calibrating temperature of a pipet is 20°C. However, should pipets be used at other temperatures, variations are usually so slight as to be negligible. (2) The minimum and maximum delivery times have been established for the various sizes and types of pipets. The size of the pipet tip regulates the rate of outflow; any alteration of delivery time may affect pipetting accuracy. (3) To meet government standards pipets must be calibrated with either distilled water or mercury. “To deliver” pipets are always calibrated with distilled water. “To contain” pipets are always calibrated with mercury.

To Contain (TC): Pipets designated as “to contain” are calibrated by introducing into them the exact weight of mercury required to give the required volume. Mercury does not wet glass. Pipets calibrated with mercury will contain, but not deliver, the stated volume of aqueous fluid (a film of water will always cling to the wall of the pipet). “To contain” pipets *must not be blown out* (except capillary micropipets). The letters “TC” are designated below the mouthpiece.

To Deliver (TD): "To deliver" pipets are calibrated by weighing the volume of distilled water that will flow from them by gravity, *with the tip against the side of the receiving vessel*. A small amount of liquid always remains in the tip and *must not be blown out*. The letters TD are designated below the mouthpiece.

To Deliver with Blow Out: Calibration of "to deliver with blow out" pipets is similar to that used for "to deliver" pipets, except that the drop remaining in the tip after delivery is blow into the receiving vessel. The letters "TD" are designated below the mouthpiece and above this a single or double etched frosted band identifies a "to deliver with blow out" pipet.

In this laboratory series you may be using three kinds of pipets: (1) volumetric (transfer), (2) measuring pipets, and (3) micropipets. Most pipets are color-coded to indicated capacity.

Volumetric pipets have a bulb midway between the mouthpiece and the tip. The bulb decreases the surface area per unit volume and diminishes the possible error resulting from water film. You should use volumetric pipets when you need a high degree. They are generally calibrated "to deliver" a specific volume. Volumetric and transfer pipets are the same.

Measuring pipets are made from straight bore tubing, have multiple graduations, and are calibrated with water. We will use both "to deliver" and "to deliver with blow out" types. Measuring pipets are guaranteed accurate only at the maximum calibration mark (accuracy depends on the uniformity of the pipet bore) and are used when a high degree of accuracy is not essential.

Micropipets (also called microcapillary pipets) have a capacity of 1 ml or less. They are of the "to contain" type and fill by capillary action. Micropipets are calibrated to dispense their entire volume and are usually used for adding a known volume of one liquid to another liquid. They will not empty by gravity, and so it is necessary to empty them either by blowing out or by using a plastic mouthpiece adapter. After expelling the contents, you should rinse the micropipet two or three times with the resulting aqueous solution to make sure all contents are mixed with the solution. Use a consistent number of rinsings for maximum reproducibility. Micropipets are color-coded to indicate their dispensing capacity; for example, green, 50 µl; white, 5 µl graduated (1–5 µl) micropipet. In these labs we will use disposable micropipets. When you finish using them, *discard them in the waste receptacle provided for disposable glass*.

Part 2: How to Pipet

You should never pipet by mouth. A pipet manipulator should be used. This is usually a rubber bulb into which the pipet is inserted and which has valves for suction and evacuation.

1. Using water and a "to deliver" pipet, draw the liquid into the pipet until the pipet contains more liquid than required. Remove the pipet from the reservoir and wipe off the tip with a tissue. Drain the pipet until it contains precisely the volume desired. Touch the tip to the side of the receiving vessel. Now deliver the appropriate volume of liquid into the vessel.

   Note: Practice dispensing a given volume with "to deliver" and "to deliver with blow out" measuring pipets, and both a volumetric pipet and a micropipet. Share a micropipet for this demonstration with other students at your bench (micropipets cost money!). However, do not re-use micropipets during the later experiments. (Why?)

2. To read the volume in a pipet, measure to the bottom of the meniscus. The meniscus should be read at *eye level*.

Part 3: Errors in Pipetting and Weighing (Work in pairs)

Question: Is there a difference in the accuracy and precision in the different types of pipets? In what way do these differences affect your choice of pipets?

Design an experiment to see how the three basic types of pipets compare in their ability to transfer the correct/true amount (accuracy) and their reliability to transfer the same amount each time (precision). Think about your design before the lab; working with your partner, refine your plan in the lab. The precision (reproducibility) of an instrument is often more important than its accuracy, since the actual performance can be calibrated against a standard.

You will be provided with the following materials: 10 ml "to deliver", 10 ml "to deliver with blowout", and 10 ml volumetric pipets; electronic balance for weighing; beakers; and water.

Write out your experimental design in your lab notebook and consult with your teaching assistant before proceeding. *Hint:* 1 ml of water weighs 1 g at standard temperature and pressure (20°C and 1 atmosphere).

Question: How much of your observed pipetting error could be the result of errors in weighing?

Design and carry out a simple experiment to test for this. You will be provided with the following materials: electronic balance (same as above); beaker.

Part 4: Dilution Series – Single Dilution Technique

You will be provided with a solution of Amaranth Red dye. The concentration is stated on the label. Amaranth Red is a dye commonly used in food coloring (it is also known as Red Dye No. 2). Many products contain this dye which you would not think contain food coloring; for example, Amaranth Red has been used to give sugar its remarkably white appearance. (*Note:* Amaranth Red is now suspected of being carcinogenic if ingested and its use in the food industry has been severely limited. Handle it with care). You are going to prepare a series of eight solutions containing the stock Amaranth Red solution at different concentrations as shown below. You will be diluting with Ringer solution – a solution of salts which is similar to body fluids and designed to keep tissues functioning in physiological preparations.

Solution	Dilution	Procedure
1	1:5	1 part Amaranth stock to 4 parts Ringer
2	1:10	1 part Amaranth stock to 9 parts Ringer
3	1:20	1 part Amaranth stock to 19 parts Ringer
4	1:30	1 part Amaranth stock to 29 parts Ringer
5	1:40	1 part Amaranth stock to 39 parts Ringer
6	1:50	1 part Amaranth stock to 49 parts Ringer
7	1:100	1 part Amaranth stock to 99 parts Ringer
8	1:500	1 part Amaranth stock to 499 parts Ringer

The term in the second column above, refers to the *concentration of the final solution*, expressed on the basis of the volume of the "stock" solution in a total final volume. Thus, a 1:5 solution contains one volume of a given solution in a total of 5 volumes of liquid. Notice that to end up with 5 volumes of the diluted solution, you take 1 volume of the stock and add 4 (not 5!) volumes of the diluent. Think of it this way: in a 1:5 (usually said as "one in five") solution, in every 5 parts there is one part of the original stock solution, and the rest is the diluent.

Notice also that 1:5 is a ratio; thus it is the same as a 2:10 or a 5:25 solution. This is useful if you are making up an arbitrary quantity of final solution. For example, to make 25 ml of a 1:5 solution, you would take 5 ml of stock, and 25 - 5 = 20 ml of diluent.

In general then, the concentration term $x{:}y$ refers to a solution which contains a volume x of a stock solution in a total volume of y; in terms of stock and diluent, the solution contains x volumes of stock and $y - x$ volumes of diluent.

Whenever you make a dilution series keep in mind the following factors:

(a) The **concentration** required. Make a table like the one above indicating the concentration, the number of parts of the stock solution needed, and the number of parts of the diluent necessary to obtain the desired concentration.

(b) The **volume** of the sample required at a given concentration. In this experiment the limiting factor is the minimum volume of solution which can be read accurately in the spectrophotometer; for this laboratory assume that volume is 5 ml so you must make up at least 5 ml of each solution. Whenever you are making dilutions, study the experiment to know how much solution you will need. Use the minimum amount of stock for the dilutions to minimize the wastage; if you need only 5 ml it is wasteful to make up 100 ml and throw the rest away.

(c) The **accuracy** of the instruments available. You may need to make a dilution of 1:5000 and the volume of the sample required is only 5 ml. If you don't have a pipet that can accurately deliver 0.001 ml (1 µl) you will need to make a greater volume of that sample. Usually you would do a *serial dilution* for such a large dilution. (Serial dilutions are discussed later.)

To make up the solutions above, you would need a table such as the one given below. You should complete the partial columns to check your understanding. Note that we have included an extra column to list the concentration of each solution in terms of weight of substance per volume of solution; you often need to know the *quantity of solute present* in addition to the volume of stock solution.

Calculate the final concentration in micrograms per milliliter, given that the stock solution of Amaranth Red dye is 0.01% w/v; this is equivalent to *0.01 g in 100 ml*, or 10 mg in 100 ml, which is the same as 0.1 mg in 1 ml, or 100 µg in 1 ml.

Tube	Concentration (vol/vol)	Amaranth stock	Ringer (ml)	Total (ml)	Concentration* ($\mu g\ ml^{-1}$)
1	1:5	1.0 ml	4.0	5	
2	1:10	0.5 ml		5	
3	1:20		7.6	8	
4	1:30	0.2 ml	5.8		
5	1:40	0.2 ml		8	
6	1:50	0.1 ml		5	
7	1:100	50.0 µl	5.0	5	
8	1:500	10.0 µl	5.0	5	

* Calculated as weight/volume; stock = 0.01% or 100 $\mu g\ ml^{-1}$.

Now make up the solutions as follows:

1. Using the appropriate pipets, make three tubes each of solutions 1 to 6, using the above table to determine volumes. Mix your solutions well. Put a square of parafilm over the mouth of the test tube before shaking (be careful not to contaminate solutions!)

2. To make solutions 7 and 8 (three tubes each), use the 50 and 10 µl capillary micropipets that are provided. These micropipets are "to contain" types and should be treated as such when you use them. They will fill by capillary action when the tip is immersed in solution. When filled to the line, they contain exactly 50 or 10 µl, respectively. Allow the fluid to rise above the line, remove the pipet from the fluid, and wipe its surface with a tissue. Then tap the tip of the pipet gently on a tissue until the liquid level corresponds precisely with the line. Expel the fluid into the solution by gently blowing. Then rinse the pipet three times with the solution by carefully sucking the solution up and down in the pipet. *Do not re-use micropipets.*

   Note: Your 10 ml pipet is not calibrated to the 0.01 ml level. So you should add 5 ml of Ringer for solutions 7 and 8, rather than the more "correct" 4.95 ml and 4.99 ml. What percentage error does this represent?

Part 5: Establishing a Standard Curve

You are now ready to read your solutions in the spectrophotometer to establish a "standard curve" which will relate optical density (OD) to concentration. Given this curve, you could then determine the OD for an unknown solution, and determine its concentration from the graph.

1. Empty the plain Ringer from your sample tube, draining as much out as possible. Refill the tube about half full with solution 8 (1:500). Read the absorbance (the optical density). Record this value.

   Note: When you are measuring a series of solutions of different concentrations, it is a good idea to start with the least concentrated. Then any contamination of the later solution by the previous one has minimal effect. (Think about it!)

2. Record the optical densities of solutions 7 through 1. Before each new solution is added, rinse out the sample tube with a small amount of distilled water, and drain well. Remember to wipe off the outside of the tube before inserting it into the spectrophotometer. Do not handle the lower part of the tube to avoid fingerprints which can affect the absorbance.

3. Plot a graph of OD versus concentration of the Amaranth Red dye solutions. Plot concentration on the horizontal axis and OD on the vertical. (Think about dependent and independent variables.) Use the calculated figures for concentration in the above table expressed as weight per volume ($\mu g\ ml^{-1}$).

Part 6: Determine the Concentrations of Unknown Solutions

Determine the concentrations of the unknown solutions of Amaranth Red (provided by your TA) by reading the OD of the solutions in the spectrophotometer and then reading the final concentration from your standard curve. *Hint:* Some of the concentrations will require diluting before you are able to read them!

Part 7: Dilution Series – Serial Dilution Technique

The "single dilution technique" is called *single* because it uses the original stock solution for *each* dilution. For example, to make a 1:100 dilution, you added 1 part of the stock solution of dye to 99 parts of Ringer solution. However, dilutions may also be prepared by *serial* dilution. To make a 1:00 solution by serial dilution you can make two 1:10 dilutions:

Solution	Concentration	Parts dye	Parts Ringer	Total parts
A	1:10	1 of stock	9	10
B	1:100	1 of A	9	10

Notice that you use Solution A to make Solution B. You only use the original stock solution to make the first dilution. If you wanted to make a 1:1000 dilution, you could make three serial 1:10 dilutions.

Solution	Concentration	Parts dye	Parts Ringer	Total parts
A	1:10	1 of stock	9	10
B	1:100	1 of A	9	10
C	1:1000	1 of B	9	10

A 1:500 dilution could be achieved in several ways (the possibilities are many!). Two examples are as follows:

Dilution	Solution	Parts dye	Parts Ringer	Total parts
1:10	A	1 of stock	9	10
1:100	B	1 of A	9	10
1:500	C	1 of B	4	5
1:5	A	1 of stock	4	5
1:500	B	1 of A	99	100

From these examples, we can write a general "formula" for calculating the proportions or "parts" of both the stock and diluent needed to prepare any dilution:

> Given a solution of concentration 1:x and knowing the desired concentration is 1:y, you would mix x parts of the given solution with y - x parts of diluent to give you y parts of the desired solution.

For example, given a solution of 1:20 and you want a solution of 1:50, you would mix 20 parts of the given solution with 50 - 20 = 30 parts of diluent, to give 50 parts of the desired solution. Notice once again that these are ratios, and can also be thought of as 2 parts plus 3 parts to give 5 parts. Reducing the terms to the smallest integers makes it easier to calculate volumes. Suppose you really wanted 20 ml of final solution, you would then simply divide the desired volume by the final number of parts, and multiply the constituent parts by this ratio. In this example, $20 \div 5 = 4$, and $4 \times 2 = 8$ and $4 \times 3 = 12$. So you take 8 ml of the given solution and add 12 ml of diluent to give you 20 ml of your desired solution.

Note that the concentration of the final solution is the product of the dilution and the original concentration. In the above example, the original concentration was 1:20. Making a dilution of 20:50 gives a final concentration of 1:20 × 20:50 = 20:1000, or 1:50. It works the same for diluting 8:20; try calculating it!

Dilution Series of Amaranth Red Dye

You are able to make a dilution series of Amaranth Red dye for a standard curve using a serial dilution as well as the single dilution. The table below outlines the protocol for setting up this dilution series. The ratio of "stock" to diluent has been worked out for you.

Complete the table below. Calculate the actual volume (in milliliters) that you will need of dye solution (either from the stock or from the previous solution) and Ringer for each solution. Remember that the final volume in each test tube must be at least 5 ml to read the sample in the spectrophotometer. Remember also that the maximum capacity of the test tube is 20 ml. Also note that some of the final volume is removed to make the next solution. *Hint:* When you are calculating the volumes, start from the end of the table and work backwards — this way you can make certain that you start with enough of Solution 1. The volumes of the last two solutions have already been calculated for you. Calculate the final concentration of Amaranth Red dye in each solution, in µg per ml.

Solution	Dilution	Parts A	Parts Ringer	Volume A (ml)	Volume Ringer (ml)	Total volume (ml)	Volume remaining (ml)	Final conc. ($\mu g\ ml^{-1}$)
1	1:5	1 of stock	4					
2	1:10	5 of soln 1 or 1 of soln 1	5 1					
3	1:20	1 of soln 2	1					
4	1:30	2 of soln 3	1					
5	1:40	3 of soln 4	1					
6	1:50	4 of soln 5	1					
7	1:100	1 of soln 6	1	3	3	6	5*	
8	1:500	1 of soln 7	4	1	4	4	5	

* Total volume of 6 minus 1 ml removed for Solution 8.