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A Versatile, Inquiry-Based Enzyme Lab: The Inhibition of Acetylcholinesterase from Bean Beetles by an Organophosphate Insecticide and Factors that Modulate this Inhibition

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Here we expand on an enzyme lab that can lend itself to a multi-session investigative approach. We use a colorimetric enzyme assay to look at the inhibitory effect of the organophosphate insecticide malaoxon on the activity of acetylcholinesterase (AChE) extracted from the bean beetle Callosobruchus maculatus. This system can be used to introduce long-term projects on which students can focus in the ensuing lab sessions. Examples include investigating whether different food sources affect sensitivity of AChE to malaoxon, and whether there are differences in sensitivity of AChE extracted from different strains of the beetles to the insecticide.

Keywords: enzyme assay, bean beetle, acetylcholinesterase

Introduction

Here we present an expanded version of the enzyme lab we presented previously (Fermin et al., 2014). This lab lends itself to a multi-session investigative approach that can be used in an introductory biology laboratory. We use an enzyme assay to look at the effect of the organophosphate insecticide malaoxon on the activity of acetylcholinesterase (AChE) in the bean beetle Callosobruchus maculatus. Bean beetles are pests of legume seeds and organophosphate insecticides have been used to control them. Malaoxon inhibits the activity of AChE and interferes with neuronal activity. The beetles are easy to maintain in the laboratory and make good model organisms to study. We provide an introduction to the animals (based on Beck and Blumer, 2011, not reproduced here) and have the students start their own cultures. The procedure involves a crude protein extraction and a colorimetric assay to determine enzyme activity. The procedure is also used to investigate whether the inhibition by the insecticide is competitive or non-competitive. It is helpful for the students to have received an introduction to enzymes and cell-cell communication in class before this lab. This system can be used to introduce long-term projects on which students can focus in the ensuing lab sessions. One example described here is investigating whether different food sources (cowpeas Vigna uncuuiculata, mung beans Vigna radiata, and perhaps even adzuki beans Vigna angularis) affect sensitivity of AChE to malaoxon. Depending on availability of resources, other projects may include whether there are differences in sensitivity of AChE extracted from different strains of the beetles to malaoxon, or whether the temperature at which the animals are incubated affects the sensitivity of AChE to malaoxon. All of these have been suggested in published research (Gbaye et al. 2011 and Gbaye et al. 2012) and some of the published data are reviewed as background information with the students in the introduction to the lab. In 2-3 regular lab periods, introductory biology students can learn about the biology of the beetles, start their own cultures, learn the basic enzyme assay, and investigate the inhibitory effect of malaoxon and its mode of inhibition, before moving on to long-term projects. The types of project outlined here can be performed in regular three-hour lab periods. The projects can be treated as inquiry-based or as guided-inquiry, depending on the goals of the instructor and the department curriculum. The students write a formal lab report on the results of the third investigation described here. By the time our students perform this set of exercises they have already been introduced to descriptive and inferential statistics, performed experiments the data from which were analyzed using student t-tests, and written formal lab

reports. The introduction to descriptive and inferential statistics is not included here. These lab exercises tie in several topics together: data processing and presentation, enzymatic reactions and enzyme inhibitors, cell-cell signaling, and biological applications to industry and their potential ecological consequences.

Depending on the goals of the course, the lab exercises could be paired with discussion of different types of scientific literature focusing on the effects of organophosphate insecticides. Examples include:

- Chapters in Rachel Carson's "Silent Spring"
- London et al. (2005) a review article focusing on the potential link between organophosphate insecticides and suicide
- Our choice for our class: Sadeghi Hashjin et al. (2013) a simple experimental paper using the same type of data analysis as our students will use that links malathion with anxiety in rodents

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Student Outline

Pests Be Gone! Or Not?!
A Versatile, Inquiry-Based Enzyme Lab: The Inhibition of Acetylcholinesterase from Bean Beetles by an Organophosphate Insecticide and Factors that Modulate this Inhibition

Objectives

After this set of lab exercises you should be able to:

1. Describe how and why organophosphate insecticides work to carry out their intended function.
2. Explain why bean beetles are a good system for studying the effect of organophosphate insecticides on acetylcholinesterase.
3. Carry out an experiment to investigate the mode of inhibition of the enzyme by the organophosphate insecticide malaoxon.
4. Design an experiment to investigate whether the food source (as an example) may affect the sensitivity of bean beetles to organophosphate insecticides.
5. Collect, analyze, and present the data from these experiments.
6. Discuss the limitations of each experiment and suggest future steps based on the results obtained.

Introduction

Bean beetles, in the genus *Callosobruchus*, are agricultural pests found in tropical and subtropical regions of Africa and Asia. They are pests of legume seeds (family Fabaceae) such as mung beans (*Vigna radiata*) and black-eyed peas (also called cowpeas, *Vigna unguiculata*). The adults do not require food or water and they spend their short lifespan (1-2 weeks) mating and laying eggs on bean seeds. The larvae then feed on the bean embryo and endosperm and thus destroy the bean crop (Beck and Blumer, 2011). Thus there is great interest in controlling or eliminating these pests and minimizing their effect on the bean harvest.

Organophosphate insecticides such as malaoxon have been used to control insect pests such as bean beetles. These insecticides work through their effect on the enzyme acetylcholinesterase (AChE). AChE is an important component of cell-cell signaling in the nervous system where it breaks down the neurotransmitter acetylcholine and helps to terminate the signal. Inhibition of AChE interferes with this process. This is how the insecticide interferes with proper functioning of the nervous system in the insects and leads to their eventual death.

Recent studies (Gbaye et al. 2011, and Gbaye et al. 2012) have suggested that there are differences in the sensitivity to organophosphate insecticides among different species in the genus *Callosobruchus* and among different geographical strains, and that these differences may be influenced by environmental factors such as temperature and food source. There are defensive chemicals in the plants that can be toxic to the insects and these insects have developed ways to overcome such chemicals from many legumes. Presumably these plant chemicals, when ingested, may induce the production of enzymes that help detoxify the insecticide as well (Gbaye et al., 2012). We are using the LB strain of the species *Callosobruchus maculatus* that were collected by Rodger Mitchell of Ohio State University from mung beans that were purchased in Columbus, Ohio in the early 1970s (Blumer, personal communication). The geographic source of the LB strain is not known.

In this lab exercise you will focus on the LB strain of the species *Callosobruchus maculatus* and will use a colorimetric enzyme assay to study the inhibitory effect of the organophosphate insecticide malaoxon on the activity of AChE extracted from these insects. You will determine whether the insecticide acts as a competitive or a non-competitive inhibitor. You will also carry out an experiment to answer one question of particular interest: whether culturing this particular beetle strain on different bean species leads to different sensitivity of the enzyme AChE to malaoxon. The Enzyme and Its Role

The Enzyme and Its Role

In synaptic signaling, a neurotransmitter such as acetylcholine (ACh) is released from the terminal end of the pre-synaptic neuron into the synaptic cleft (the space between the two neurons or between a neuron and a muscle cell). The neurotransmitter then binds to specific receptors on the post-synaptic membrane where it will relay the signal. The enzyme acetylcholinesterase (AChE) is present in the post-synaptic membrane and breaks down ACh into acetate and choline as shown in Figure 1.

This helps both maintain a low concentration of ACh in the synaptic cleft and terminate the signal. Choline is taken up by the pre-synaptic neuron and used to make ACh again. The post-synaptic membrane can then be ready to receive a future signal. See Figure 1. If AChE is not acting properly or its activity is inhibited, then concentrations of ACh in the synaptic cleft can remain high, and the ACh receptors on the post-synaptic membrane can be saturated with ACh. This interferes with proper functioning of the neurons that could lead to death.

The Enzyme Assay

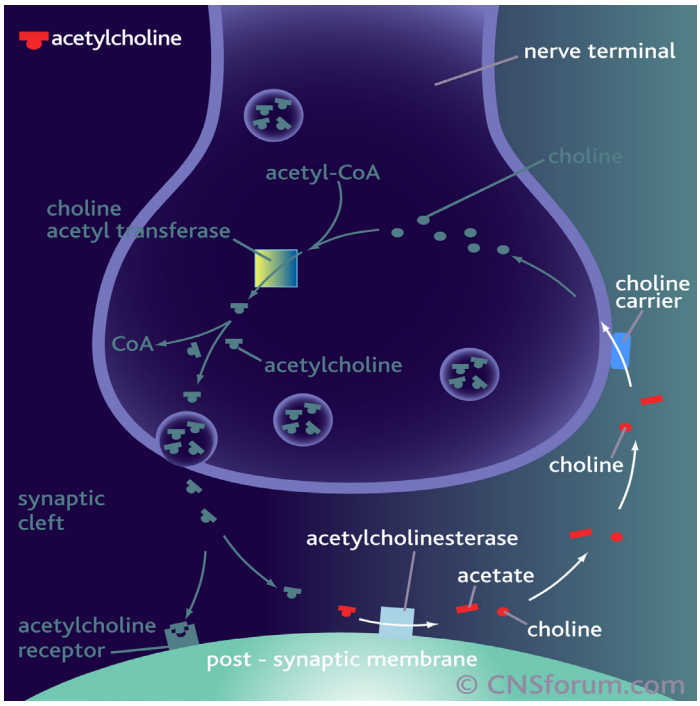
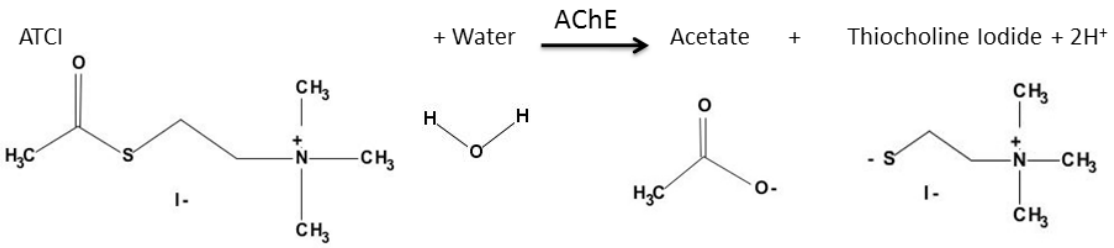
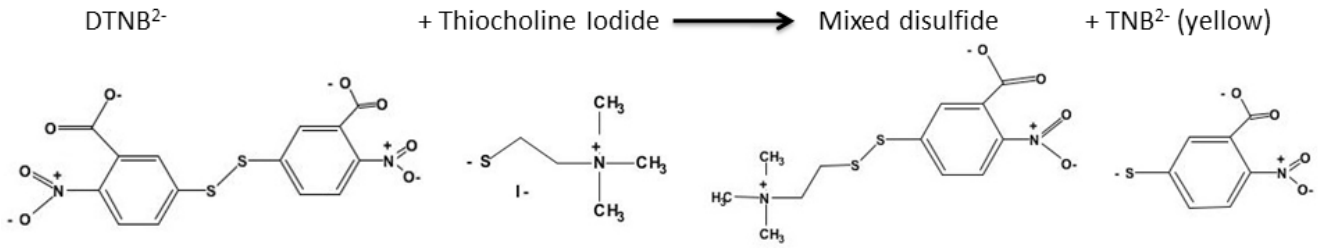


Figure 1. Synaptic signaling involving the neurotransmitter acetylcholine.
Image source: The Lundbeck Institute, CNS Forum (2002-2011). The mechanism of action of acetylcholinesterase. Source: http://www.cnsforum.com/educationalresources/imagebank/dementia_cholinergic/rcpt_sys_ach_esterase (Note: The CNS forum grants permission to download and use this image "including in presentations, articles and publications, provided that the copyright notice on the images is not hidden nor removed.")

The enzyme assay we will use is based on the work done by Ellman et al. (1961), Ffrench-Constant and Bonning (1989), Spencer et al. (1998), and Gbaye et al. (2012). Basically we take advantage of the type of reaction carried out by the enzyme AChE by supplying substrates other than ACh to the enzyme in vitro. In the presence of the enzyme AChE, the compound ATCI (acetylthiocholine iodide) is hydrolyzed to produce acetate and thiocholine.



The thiol (R-SH) group of thiocholine can react with the indicator compound DTNB (5,5-dithio-bis(2-nitrobenzoic acid)) to form TNB- (5-thio-2-nitrobenzoate), which ionizes to the TNB2- dianion in water at mildly alkaline pH. This dianion is yellow and its presence can be detected using a spectrophotometer.



The more yellow TNB2- products accumulate in the test tube, the higher the optical density (absorbance) of the solution in the test tube will be at a specific wavelength. If an inhibitor inhibits the enzyme, then the rate of reaction will be slower and the optical density (absorbance) of the solution in the test tube will be lower after a set amount of time compared to a similarly prepared test tube without such an inhibitor. Thus, we can use a simple bioassay using this indicator dye to determine whether the insecticide of interest inhibits the enzyme AChE.

In this lab exercise you will use the enzyme assay described above to study the inhibitory effect of the organophosphate insecticide malaoxon on the activity of AChE extracted from bean beetles. To get used to the technique and how the insecticide inhibition is measured you will first carry out a simple experiment to document that a known concentration of the insecticide does in fact inhibit the rate of AChE extracted from these insects. In your second investigation you will use the same technique to determine whether the insecticide acts as a competitive or a non-competitive inhibitor. This will set the groundwork for us to determine whether culturing the beetles on different legume seeds can affect the sensitivity of AChE to malaoxon. Since it takes 4-5 weeks for the adults to emerge, we need to start the cultures on the new legume seeds now before we perform any other part of this lab exercise.

Setting up Cultures

Materials

- Bean beetles, Callosobruchus maculatus, the LB strain
- Seeds of different species of legumes (Vigna radiata mung beans and V. unguiculata black-eyed peas = cowpeas)
- Lidded plastic containers with pin-holes in the lids
- Small paint brushes
- Dissecting microscopes
- Incubators at 30C
- Petri dishes

Procedure

- Familiarize yourselves with the life cycle of the animal. Observe the available cultures. You may use a dissecting microscope to better view the eggs and the color patterns of the male and female adults.
- Look for eggs on the beans. Note that some beans may have more than one egg.
- Look for openings in the beans through which the adults have emerged.
- Look for the adults.
- Identify the adult males and females. How can you tell them apart?
- Add 50 mL of seeds of the selected legume species to a new snap-lid container. Make sure there are pin-holes in the lid to allow for gas exchange.
- Label the container with a piece of tape on which you write your names and the date the culture is started.
- Select 5 adult males and 5 adult females. Use the available brushes to move the adults to the new container.
- Place the container in the incubator.
- In a few days check the culture to make sure there are eggs present on the seeds. Check your culture weekly to look for adults. The adults should emerge in a few weeks.

First Investigation: The Effect of Malaoxon on the Activity of AChE

What is the class hypothesis with regards to the effects of malaoxon on the activity of AChE?

Alternative:

Null:

What is the experimental prediction?

Materials

- Bean beetles, Callosobruchus maculatus
- Small paint brushes
- Dissecting microscopes
- Homogenizing buffer: 50 mM Tris (pH 7.5), 1% Triton X100
- Reaction substrate: 50 mM Tris (pH 7.5), 10 mM acetylthiocholine iodide (ATCI), 1% Triton X100
- Reaction substrate plus inhibitor: 50 mM Tris (pH 7.5), 10 mM acetylthiocholine iodide (ATCI), 1% Triton X100, 2 mM malaoxon
- Indicator DTNB: 50 mM Tris (pH 7.5), 1.5 mM 5-5-dithio-bis(2-nitrobenzoic acid), 1% Triton X100
- Spectrophotometer
- Spectrophotometer cuvettes (semimicro, 1.5 mL)
- Centrifuge
- Water baths set at 30C
- Petri dishes
- Disposable pellet pestles
- Vortexer
- 1.5 mL microcentrifuge tubes
- Ice bucket
- Micropipettors and tips

Basic Procedure

Follow the basic steps for the enzyme assay to quantify the inhibitory effect of the insecticide on the activity of AChE extracted from the beetles as outlined in the steps below and shown in Figure 2.

- Wear gloves and goggles. There should be no exposed skin: covered legs and arms.
- Use 1 beetle per person: 4 beetles per group, if enough beetles are available. Make sure to determine the gender.
- Take a clean 1.5 mL microcentrifuge tube. Label the tube by writing 1A on the cap and your group number on the side using a permanent marker.
- Select one adult beetle. Make sure to determine the gender. Put the animal in tube 1A. This is beetle #1 for your group.
- Repeat steps 3 and 4 for beetles #2, 3, and 4 (tube 2A, 3A, and 4A).
- Use a micropipettor with the correct tip to add 900 µl of homogenizing buffer to the tube. Keep the tube on ice.
- Use the pestles provided to rapidly homogenize the tissues for 3-5 seconds. Use a separate clean pestle for each beetle. After you are done, you can rinse and dry the pestles and return them to the container for used pestles.
- Close the tube by snapping the cap shut. Put the tubes in the centrifuge. Make sure the centrifuge is balanced. Centrifuge for 5 minutes.
- Label 2 clean microcentrifuge tubes: 1B and 1C (or 2B and 2C, etc.) on the cap and your group number on the side.
- Transfer the supernatant from tube 1A into tube 1B (and from tube 2A into tube 2B, etc.). Avoid transferring the animal parts. Keep the tubes on ice.

- Close the cap. Put the B tubes (1B, 2B, etc.) in the centrifuge. Make sure the centrifuge is balanced. Centrifuge for 5 minutes.
- Transfer the supernatant from tube 1B into tube 1C (and from tube 2B into tube 2C, etc.). Avoid transferring the cloudy white layer from the top of the tubes 1B etc. You should have at least 0.7 mL (700 µl) of solution in each tube. Keep the tubes on ice.
- Close the tube cap. Use the vortexer to vortex tube 1C (and 2C, etc.) for 30 seconds for the solution to be mixed properly.
- Centrifuge for 10 seconds to remove any bubbles.
- Label 2 clean microcentrifuge tubes: 1D and 1E (2D and 2E, etc.) on the cap and your group number on the side.
- Mix the solution in tube 1C (and 2C, etc.) before performing the next step. You need to ensure that the portions of the solution from tube 1C that you put in tubes 1D and 1E are similar.
- Follow the following steps and Table 1 to set up the reaction tubes and the blanks for the enzyme assay.
- Use a micropipettor to transfer 320 µl of the supernatant from tube 1C to tube 1D. Keep the tube on ice.
- Use a micropipettor to transfer 320 µl of the supernatant from tube 1C to tube 1E. Keep the tube on ice.
- Repeat for tube 2C, 2D, and 2E, etc. Keep the tubes on ice.
- Use a micropipettor to add 48 µl of the "reaction substrate" (50 mM Tris (pH 7.5), 10 mM acetylthiocholine iodide (ATCI), 1% Triton X100) to the D tubes (1D, 2D, etc.). Keep the tubes on ice.
- Use a micropipettor to add 48 µl of the "reaction substrate plus inhibitor" (50 mM Tris (pH 7.5), 10 mM acetylthiocholine iodide (ATCI), 1% Triton X100, 2mM malaoxon) to the E tubes (1E, 2E, etc.). Keep the tubes on ice.
- For the entire group (not individually) label two microcentrifuge tubes B-M and B+M. These will serve as the blanks for the spectrophotometer.

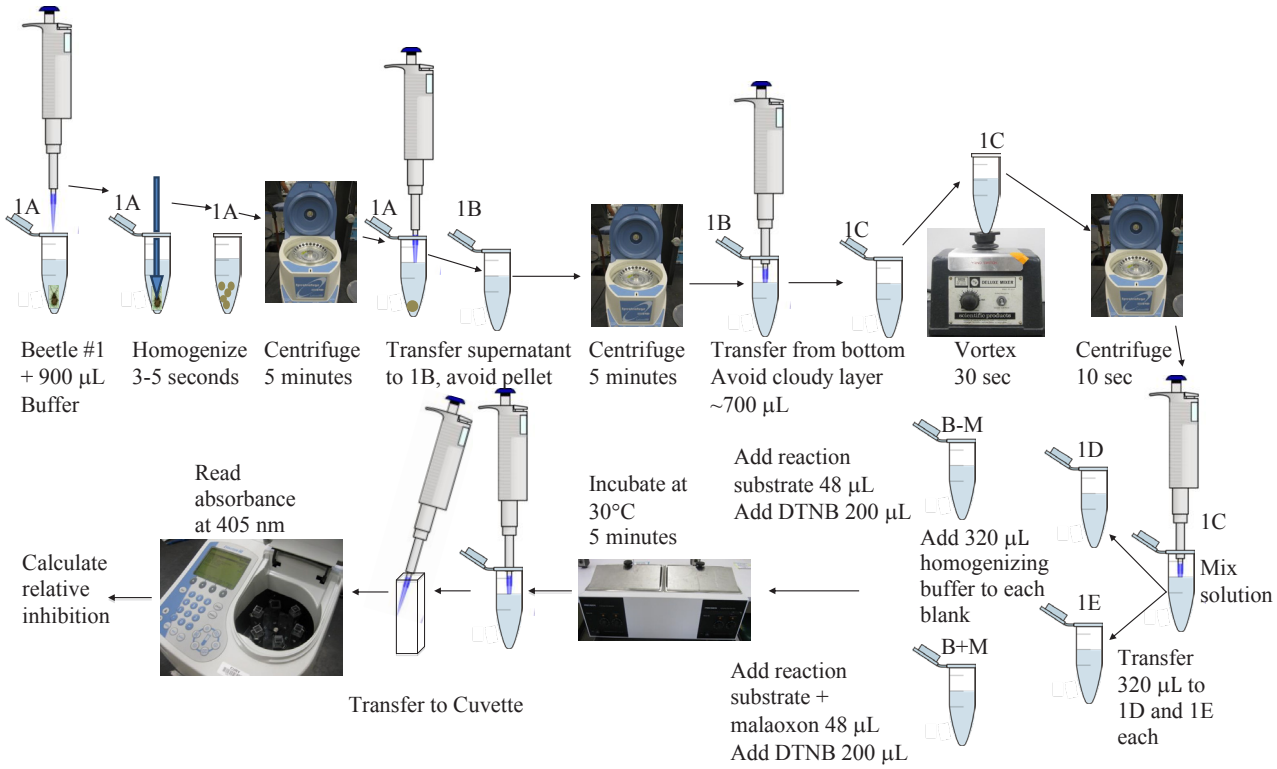


Figure 2. The basic procedure for the enzyme bioassay.

- Prepare a Blank tube that includes the following; this is the blank B–M for solution 1D (and 2D, etc.).
- 320 µl of homogenizing buffer
- 48 µl of "reaction substrate"
- Prepare a Blank tube that includes the following; this is the blank B+M for solution 1E (and 2E, etc.).
- 320 µl of homogenizing buffer
- 48 µl of "reaction substrate plus inhibitor (malaoxon)"
- Use a micropipettor to add 200 µl of the indicator DTNB to both tubes 1D and 1E (2D and 2E, etc.) and the blanks. Keep the tubes on ice until you are ready for the incubation. Why is it important to add the DTNB after the insecticide?

Table 1. Chemical components of the D, E, and blank tubes for the enzyme assay to determine the effect of the inhibitor (malaoxon) on the activity of the AChE enzyme. Add in the order from left to right. Add the DTNB at the end to all tubes.

Tube	320 µl	48 µl	200 µl
D tubes	Supernatant from tube C	Reaction Substrate	DTNB
E tubes	Supernatant from tube C	Reaction Substrate plus inhibitor (malaoxon)	DTNB
B-M	Homogenizing buffer	Reaction Substrate	DTNB
B+M	Homogenizing buffer	Reaction Substrate plus inhibitor (malaoxon)	DTNB

- Incubate the tubes 1D (and 2D, etc.) and 1E (and 2E, etc.) and the blanks (B-M and B+M) in an incubator or in the water bath (your instructor will show you how) at 30C for 5 minutes. Every minute, gently shake the tubes a few times.
- Take 2 spectrophotometer cuvettes (semimicro, 1.5 mL) per beetle. Label them as 1D (and 2D, etc.), 1E (and 2E, etc.) plus your group number. Make sure to label them on the sides that do not interfere with light absorption; your lab instructor will show you how to do this.
- For the entire group label two other spectrophotometer cuvettes (semimicro, 1.5 mL): B–M and B+M and group number.
- Use a micropipettor to transfer the solution from microcentrifuge tube 1D to the clean spectrophotometer cuvette labeled 1D. Do this very gently, and touch the tip of the pipette to the side of the cuvette to avoid forming any bubbles. Discard the tip. Repeat for tubes 2D, 3D, and 4D.
- Use a micropipettor to transfer the solution from microcentrifuge tube 1E to the clean spectrophotometer cuvette labeled 1E. Discard the tip. Repeat for tubes 2E, 3E, and 4E.
- Use a micropipettor to transfer the solution from microcentrifuge tube B-M to the clean spectrophotometer cuvette labeled B-M. Repeat for B+M.
- For each cuvette measure the absorbance at 405 nm using the spectrophotometer provided. Follow the instructions below:
    - o Turn on the spectrophotometer. Allow it to warm up for a few minutes.
    - o The lab instructor will show you how to program the spectrophotometer.
    - o Place the Blank B–M cuvette and your D cuvettes (1D, 2D, etc.) in the correct slots in the spectrophotometer.
    - o Record the absorbance readings at 405 nm in Table 2 below.
    - o Place the Blank B+M cuvette and your E cuvettes (1E, 2E, etc.) in the correct slots in the spectrophotometer.
    - o Record the absorbance readings at 405 nm in Table 2 below.

- Note absorbance is a ratio, and thus unitless. Typically it is written with AU = absorbance units.
- The difference between the absorbance readings for solutions D and E for any given beetle shows you the effect of the insecticide, if any, on the enzyme extracted from the animals.
- We are interested in whether malaoxon inhibits the reaction. However, since the beetles are different sizes and genders, we have to look at the relative change in enzyme activity, not the absolute numbers. For a given beetle, relative inhibition (%) is calculated as follows:

100 (Absorbance for tube D – Absorbance for tube E) / Absorbance for tube D

- Use the class data to determine the average effect of the insecticide on the activity of the enzyme AChE. Use the relative inhibition values. Calculate the mean and standard deviation for the data.
- Your instructor will tell you how to discard your tubes and chemicals.

Second Investigation: Does the Insecticide Act as a Competitive or Non-competitive Inhibitor of AChE?

Alternative hypothesis 1:

Alternative hypothesis 2:

Several different concentrations of malaoxon are available for your use. How could manipulating the concentration of the insecticide help us to test the hypotheses above?

Write the experimental prediction for each alternative hypothesis you wrote above:

Experimental prediction for alternative hypothesis 1:

Experimental prediction for alternative hypothesis 2:

Table 2. Measured absorbance values and relative inhibition of the AChE enzyme activity without or with the organophosphate insecticide (samples D and E, respectively).

Group #	Beetle #	Gender	Sample	Absorbance (AU)	Relative inhibition (%)
			D		
			E		
			D		
			E		
			D		
			E		
			D		
			E		
			D		
			E		
			D		
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			E		
			D		
			E		
			D		
			E		
			D		
			E		
Class Mean					
Standard Deviation					

Conduct the experiment using the basic bioassay as outlined below, collect the data, share the data with the class, and decide how you will graphically present the data. Do the data in your graph look like they support alternative hypothesis 1 or 2? Explain.

Procedure

- The procedure is outlined in the steps below and shown in Figure 3. Wear gloves and goggles. There should be no exposed skin: covered legs and arms.
- Use a permanent marker to label four 1.5 mL microcentrifuge tubes: A, B, C, and D on the caps, group number on the side.
- Select one adult beetle. Make sure to determine the gender. Put the animal in tube A.
- Repeat for tubes B, C, and D.
- Use a micropipettor with the correct tip to add 900 µL of homogenizing buffer to each of the tubes. Keep the tubes on ice.
- Use the pestles provided to rapidly homogenize the tissues for 3-5 seconds. Use a separate clean pestle for each beetle. Rinse and dry the used pestles and put them in a container for used pestles.
- Close the tube caps. Place the tubes in the centrifuge and centrifuge the four tubes for five minutes. Make sure the centrifuge is balanced.
- Use a permanent marker to label a clean, **2 mL** microcentrifuge tube as tube E on the cap and write your group number on the side. The 2 mL microcentrifuge tube differs from the 1.5 mL microcentrifuge tubes in size and shape.
- After the centrifugation, transfer 480µL of the supernatant from each of tubes A-D to tube E. Be careful not to transfer the animal body parts. Keep the tubes on ice.
- Close the tube cap. Centrifuge tube E for five minutes. Make sure the centrifuge is balanced.
- Use a permanent marker to label a clean, **2 mL** microcentrifuge tube as tube F on the cap and write your group number on the side. Transfer the contents of tube E to tube F. Avoid transferring the cloudy white layer from the top of tube E. You should have at least 1.9 mL (1900 µl) of solution in tube F. Keep the tubes on ice.
- Close the tube cap. Use the vortexer to vortex tube F for 30 seconds for the solution to be mixed properly.
- Centrifuge for 10 seconds to remove any bubbles. Make sure the centrifuge is balanced.
- Use a permanent marker to label five **1.5 mL** microcentrifuge tubes as 0, 0.5, 1, 2, and 3 on the cap and write your group number on the side. These are for the different concentrations of the insecticide malaoxon you will be using.
- Follow the **instructions below and Table 3** to add the correct amount of each solution to each tube including the blanks.
- Use a micropipettor to transfer 320 µL of the supernatant from tube F into each of the five tubes labeled 0, 0.5, 1, 2, and 3.
- Use a micropipettor to add 48 µl of the "reaction substrate" to the tube labeled 0. Keep the tubes on ice.
- Use a micropipettor to add 48 µl of the "reaction substrate with 0.5 mM malaoxon" to the tube labeled 0.5. Keep the tubes on ice.
- Use a micropipettor to add 48 µl of the "reaction substrate with 1.0 mM malaoxon" to the tube labeled 1. Keep the tubes on ice.
- Use a micropipettor to add 48 µl of the "reaction substrate with 2.0 mM malaoxon" to the tube labeled 2. Keep the tubes on ice.
- Use a micropipettor to add 48 µl of the "reaction substrate with 3.0 mM malaoxon" to the tube labeled 3. Keep the tubes on ice.
- For each concentration of malaoxon you need a different blank. Two or more groups can share the blanks. Talk to your neighboring group(s) and decide who will make which blank. Here is the list of the blanks the you will need:

- o In a microcentrifuge tube prepare a Blank that includes the following, the "B-0" blank for solutions with no malaoxon (tubes 0).
  - 320 µl of homogenizing buffer
  - 48 µl of "reaction substrate"
- o In a microcentrifuge tube prepare a Blank that includes the following, the "B-0.5" blank for solutions with 0.5 mM malaoxon (tubes 0.5).
  - 320 µl of homogenizing buffer
  - 48 µl of "reaction substrate plus 0.5 mM malaoxon"
- o In a microcentrifuge tube prepare a Blank that includes the following, the "B-1" blank for solutions with 1.0 mM malaoxon (tubes 1).
  - 320 µl of homogenizing buffer
  - 48 µl of "reaction substrate plus 1.0 mM malaoxon"
- o In a microcentrifuge tube prepare a Blank that includes the following, the "B-2" blank for solutions with 2.0 mM malaoxon (tubes 2).
  - 320 µl of homogenizing buffer
  - 48 µl of "reaction substrate plus 2.0 mM malaoxon"
- o In a microcentrifuge tube prepare a Blank that includes the following, the "B-3" blank for solutions with 3.0 mM malaoxon (tubes 3).
  - 320 µl of homogenizing buffer
  - 48 µl of "reaction substrate plus 3.0 mM malaoxon"

• Use a micropipettor to add 200µL of DTNB into each of the tubes. **Do this step last for all tubes.** Keep the tubes on ice until you are ready for the incubation.

• Incubate the blanks and the reaction tubes at 30C for 5 minutes in the incubator or the water bath as instructed. Every minute, gently shake the tubes a few times.

• Take 5 spectrophotometer cuvettes (semimicro, 1.5 mL). Label them as 0, 0.5, 1, 2, and 3 for your group. Make sure to label them on the sides that do not interfere with light absorption; your lab instructor will show you how to do this.

• Label another 5 spectrophotometer cuvettes (semimicro, 1.5 mL) for the 5 blanks that you and your neighboring group(s) are sharing.

• Use a micropipettor to transfer the solution from tube 0 to the clean spectrophotometer cuvette labeled 0. Do this very gently, and touch the tip of the pipette to the side of the cuvette to avoid forming any bubbles. Discard the tip.

• Repeat for the other reaction tubes (0.5, 1, 2, and 3) for your group and the five shared blanks.

• For each cuvette measure the absorbance at 405 nm using the spectrophotometer provided. Follow the instructions below:

- o Turn on the spectrophotometer. Allow it to warm up for a few minutes.
- o The lab instructor will show you how to program the spectrophotometer.
- o **For each concentration** of malaoxon, place the Blank cuvette and your cuvette and your neighboring group's cuvette in the correct slots in the spectrophotometer. For example, do this for the blank with 0 malaoxon (B-0) along with cuvette 0 from your group and cuvette 0 from the neighboring group.
- o Record the absorbance readings at 405 nm in Table 4 below.
- o Repeat for the other concentrations of malaoxon and record the data.

- Your instructor will tell you how to discard your tubes and chemicals.
- The difference between the absorbance readings for solutions with no malaoxon and solutions containing different concentrations of malaoxon shows you the effect of the different concentrations of the insecticide, if any, on the enzyme extracted from the animal.
- Calculate the relative inhibition (%) due to X mM malaoxon as follows:

100 (Absorbance for tube 0 – Absorbance for tube X) / Absorbance for tube 0

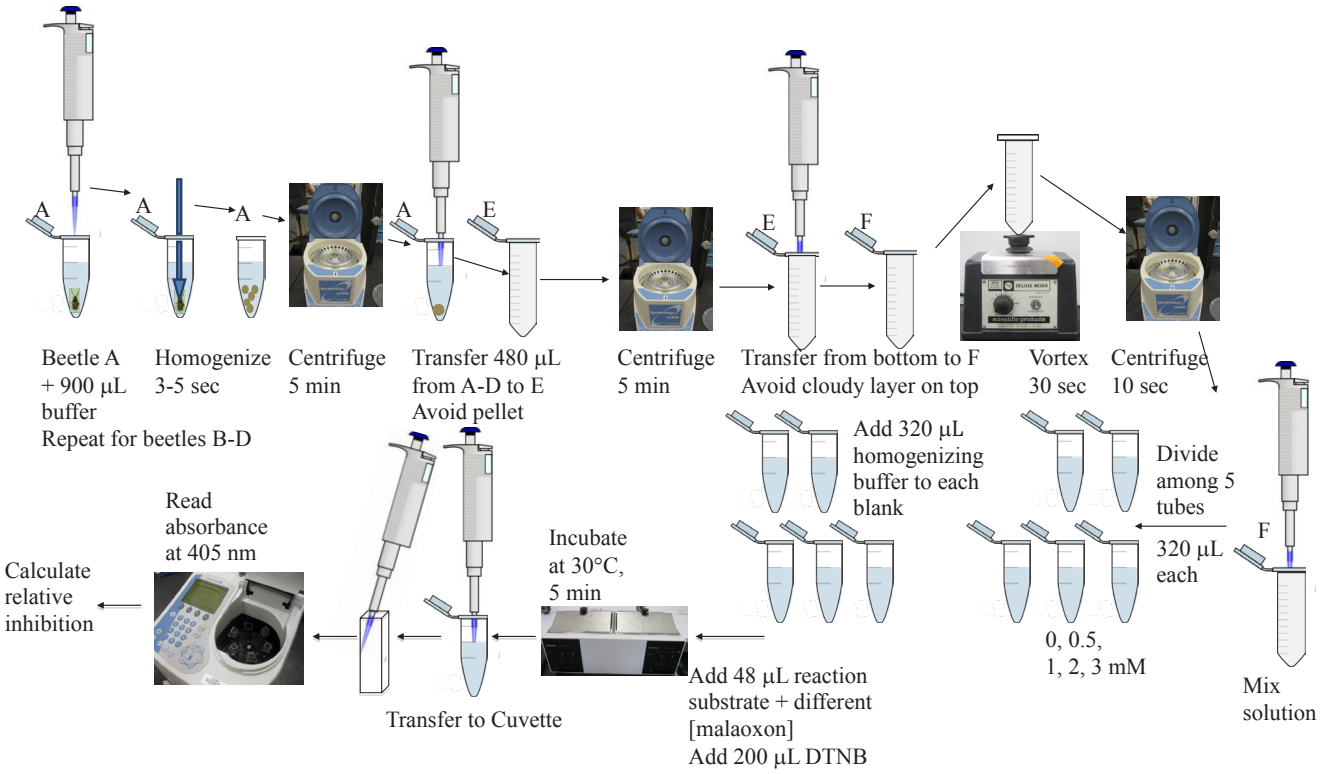

Figure 3. The procedure to determine whether the insecticide acts as a competitive or a non-competitive inhibitor.

Table 3. Chemical components of the tubes for the AChE enzyme assay using different concentrations of the inhibitor malaoxon. Add in the order from left to right. Add the DTNB to all tubes at the end.

Tube	320 µl	48 µl	200 µl
0	Supernatant from tube F	Reaction Substrate	DTNB
0.5	Supernatant from tube F	Reaction Substrate plus 0.5 mM malaoxon	DTNB
1	Supernatant from tube F	Reaction Substrate plus 1.0 mM malaoxon	DTNB
2	Supernatant from tube F	Reaction Substrate plus 2.0 mM malaoxon	DTNB
3	Supernatant from tube F	Reaction Substrate plus 3.0 mM malaoxon	DTNB
B-0	Homogenizing Buffer	Reaction Substrate	DTNB
B-0.5	Homogenizing Buffer	Reaction Substrate plus 0.5 mM malaoxon	DTNB
B-1	Homogenizing Buffer	Reaction Substrate plus 1.0 mM malaoxon	DTNB
B-2	Homogenizing Buffer	Reaction Substrate plus 2.0 mM malaoxon	DTNB
B-3	Homogenizing Buffer	Reaction Substrate plus 3.0 mM malaoxon	DTNB

• Share your data with the class. Use the class data to determine the effect of the different concentrations of malaoxon on the activity of the enzyme. How will you analyze and present your data? Based on the class data, what are your conclusions?

Table 4. Measured absorbance values and relative inhibition of the AChE enzyme activity using different concentrations of the organophosphate insecticide malaoxon as an inhibitor.

Concentration of malaoxon (mM)	Absorbance (AU)	Relative inhibition (%)
0		
0.5		
1.0		
2.0		
3.0		

Third Investigation: The effect of bean type on the sensitivity of AChE to malaoxon

What is the question you are asking?

What are your hypotheses?

Alternative:

Null:

What is the justification for your hypothesis?

What are the variables?

Independent

Dependent

Standardized

What are the levels of treatment? What is the control treatment?

What are the replications and sample sizes?

What is/are the organisms being used for this study?

What is the prediction for the experiment?

Materials

In addition to the materials used in the **first investigation:**

- Bean beetles, *Callosobruchus maculatus*, the LB strain grown on different species of legume (Note the species you are using)

Basic Procedure

Follow the basic procedure you used in the first investigation. Multiple groups are performing the same experiment. Each group will use two beetles from each of the two legume cultures. Keep track of which beetles were grown on which species of legume. Record your group data in a table similar to Table 5 below.

Table 5. Measured absorbance values and relative inhibition of the AChE enzyme activity without or with the organophosphate insecticide (samples D and E, respectively) for beetles grown on different food sources (legume species).

Legume	Beetle #	Gender	Sample	Absorbance (AU)	Relative inhibition (%)
			D		
			E		
			D		
			E		
			D		
			E		
			D		
			E		

Analyzing and Presenting the Data for the Experiment

Use the class data to determine the average effect of the insecticide on the activity of the enzyme AChE for the beetles grown on different legume species. Use the relative inhibition values. Calculate the mean and standard deviation for the class data and record in Table 6.

Table 6. The relative inhibition of the AChE enzyme activity caused by the insecticide for the beetles grown on different legume species. The values are means and standard deviations for the class data.

Legume Species		
Class Mean (%)		
Class Standard deviation (%)		

Make a column graph of the means ± s for the relative inhibition data for the two treatments. Do the means look different?

Perform a simple t-test to determine whether the class hypothesis is supported. Record the results of your t-test in Table 7 below.

Do the results of the t-test support or reject your alternative hypothesis? How confident are you? Explain. Include in your lab report.

Table 7. The results of the t-test for the comparison of relative inhibition of the enzyme caused by the insecticide for the beetles grown on different legume species.

D.F.	
t-critical for 95% confidence level	
|t-calculated|	
Confidence level	

Materials

See Table 8.

Table 8. A list of materials for a class of 24 Students.

Common supplies		**For a class of 24**
15-mL Falcon Tubes (for reagents)		12
Spectrophotometer (depends on the number of groups)		3
Spectrophotometer cuvettes (semimicro, 1.5 mL)		2 packs of 100
KimwipesTM		6 boxes
30°C water bath		1
Waste solution container (100 mL or larger)		1
Bio hazardous waste disposal bag		1
Container for used pestles		1
Microcentrifuges (10,000-14,000 rpm)		3
At each station	**Per group**	**For a class of 24**
P1000 micropipettors	2	12
P200 micropipettors	2	12
Pipet tips Yellow; 1 to 200 µL	1 Pack	6
Pipet tips Blue; 101 to 1000 µL	1 Pack	6
Waste container for used tips	1	6
Disposable pellet pestles	12	72
Fine-point permanent markers, different colors	2	12
Small paint brushes	4	24
Ice bucket	1	6
Dissecting microscope	1	6
100 mm petri dish	1	6
Microcentrifuge tube rack	1	6
Floating Microtube Rack	1	6
Vortexer	1	6
1.5 mL Microcentrifuge tube	75	450
2 mL Microcentrifuge tube	10	60
Adult bean beetles	8	50
Solutions	**Per group**	**For a class of 24**
14 mL Homogenizing Buffer	1	6
600 µL Reaction substrate	1	6
600 µL Reaction substrate plus inhibitor (2 mM)	1	6
10 mL Indicator DTNB	1	6
200 µL Reaction substrate plus inhibitor (0.5 mM)	1	6
200 µL Reaction substrate plus inhibitor (1 mM)	1	6
200 µL Reaction substrate plus inhibitor (3 mM)	1	6

Notes for the Instructor

This laboratory exercise is an extension of the poster we presented during the ABLE conference in 2013 (Fermin et al., 2014). Here we add an additional exercise (third investigation) and provide detailed instructions for the methods in addition to the flow charts (modified from what was presented previously). The lab was designed for an Introductory Biology class. Our class typically has 300+ students with multiple lab sections with 20 students per lab. By the time this set of experiments begins our students have done multiple case studies of parsing experiments, and have performed a 3 week lab sequence in which they have designed experiments, carried out the experiments, analyzed the data with descriptive and inferential statistics (simple student t-tests), and written lab reports with the option to rewrite. The students have reviewed the basics of protein structure and enzymes and cell-cell communication in class.

Introducing the Students to the Lab

We introduce the beetles to the students during the microscopy lab (early in the semester). The students learn about the life cycle of the beetles, observe them under the dissecting scope, and learn to distinguish males and females. The students start their own cultures following the recommendations from the bean beetles handbook (Beck and Blumer, 2011).

Later in the semester, after the students have reviewed basics of enzymes and cell-cell signaling in class, the first two investigations are carried out in lab. Alternatively, one could use the lab as a means of introducing these topics. Depending on the schedule, the third investigation is carried out later in the semester.

Currently, the third investigation is a directed investigation. We focus on the effect of food source on the sensitivity of the enzyme to the insecticide following the published research by Gbaye et al. (2012). We start out with the LB strain that has been growing on mung beans for many generations (more than 20). The third investigation could be designed to involve more independent work by allowing the students to pick their focus: differences among the different strains of beetle available (FM and LB are available from Larry Blumer at Morehouse College, more strains may be available from the folks at Kenyon College), differences between larvae and adults, differences due to incubation temperature (if you have more than one incubator), number of generations grown on different food sources, to mention a few possibilities.

Pre-Laboratory Preparation of Material

You need to have a large population of adult beetles for each investigation. We have multiple cultures started at different times in our incubator so that adults are available for the first and second investigations. Since the students start their own cultures for the third investigation, we schedule the third investigation 5 weeks after the time the students culture their beetles. With our incubator, 5 weeks is the correct time interval to have adults. You should try growing the cultures in your lab in your incubator to get an idea of what the time interval between adult generations will be for you.

The solutions can be made ahead of time and stored at room temperature or in the refrigerator. We typically use the indicator solution and the reaction substrates within a week. The solution volumes used during the procedure are set for a final volume that works with the semimicro, 1.5 mL spectrophotometer cuvettes. If you are using standard cuvettes (4.5 mL), then you need to adjust the volumes and redesign the procedure, or do a final dilution and then correct for it.

Sample Results

We tried this project in 4 lab sections of our introductory biology class (Bio 10100 at City College of New York) during the spring (2 sections) and summer (2 sections) of 2013. We tried it in all 30 lab sections of the large introductory biology class during the 2013-2014 academic year. The biggest challenges were:

- Pipetting correct amounts of solutions using micropipettors.
- Making sure the solutions that are divided between microcentrifuge tubes or spectrophotometer cuvettes were uniformly mixed and there were no solid objects floating in the solutions that would interfere with absorption measurements (problems with disturbing the pellets after centrifugation).

Sample data from the students are presented below. We do not perform regression analysis or ANOVA in this class. Therefore, no statistical test was used for Figure 4 type data. Depending on the level of the course in which this exercise is used, more advanced statistical analysis can be used. We did perform a simple t-test for Figure 5 type data. We introduce descriptive statistics and t-tests early in the semester and by the time of this lab sequence the students have already performed t-tests on two separate lab sequence exercises. Note that the error bars in the graphs represent standard deviation (a topic reviewed in lab) and not standard error (not reviewed in this class).

Table 9. Relative inhibition of activity of AChE (acetylcholinesterase) caused by the insecticide malaoxon. AChE was extracted from bean beetles grown on black-eyed peas. Relative inhibition calculated as:

$$100\times \frac{\text{(Absorbance without malaoxon – Absorbance with malaoxon)}}{\text{Absorbance without malaoxon}}$$

Beetle #	Gender	Relative Inhibition (%)
1	F	49.6
2	M	30.2
3	M	34.2
4	M	17.7
5	M	25.0
6	F	41.2
7	F	12.5
8	M	3.42
Mean		26.7
Standard Deviation		15.3

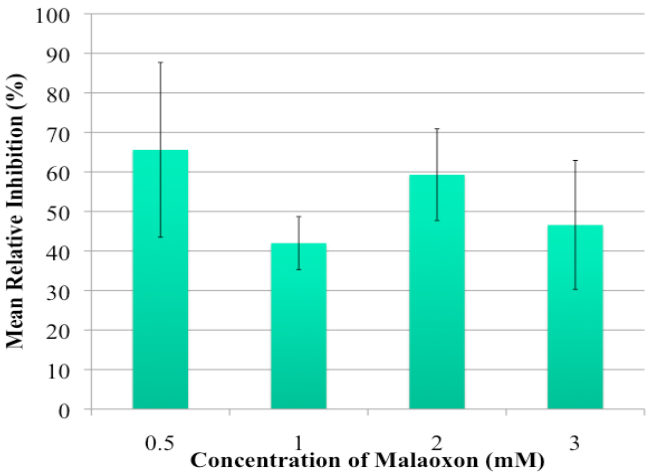

Figure 4. The effect of different concentrations of the insecticide malaoxon on the AChE enzyme activity. Columns represent mean relative inhibition caused by the insecticide, and error bars represent standard deviations. Sample size was 4 (see description of lab exercise for sample preparation).

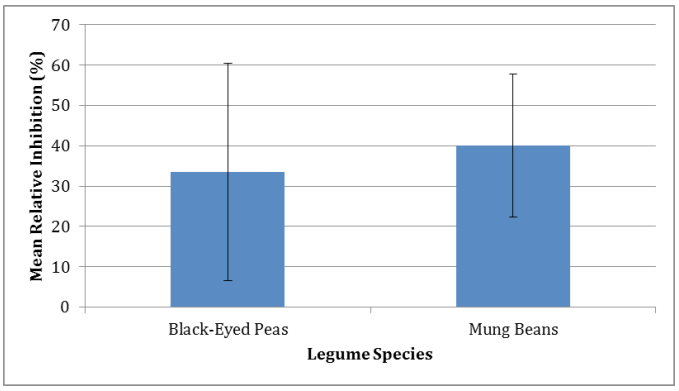

Figure 5. The effect of food source on which beetles were grown on the relative inhibition of AChE caused by Malaoxon. Columns represent mean relative inhibition caused by the insecticide, and error bars represent standard deviations. Sample size was 10 for Black-Eyed Peas and 11 for Mung Beans. There were no significant differences at 95% confidence level.

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Appendix A
Recipe for Making Stock Solutions

Malaoxon (inhibitor)
Stock Malaoxon purchased from Sigma comes as 3.72 M

1M TRIS-HCl (Tris[hydroxymethyl]-aminomethane)
60.57 g in 400 mL deionized water, add drops of HCl until pH is 7.5, then add deionized water to 500 mL. Autoclave and store at room temperature.

Homogenizing Buffer (HB): 50 mM TRIS-HCl, pH 7.5, 1% Triton X100
To make 500 mL:
25 mL 1M TRIS-HCl, pH 7.5
0.5 mL Triton X100
Deionized (or distilled) water to 500 mL
Stirring (20 min)
Store at 4°C

Reaction Substrate: 10 mM Acetylthiocholine iodide (MW 289.1), 50 mM TRIS-HCl, pH 7.5, 1% Triton X100
To make 10 mL:
28.91 mg of ATCI
10 mL HB
Stirring (3 min)
Store at 4°C

Reaction Substrate Plus Inhibitor (2 mM): 10 mM Acetylthiocholine iodide, 50 mM TRIS-HCl, pH 7.5, 1% Triton X100, 2 mM Malaoxon
To make 10 mL:
28.91mg of ATCI
10 ml HB
5 µL Malaoxon
Stirring (3 min)
Store at 4°C

Indicator DTNB: 1.5 mM 5,5-Dithio-bis(2-nitrobenzoic acid): MW 396.35
To make 10 mL:
5.9 mg of DTNB
10 mL HB
Stirring (15 min)
Store 4°C in dark bottle

Reaction Substrate with 3 mM Malaoxon
To make 5 mL:
4.996 mL Reaction substrate
4 µL Malaoxon
Mix and store at 4°C

Reaction Substrate with 0.5 mM Malaoxon
To make 2 mL:
0.333 mL Reaction substrate with 3 mM malaoxon
1.666 mL Reaction substrate
Mix and store at 4°C

Reaction Substrate with 1 mM Malaoxon
To make 2 mL:
0.666 mL Reaction substrate with 3 mM malaoxon
1.333 mL Reaction substrate
Mix and store at 4°C

Appendix B
Supplies Shopping List

Sigma-Aldrich

5,5-Dithio-bis(2-nitrobenzoic acid)	D218200-1G
Malaoxon (liquid, 95% purity, 3.72 M)	36142-100MG
Acetylthiocholine iodide	A5751-1G
Triton™ X-100	X100-100ML

General Supplies

FisherbrandTM Disposable Pellet Pestles 12-141-364
Case of 100
Can be reused if washed thoroughly and subjected to UV in a sterilizer for 10 min.

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