Tested Studies for Laboratory Teaching
Proceedings of the Association for Biology Laboratory Education
Vol. 35, 436-444, 2014

Pests Be Gone! Or Not?! Looking at the Effect of An Organophosphate Insecticide on Acetylcholinesterase Activity in the Bean Beetle

Hector Fermin, Gurcharan Singh, Rahat Gul, Min Gyu Noh, Violeta Contreras Ramirez, and Fardad Firooznia

City College of New York, Department of Biology, MR-526, 160 Convent Ave., New York NY 10031 USA
(**hector@sci.ccny.cuny.edu; gsingh20@citymail.cuny.edu; rgul01@citymail.cuny.edu; mnoh00@citymail.cuny.edu; vcontre01@citymail.cuny.edu; ffirooznia@ccny.cuny.edu**)

Here we describe 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*. Malaoxon inhibits the activity of AChE and interferes with neuronal activity. 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. 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.

Keywords: enzyme assay, bean beetle, acetylcholinesterase

Link to Original Poster

http://www.ableweb.org/volumes/vol-35/poster?art=52

Introduction

Here we present a new enzyme lab that uses a simple, colorimetric assay to investigate the effect of malaoxon, an insecticide, on acetylcholinesterase (AChE) extracted from bean beetles (*Callosobruchus maculatus*).Furthermore, we use this assay system to determine the mode of inhibition of malaoxon (a non-competitive inhibitor). Bean beetles are pests of legume seeds and organophosphate insecticides have been used to control them. The beetles are easy to maintain in the laboratory and make good model organisms to study. This lab exercise ties together several course topics: organismal biology, enzymes, cell-cell signaling, and applied biology and its ecological and unintended consequences. In two 3-hour long lab periods, students can learn about the biology of the beetles, learn the basic enzyme assay, and investigate the inhibitory effect of malaoxon and its mode of inhibition. The data collected by the class can be analyzed statistically and presented in the format of a lab report. 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
- 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

Student Outline

Objectives

After this lab exercise 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. Collect, analyze, and present the data from such an experiment.
5. Discuss the limitations of such an experiment and suggest future steps based on the results obtained.

Introduction

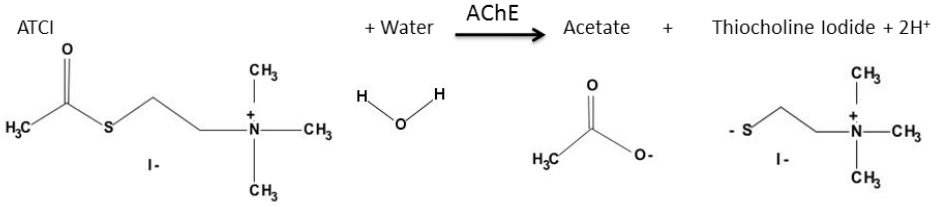
Bean beetles, in the genus *Callosobruchus*,are agricultural pests found in tropical and subtropical regions of Africa and Asia. They are pests of legume seeds (family Fabaceae) such as mung beans (*Vigna radiata*) and black-eyed peas (also called cowpeas, *Vigna unguiculata*). The adults do not require 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.

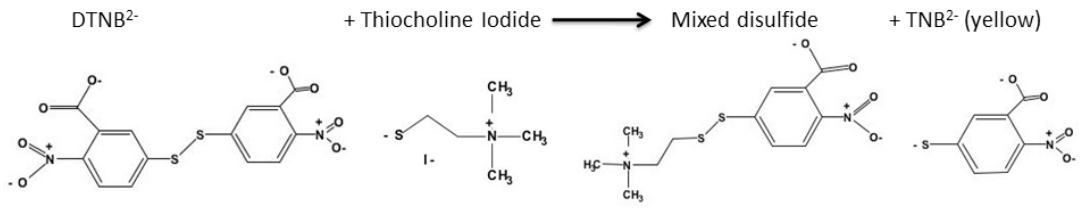
In this lab exercise you will use the species *Callosobruchus maculatus* and a colorimetric enzyme assay to study the inhibitory effect of the organophosphate insecticide malaoxon on the activity of AChE extracted from these insects. You will determine whether the insecticide acts as a competitive or a non-competitive inhibitor.

The Enzyme Assay

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 TNB^{2-} dianion in water at mildly alkaline pH. This dianion is yellow and its presence can be detected using a spectrophotometer.



The more yellow TNB^{2-} 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.

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

Write the alternative and null hypotheses and the experimental prediction.

Materials

- Bean beetles, *Callosobruchus maculatus*
- Small paint brushes
- Dissecting microscopes
- Homogenizing buffer: 50 mM Tris (pH 7.5), 1% Triton X-100
- Reaction substrate: 50 mM Tris (pH 7.5), 10 mM acetylthiocholine iodide (ATCI), 1% Triton X-100
- Reaction substrate plus inhibitor: 50 mM Tris (pH 7.5), 10 mM acetylthiocholine iodide (ATCI), 1% Triton X-100, 2 mM malaoxon
- Indicator DTNB: 50 mM Tris (pH 7.5), 1.5 mM 5-5'-dithio-bis-2-nitrobenzoic acid, 1% Triton X-100
- Spectrophotometer
- Spectrophotometer vis (visible spectrum) cuvettes
- Centrifuge
- Water baths set at 30°C
- 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 Figure 1.

1. Wear gloves and goggles. There should be no exposed skin: covered legs and arms.
2. Use 1 beetle per person: 4 beetles per group, if enough beetles are available. Make sure to determine the gender.
3. Follow the procedure outlined in Fig. 1. In between steps keep the tubes on ice.
4. For the entire group (not individually) make the blank tubes B-M and B+M according to Table 1. Tube B-M is the blank for solution 1D (and 2D, etc.), while tube B+M is the blank for solution 1E (and 2E, etc.).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

5. Measure the absorbances at 405 nm (with appropriate blanks). Record the data in Table 2 below.
6. Absorbance is a ratio, and thus unitless. Typically it is written with AU = absorbance units.
7. The difference between the absorbance readings for solutions D and E (measured with appropriate blanks) for any given beetle shows you the effect of the insecticide, if any, on the enzyme extracted from the animals.
8. 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 x (Absorbance for tube D – Absorbance for tube E) / Absorbance for tube D

9. 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.
10. Your instructor will tell you how to discard your tubes and chemicals.

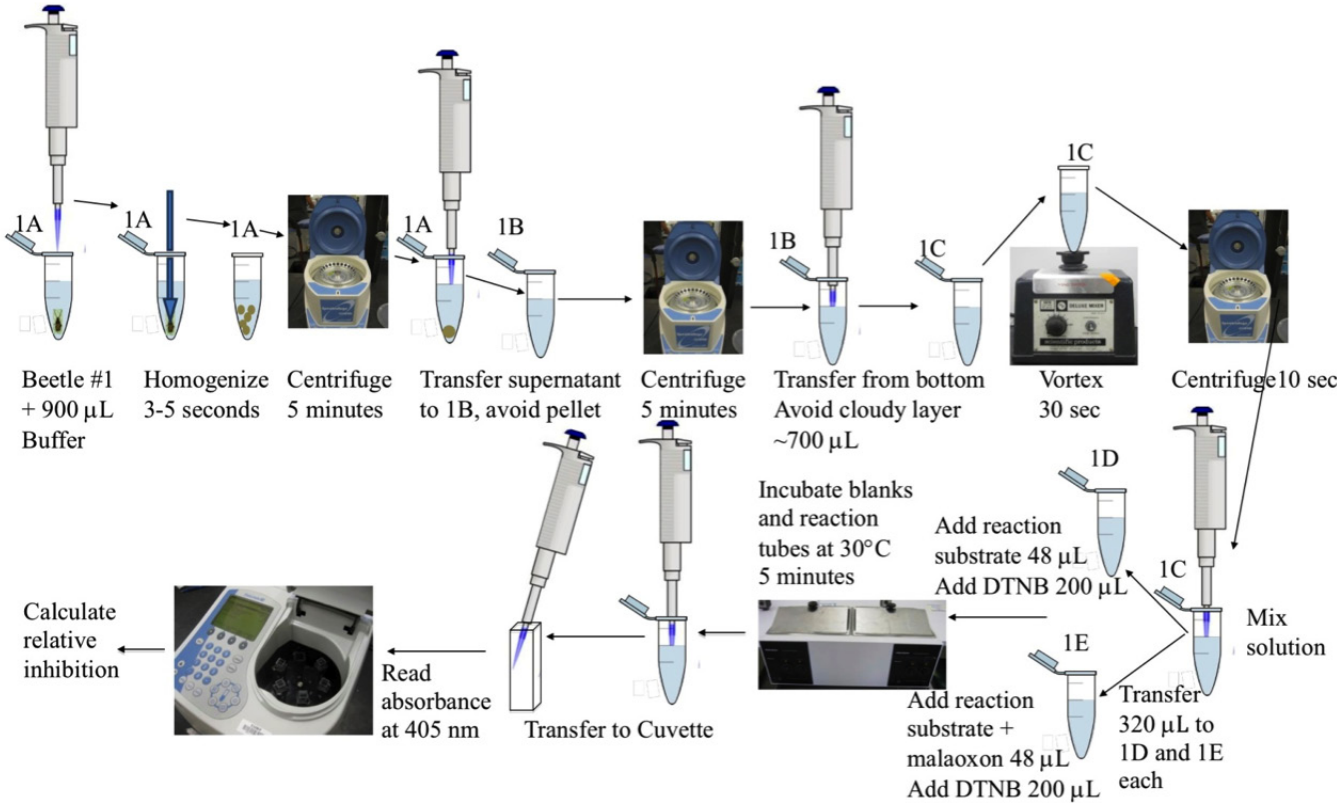


Figure 1. The basic procedure for the enzyme bioassay.

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

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

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:

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.

Procedure

1. The procedure is outlined in Fig. 2.
2. 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. The blanks are listed in Table 3. For example, for 0.5 mM malaoxon, you will be using the B-0.5 blank.
3. Record the absorbance readings at 405 nm in Table 4.
4. Your instructor will tell you how to discard your tubes and chemicals.
5. 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 animals.
6. Calculate the relative inhibition (%) due to X mM malaoxon as follows:

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

7. 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 3. Chemical components of 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

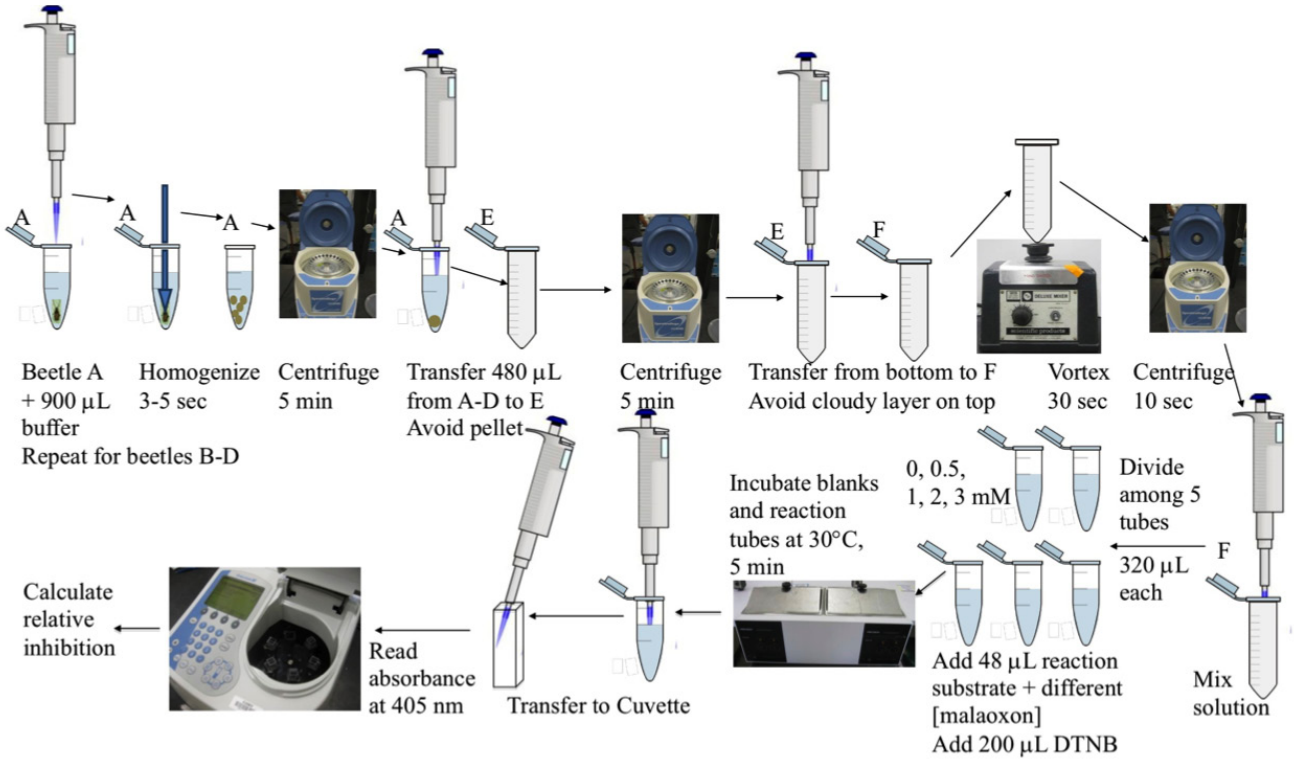


Figure 2. The procedure to determine whether the insecticide acts as a competitive or a non-competitive inhibitor.

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		

References

Beck, C. W., and L. S. Blumer. 2011. A handbook on bean beetles, *Callosobruchus maculatus*. **www.beanbeetles.org**.

Carson, R. 1962. Silent Spring. Mariner Books, Boston, Massachusetts, 378 pages.

Ellman, G. L., K. D. Courtney, V. Andres, and R. M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, 7: 88-95.

Ffrench-Constant, R. H., and B. C. Bonning. 1989. Rapid microtitre plate test distinguishes insecticide resistant acetylcholinesterase genotypes in the mosquitoes *Anopheles albimanus*, *An. nigerrimus* and *Culex pipiens*. *Medical and Veterinary Entomology*, 3: 9-16.

Gbaye, O. A., G. J. Holloway, and A. Callaghan. 2012. Variation in the sensitivity of *Callosobruchus* (Coleoptera: Bruchidae) acetylcholinesterase to the organophosphate insecticide malaoxon: effect of species, geographical strain and food type. *Pest Management Science*, 68: 1265-1271.

London, L., A. J. Flisher, C. Wesseling, D. Mergler, and H. Kromhout.2005. Suicide and Exposure to Organophosphate Insecticides: Cause or Effect? *American Journal of Industrial Medicine*, 47:308–321.

Sadeghi Hashjin, G., F. Sadeghi Dizaj, H. Attaran, and M. K. Koohi. 2013. Malathion induces anxiety in the male adult mouse. *Archives of Medical Science*, 9(2): 368-371.

Spencer, A. G., N. R. Price, and A. Callaghan. 1998. Malathion-specific resistance in a strain of the rust red grain beetle *Cryptolestes ferrugineus* (Coleoptera: Cucujidae). *Bulletin of Entomological Research*, 88: 199-206.

Notes for the Instructor

This laboratory exercise was prepared 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 experiment, analyzed the data with descriptive and inferential statistics (simple t-tests), and written a lab report with a rewrite.

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 when we need them. 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.

The solutions can be made ahead of time and stored at room temperature or in the refrigerator (see below). We typically use the indicator solution and the reaction substrates within a week.

Reagents per group

1. First Investigations: Basic Procedure
    - 3 ml Homogenizing Buffer
    - 200 µL Reaction substrate
    - 200 µL Reaction substrate plus inhibitor (2 mM malaoxon)
    - 1.5 ml Indicator DTNB

2. Second Investigation
    - 5 ml Homogenizing Buffer
    - 200 µl Reaction substrate
    - 200 µl Reaction substrate plus inhibitor(0.5,1,2, and 3 mM Malaoxon)
    - 3 ml Indicator DTNB

Reagents

- Malaoxon. Stock Malaoxon purchased from Sigma-Aldrich comes at 3.729 M (purity 95.2%, density 1.231 g/ml).
- 1 M TRIS-HCl (Tris[hydroxymethyl]-aminomethane). To make 500 ml: Dissolve 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 X-100. To make 500 ml, add 0.5 ml Triton X-100 to 25 ml 1M TRIS-HCl, pH 7.5, add deionized (or distilled) water to 500 ml. Stir for 20 minutes. Store at 4°C.
- Reaction substrate: 10 mM Acetylthiocholine iodide (MW 289.1), 50 mM TRIS-HCl, pH 7.5, 1% Triton X-100. To make 10 ml: Dissolve 28.91 mg of ATCI in 10 ml HB, stir for a few minutes. Store at 4°C.
- Reaction substrate plus inhibitor: 10 mM Acetylthiocholine iodide, 50 mM TRIS-HCl, pH 7.5, 1% Triton X-100, 2 mM malaoxon. To make 10 ml: Dissolve 28.91 mg of ATCI in 10 ml HB, add 5.3 µL Malaoxon and stir for a few minutes. Store at 4°C.
- Indicator DTNB: 1.5 mM DTNB (5-5'-dithio-bis-2-nitrobenzoic acid): MW 396.35. To make 10 ml: Dissolve 5.9 mg of DTNB in 10 ml HB and stir for 15 minutes. Store at 4°C in a dark bottle.

Acknowledgments

We thank the Department of Biology and Division of Science at CCNY, Mariano Soley (lab instructor) and the students who participated in the trial run of this lab exercise, and Chris Beck and Larry Blumer for beetles, instructions, and other help. Funding was provided by NSF DUE-0815135, and DUE-0814373 to Morehouse College and Emory University. **Disclaimer**: Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

About the Authors

Gurcharan Singh, Rahat Gul, Min Gyu Noh, and Violeta Contreras Ramirez are undergraduate students at The City College of New York. Gurcharan and Rahat are majoring in Biology; Min Gyu is majoring in Biochemistry; and Violeta is pursuing a dual major in Biology and Psychology with a combined BS/MS in Biology.

Mission, Review Process & Disclaimer

The Association for Biology Laboratory Education (ABLE) was founded in 1979 to promote information exchange among university and college educators actively concerned with teaching biology in a laboratory setting. The focus of ABLE is to improve the undergraduate biology laboratory experience by promoting the development and dissemination of interesting, innovative, and reliable laboratory exercises. For more information about ABLE, please visit **http://www.ableweb.org/.**

Papers published in *Tested Studies for Laboratory Teaching: Peer-Reviewed Proceedings of the Conference of the Association for Biology Laboratory Education* are evaluated and selected by a committee prior to presentation at the conference, peer-reviewed by participants at the conference, and edited by members of the ABLE Editorial Board.

Citing This Article

Fermin, H., G., Singh, R. Gul, M.G. Noh, V.C. Ramirez, and F. Firooznia. 2014. Pests Be Gone! Or Not?! Looking at the Effect of An Organophosphate Insecticide on Acetylcholinesterase Activity in the Bean Beetle. Pages 436-444 in *Tested Studies for Laboratory Teaching,* Volume 35 (K. McMahon, Editor). Proceedings of the 35th Conference of the Association for Biology Laboratory Education (ABLE), 477 pages. http://www.ableweb.org/volumes/vol-35/?art=52