Chapter 6

The Electroretinogram of the Horseshoe Crab, *Limulus polyphemus:* A Laboratory Exercise in Sensory Physiology

Robert A. Linsenmeier [1]***, Charles M. Yancey*** [2]***, and Wesley W. Ebert*** [3]

[1] Department of Neurobiology and Physiology, and Department of Biomedical Engineering,
[2] Interdepartmental Graduate Program in Neuroscience, and
[3] Section of Biological Sciences
Northwestern University
O.T. Hogan Hall
2153 Sheridan Road
Evanston, IL 60208

Robert Linsenmeier is an Assistant Professor in the departments of Neurobiology and Physiology, and Biomedical Engineering at Northwestern University. He received his B.S. in Chemical Engineering from Carnegie-Mellon and his M.S. and Ph.D. (1978) from Northwestern. After spending four years as an Assistant Research Physiologist at the University of California-San Francisco, he returned to Northwestern, where he teaches an animal physiology course and a biomedical engineering laboratory course. His research interests are in the neural circuitry of the retina and the microenvironmentof the retina.

Charles M. Yancey received his B.A. and M.S. degrees from Northwestern University in 1983 in biology with an emphasis in sensory physiology. He is currently a fellow in Northwestern's combined M.D./Ph. D. program and has recently completed his Ph.D. dissertation. He has been a laboratory teaching assistant in animal physiology and medical neuroscience. His research interests are focused on the role of altered retinal oxygenation and metabolism in ocular disease.

Wesley W. Ebert received his B. S. degree from the University of California-Davis and his M.S. degree from the University of Wisconsin-Madison. He is currently a Lecturer at Northwestern University in the Section of Biological Sciences, with responsibility for coordination of the Ecology and Evolutionary Biology Department's teaching labs. His earlier responsibilities included operation of the teaching labs for the Neurobiology and Physiology Department.

THE ELECTRORETINOGRAM OF THE HORSESHOE CRAB, *Limulus polyphemus:* A LABORATORY EXERCISE IN SENSORY PHYSIOLOGY

INTRODUCTION

Over the past several years, we have developed two versions of a laboratory exercise in which we record the electroretinogram (ERG) from the large, lateral, compound eye of *Limulus polyphemus,* the horseshoe crab. The simpler version is for undergraduates (mostly juniors) taking their first course in Neurobiology and Physiology. It is designed to be completed in one four-hour laboratory period. Students in this course are also expected to read relevant sections of the textbook, Animal Physiology (Second Edition) by R. Eckert and D. Randall (W. H. Freeman and Company; San Francisco, CA 1983). The advanced version is for juniors, seniors, and graduate students in Biomedical Engineering, and takes two four-hour laboratory periods. The simpler version, as given to the students, is presented here as an Appendix. Ideas from the advanced version that may be of interest to instructors and preparators are included in the following sections.

The objectives of both exercises are 1) to give the students some technical experience in working with an animal preparation and in making electrophysiological recordings; 2) to enable the students to leave the laboratory with a set of data which they can analyze; and, hopefully, 3) to teach some principles of sensory physiology. While the system under study is vision, many of the concepts apply to other sensory systems as well.

One of the great advantages of this experiment is the variety of investigations that can be done after a relatively simple preparation. All students are expected to study the dependence of response amplitude on the intensity and duration of the stimulus, the variation of response latency with stimulus intensity, and the time course of dark adaptation following a prolonged period of illumination. Many students also investigate the shift in visual sensitivity that occurs during steady illumination and the effect on response amplitude of trading off stimulus duration for intensity. The data lend themselves to quantitative analysis, particularly linear regression and determination of time constants, but these are not stressed in our simpler version.

We are aware of only two undergraduate laboratory exercises that deal with visual physiology in an animal preparation. These are an exercise on the frog electroretinogram in Experimental Neurobiology by Oakley and Schafer (1978). and one on the visual system of the horseshoe crab in Twenty-six Afternoons of Biology by Wald et al. (1968). A slightly modified version of the Wald experiment has also been published by Packer (1967) in Experiments in Cell Physiology.

We feel that it is advantageous to expose students to a wide range of animal preparations, particularly when one can use an animal as interesting as the horseshoe crab. Direct conclusions about the mammalian visual system have been drawn from research on horseshoe crabs despite the enormous evolutionary gap, and students should appreciate the similarities. We also suspect that the horseshoe crab preparation is hardier than that of the frog. Furthermore, the

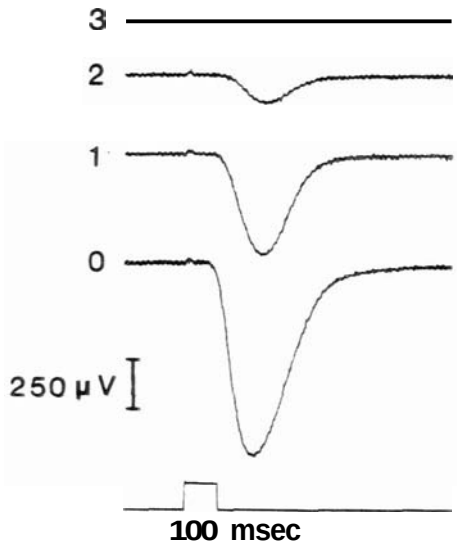

Figure 1. *Limulus* ERG in response to 100 msec flashes. Log attenuation of the stimulus (number of 1.0 log unit neutral density filters in the light path) is shown next to each trace. (For this experiment, 0 log attenuation was approximately 4 $\mu W/cm^2$.) These responses were recorded on FM tape, digitized by a PDP 11/23 computer, and plotted.

Studies of the ERG of the horseshoe crab apparently began with the work of H. K. Hartline (1927). who eventually pursued work on this visual system at the level of single–cell recordings. In 1964, Hartline won the Nobel prize in Physiology and Medicine for his work on visual systems, an important part of which involved *Limulus polyphemus.* Hartline showed that the ERG could be recorded from many kinds of arthropods, but that the ERG of the horseshoe crab was one of the simplest - considerably simpler, in fact, than the insect ERG, which may have several components. Little quantitative work has been done on the ERG of the horseshoe crab, since one can address questions of visual physiology more directly in this animal by recording from the optic nerve or from photoreceptors. Two studies on the ERG are of interest, however, and these will lead to other literature.

Chapman and Lall (1967) measured the spectral sensitivity of the ERG recorded from the lateral eye. They suggested that only a single type of receptor, with a peak sensitivity at 525 nm, as present. Their article includes some response waveforms and intensity–response functions. More recently, Barlow and coworkers have used the ERG to monitor several circadian changes in the structure and function of the lateral eye (e. g., Barlow 1984). They show that the sensitivity of the eye is at least a factor of ten greater at night than during the day, and that the response is graded over four to five log units of illumination. If the optic nerve is cut, the rhythms disappear, indicating that efferent fibers to the eye carry the circadian information.

A great deal of information relevant to this exercise can also be obtained from studies of action potentials recorded from individual axons of the optic

ERG of the horseshoe crab is a very simple wave, not a complicated set of potentials like the frog ERG. Thus, there is no question about what to measure as the response amplitude. In addition, the horseshoe crab ERG is generated primarily by the photoreceptor cells themselves (Barlow, 1984). It is much more difficult to explain the origin of the various components of the vertebrate ERG, some of which are probably generated by glial cells and retinal pigment epithelial cells (e.g., Armington, 1974).

Wald's exercise on the horseshoe crab gives a good starting point, but most faculty would find it difficult to implement since the write-up is really only an outline. Our intent is to give a more complete description than Wald *et al.* provide so that one can teach the laboratory without being a specialist in visual physiology.

BACKGROUND

Limulus polyphemus has impressive visual capabilities. Under dark-adapted conditions, one photon of light can lead to an action potential in an optic nerve fiber, so that its sensitivity is at least capable of matching that of humans (Kaplan and Barlow, 1976). The horseshoe crab has ultraviolet receptors in the median ocelli (Chapman and Lall, 1967), and it has at least six different photosensitive systems: 1) the large, lateral, compound eyes, 2) the pair of median ocelli at the front of the carapace, 3) ventral photoreceptors near the front legs which are not organized into an eye, 4) photoreceptors in the telson (tail) that also act as mechanoreceptors and can shift the phase of the circadian clock (Hanna, Horne, Renninger, Kaplan, and Barlow, 1985). 5) rudimentary lateral eyes that can be seen under the cuticle just behind and medial to the lateral compound eyes as masses of white connective tissue containing photoreceptors, and 6) an endoparietal eye, similar to the rudimentary lateral eyes in structure and just rostral to the median ocelli (Millecchia, Bradbury, and Mauro. 1966). What the horseshoe crab uses all of these eyes for is not entirely clear. They are probably not used for feeding, but vision does seem important in mating (Barlow, Powers, and Kass, 1987).

The ERG

The electroretinogram is a potential that results from currents generated by retinal cells and conducted to the surface of the eye. It bears the same relation to the neurons of the retina that the electrocardiogram has to the electrical activity of cardiac muscle. In humans, the ERG is recorded clinically between an electrode embedded in a contact lens and a reference electrode at another point on the head. In animals, it can be recorded in the same way, but one electrode is often placed inside the eye in order to have recordings that are larger in amplitude and more stable. In the experiment presented here, one electrode is placed in contact with the cornea and a reference electrode is placed in sea water underneath the isolated eye. As shown in Figure 1, the horseshoe crab has a corneal negative ERG. This is because depolarization of the *Limulus* photoreceptors during illumination causes a sodium current to flow inward at the distal (top) portion of the cell. Extracellular current then flows from the proximal to the distal end of the cell, and the extracellular voltage at the distal end of the cell, which is nearest the corneal electrode, is therefore more negative than the voltage at the reference electrode.

nerve of the horseshoe crab. An introductory article on this subject is by Miller, Ratliff, and Hartline (1961). Hartline and McDonald (1947) wrote a very useful article on the time course of dark adaptation following different conditions of light adaptation and on sensitivity adjustments during light adaptation. In the 1970s and 1980s. the horseshoe crab has continued to be an important animal in vision research. Most of the very recent articles are cited by Barlow, Kaplan, Renninger, and Saito (1987).

MATERIALS REQUIRED

Figure 2 illustrates the set–up that we use at Northwestern University for the simpler version of the exercise; it can be readily modified to fit individual needs. The only piece of apparatus missing from this diagram is the Physioscribe, a chart–recorder described below. Figure 3 is a schematic showing the equipment used in the more advanced version of the exercise. The materials required for the simpler version are listed below. The actual preparation is described in the Appendix.

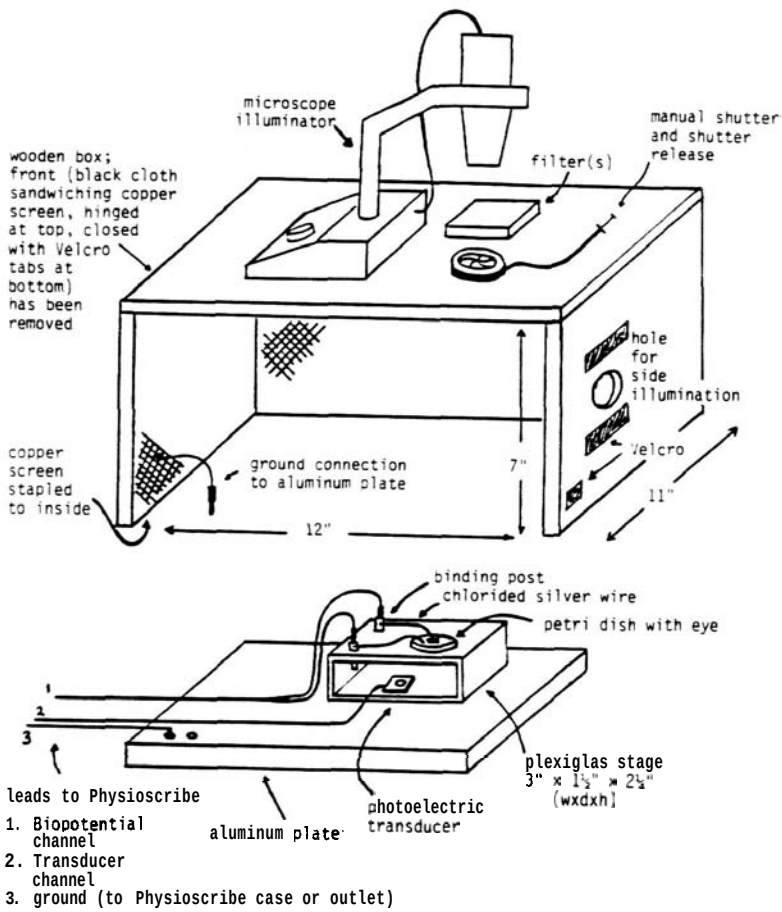

Figure 2. Basic set–up for *Limulus* ERG experiment.

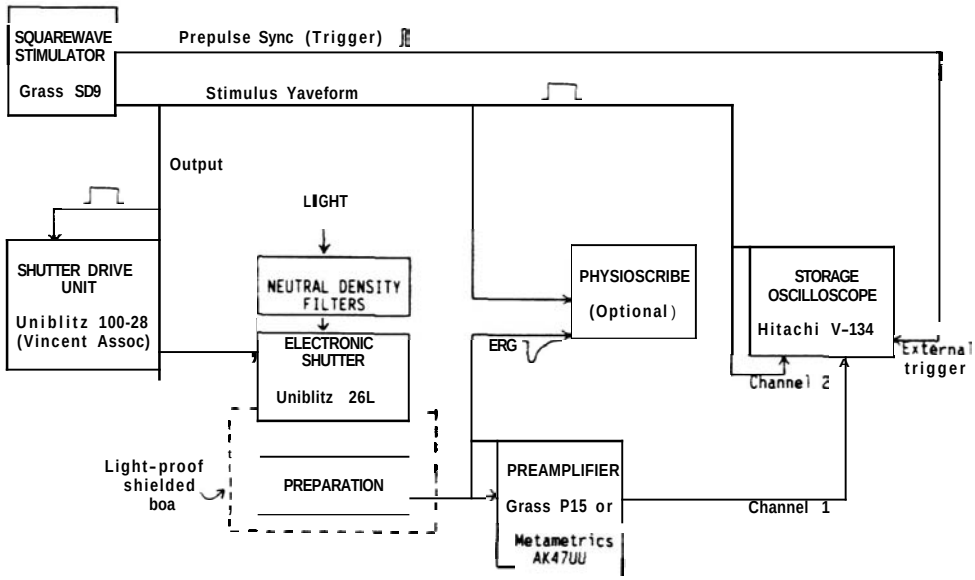

Figure 3. Schematic for advanced version of the ***Limulus*** ERG experiment.

Electrophysiological equipment

1. Physioscribe with Time and Event channel. Biopotential channel, and Transducer channel; three pens with ink bottles connected to the three channels; chart paper for Physioscribe.

2. Wooden, light–proof box with no bottom and a front made by stapling copper screen between two pieces of black cloth. The front is stapled to the top edge of the box so that it opens upward. The interior of the box is painted black and lined with copper screen to which a grounding wire is attached. There should be a hole in the top of the box to hold the shutter and a closeable hole (black cloth attached by Velcro) in one side for the light–adaptation experiments.

3. Aluminum plate (half–inch thick) with several holes drilled to receive banana plugs of grounding wires; fits beneath box.

4. Camera shutter with cable release mounted in hole on top of box.

5. Neutral density filters: three 1.0 log unit filters and one 0.5 log unit filter. These are stacked on top of shutter in various combinations.

6. Two dissecting microscope illuminators; one mounted on top of box with light directed down through shutter into interior; the other directed through the side hole during the light–adaptation experiment. (Light sources other than microscope illuminators could be used as well.)

7. Plexiglas stage with two banana jacks. The eye preparation sits in a

petri dish on top of the stage. Two Ag/AgCl electrodes lead from the banana jacks to the preparation; the jacks are connected to the Biopotential channel of the Physioscribe.

8. Photoelectric transducer. This is placed below the Plexiglas stage and connected to the Transducer channel of the Physioscribe.

9. Grounding lead from aluminum plate to case of Physioscribe or power outlet.

Equipment for dissection and mounting of eye

1. 125 ml Erlenmeyer flask containing approximately 75 ml seawater from aquarium; Pasteur pipette with bulb.

2. 1 ml plastic hypodermic syringe with 25G 5/8" needle.

3. Scalpel, forceps, razor blade.

4. Bottom of small (60 x 15 mm) glass petri dish.

5. Lump of plasticene (modeling clay) – approximately 1 cm diameter.

6. Silicone stopcock grease and small spatula.

7. Binocular dissecting microscope with illuminator.

Animals

1. *Limulus polyphemus* (2"–4" carapace width), secured to a board with T–pins; one animal per pair of students. The second eye may be used later by another pair.

2. Conditioned marine aquarium; equipped for refrigeration, aeration, and filtration. Alternatively, animals could be kept for at least a few days in unfiltered, unaerated aquaria in a cold room at 10-15°C.

PREPARATION OF MATERIALS

1. *Limulus polyphemus*

These animals are so hardy that they are shipped almost dry from some of the suppliers. We routinely order them from Marine Biological Laboratories at Woods Hole. Massachusetts. We keep them in a marine aquarium at 12-15°C where they will be fine for months if the aquarium is in good shape. (If animals are obtained from a gulf coast supplier, they should be kept at room temperature.) Animals kept in an inadequately–prepared aquarium may not respond well. Horseshoe crabs are scavengers, eating soft–bodied invertebrates, benthic algae, and detritus. They can be fed any number of marine invertebrate foods, or things such as chopped fish, but they seem to do fine for a short time with no feeding.

We have identified four suppliers for horseshoe crabs, but have actually ordered from only the first two.

1. Marine Biological Laboratory (617) 548–3705 ext. 325
Department of Marine Resources
Woods Hole, Massachusetts 02543

L95 *Limulus* (small 2"–4"); one–half dozen (minimum order) $12.50. Animals are kept in aquaria at MBL, are shipped as necessary, and arrive in excellent shape. Keep at 12-15ºC.

2. Carolina Biological Supply Co. (800) 334–5551
2700 York Road
Burlington, North Carolina 27215

16–2978 Horseshoe crab $3.35 ea or $16.50/5. Animals are collected and shipped after receipt of order. Exact delivery dates cannot be guaranteed. Sizes vary, but all are usable for the exercise. Keep at room temperature.

3. Ward's Natural Science Establishment (800) 962–2660
5100 West Henrietta
P. O. Box 92912
Rochester, New York 14692–9012

87 W 7530 Horseshoe crab $3.25 ea. Keep at room temperature.

4. Gulf Specimen Company, Inc. (904) 984–5297
P.O. Box 237
Panacea. Florida 32346

Ar–1070 *Limulus polyphemus* small (2.5–8.0 cm) $4.00 ea ($1.00 extra per animal to select for clear lateral eyes for electrophysiological recording). Keep in room temperature aquarium.

Physioscribe and Physioscribe problems

The Physioscribe is a chart mover and recorder equipped with a number of channels and produced by the Stoelting Corporation, Chicago, Illinois. In addition to the Time and Event channel, the Biopotential channel, and the Transducer channel which are needed for this exercise, a Stimulator module and other input channels are available, together with the necessary leads and cables. Two suppliers are:

E. J. McGowan and Associates (312) 530–5556
310 Lake Street Suite 110
Elmhurst, Illinois 60126

Carolina Biological Supply Co. address given above

We have found the Physioscribe to be a rugged and reliable piece of laboratory apparatus. Several phenomena, however, are worth mentioning. The extent to which these would apply to other student–model strip–chart recorders is not known.

First, it is important that all three pens are aligned so that they cross the same vertical line on the strip–chart at the same time. In the

worst case, the pen on the biopotential channel could be longer than that on the transducer channel. The record would then appear to have a response that preceded the stimulus.

Second, we have sometimes observed "cross-talk" between channels, especially when the sensitivity of the biopotential channel is high. The result is that timing pulses recorded on the time and event channel or light signals recorded on the transducer channel may cause spurious deflections on the biopotential channel which could be misinterpreted as ERGs. The latter circumstance can be reduced or eliminated by keeping the sensitivity of the transducer channel at the lowest level at which the dimmest light flash (*i.e.*, that attenuated by 3.5 log units) will just barely register.

Third, one records response amplitudes that span a large range in some of these experiments. If the sensitivity is high enough to see the smallest response, the pen may be driven to saturation for large responses. Once the pen deflection reaches about 30 mm, it is probably a good idea to decrease the sensitivity. Unfortunately the Physioscribes are not calibrated, so it is difficult to know exactly what the sensitivity is. If the same flash is delivered before and after adjusting the sensitivity, the ratio of the two recorded response amplitudes can be taken to be the ratio of the sensitivities.

Finally, because the recording modules do not appear to be adequately grounded to the case of the Physioscribe, we find it necessary to run an additional wire from a convenient location on one of the modules (we use the nut on the input jack of the stimulator module) to the ground plug of the wall outlet. Failure to do this results in 60 Hz interference on the biopotential channel.

If one were to use a different recorder, it would be necessary to make sure that the low frequency cutoff of the channel used for the ERG is rather low, on the order of 0.1 Hz, so that it reproduces the ERG relatively faithfully.

3. Photoelectric transducers

We obtain these from:

Carolina Biological Supply Co. address given above

69-7567; $127.50 each.

4. Shutters and cable releases

American Science Center, Inc. (312) 763-0314
5700 Northwest Highway
Chicago, Illinois 60646

AGC Prontor II shutter; $2.00 each. These are cheap, second-quality shutters that we found at ASC. They work well enough if lightly lubricated. Cable releases can be obtained at any camera shop.

5. Filters

Cadillac Plastic and Chemical Co. (312) 342–9200
1924 North Paulina
Chicago, Illinois 60622

Quarter–inch thick smoky gray plastic which attenuates light by 0.5 and 1.0 log unit is commercially available. We purchased scraps and cut them to 6 cm squares.

6. Other items

The light–proof box, the aluminum plate, and the Plexiglas stage were of our own design, and constructed in the shops at Northwestern University.

7. Ag/AgCl electrodes

For electrodes, we use pure, uncoated 0.01" or 0.015" diameter silver wire. Three sources are listed below.

Medwire Corporation (914) 664-5300
121 South Columbus
Mt. Vernon, New York 10553

A–M Systems, Inc. (800) 426-1306
1220 75th Street S.W.
Everett, Washington 98203

World Precision Instruments, Inc. (203) 469-8281
375 Quinnipiac Avenue
New Haven, Connecticut 06513

AGW 1010 30' of 0.01" @ $50.00 January 1987

In order to have stable recordings. Ag/AgCl electrodes are desirable. The principle of this electrode is described by Ferris (1974), among others. Essentially, one wants a reversible, non–polarizable electrode that will not develop a potential of its own with respect to the fluid it is in. To prepare Ag/AgCl electrodes, silver wires are made the anode and current is passed. As a power supply. we use a Hewlett–Packard 6202B DC power supply which has a maximum output of 50V and 1A. This is set to provide 4 volts, but this is not critical. Batteries could also be used for power. An ammeter in the circuit is convenient. The microgator clips (smooth alligator clips) that are used to hold the wires in solution rust after some time, and we monitor the current in order to know whether the wires are making contact. The chloriding bath is simply 0.9% NaCl. A silver cathode is not necessary since the reaction at the cathode is not important, but silver prevents contamination of the bath.

Before chloriding, wires are cleaned using soap to remove any grease and then rinsed in distilled water. Four wires are twisted together at the end where they contact the microgator clip. The cluster is suspended in the solution so that the free ends do not touch each other or the cathode. The bath containing the wires is placed in a dark box since the Ag/AgCl is

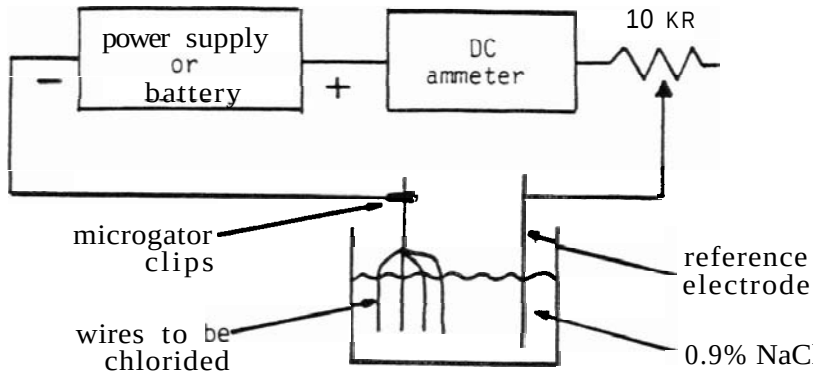

Figure 4. Schematic of apparatus for preparing Ag/AgCl electrodes.

sensitive to light. A current of about 1 mA is applied for 30 minutes to create a uniform dark grey AgCl layer. The set-up is shown in Figure 4. After chloriding, wires are rinsed with distilled water, inspected for unchlorided sections, and stored in the dark. Only the portion of the electrode that touches fluid needs to be chlorided. A pair of electrodes should last through at least two laboratory periods, but if they become scratched or if noise or drift is a problem, they should be replaced.

INTACT ANIMAL PREPARATION

We have used the isolated eye preparation as described in the two versions of this exercise for several years with considerable success. It is, however, feasible to record the ERG from an intact animal. This is the preferred technique for doing research on the horseshoe crab, and it may be preferable for student exercises as well, since it would eliminate the dissection with its attendant technical difficulties. An intact animal preparation would also be more satisfactory for those students who find excision of the eye from the living animal to be aesthetically and morally objectionable.

Our preliminary experience with this preparation is good, although we have tried it on just one occasion using the advanced version of the exercise. We have not yet optimized the conditions for the intact-eye preparation, and therefore, we will not present it here. We will, however, offer the following preliminary observations.

Equipment for intact animal preparation

1. All of the electrophysiological equipment listed previously can be utilized with the intact-eye preparation with the following exceptions. The plexiglas stage must be replaced with some sort of a stage that will hold the entire horseshoe crab - securely pinned to a board - at a suitable angle so that the eye is appropriately oriented toward the light. One of the two silver-chlorided electrodes should be replaced with a wick electrode. Same sort of a clamp will be necessary to hold the wick electrode

and to support the two banana jacks into which the Biopotential channel lead will plug.

2. None of the equipment listed for dissection and mounting will be necessary, save for the razor blade to scrape the surface of the eye. In addition, a sharp pin will be required to pierce the carapace for placement of the reference electrode.

Preparation of materials

1. It is most important that the crab be immobile. In order to accomplish this, the animal is attached to a small wooden board in the same way as for dissection of the lateral eye. A folded paper towel wetted with sea water is placed on the board beneath the animal. A T-pin is inserted through the edge of the carapace on each side, and these are hammered into the board. It is better to put the pins at an angle (*i.e.,* not vertical) so that the animal can not push up with its legs and slide up along the pins. It will also help to push down on the center of the carapace so that the animal is spread out a bit laterally. A third pin is inserted through the back hinged section (abdomen) so that the hinge joint is immobilized. We have considered using heavy-duty rubber bands instead of pins, but have no experience with this yet.

2. Once the eye is scraped (see below), there may be fluid coming out onto the surface, allowing use of a chlorided silver wire electrode as in the isolated eye preparation. It seems preferable, however, to make sure that there is a fluid bridge by using a wick electrode (Barlow, 1984). These can be made by threading a piece of silk or cotton thread through a plastic tip for an automatic pipetter, and attaching a rubber Pasteur pipette bulb to the back end of the tip. A hole is made in the bulb with a pin and a chlorided silver wire is fed through, leaving enough outside the bulb to attach to the binding post on the stage. To fill the electrode, hold the thread against the outside of the pipette tip, squeeze the rubber bulb and draw in artificial sea water. The amount is not critical, provided that the wire is in the fluid and that the wick itself is wet. Bubbles in the pipette tip will not matter. Cut the wick so that about 2 mm sticks out of the pipette tip.

American Scientific Products, 1210 Waukegan Road, McGaw Park, Illinois, 60085-6788, has been our source for both amber latex Pasteur pipette bulbs (P5002-1) and for polypropylene pipette tips (P5059-80R or P5059-80D; 2", 2-200 µl capacity). Note that pipette tips with ribbed tops will not work.

Preliminary procedural tips

1. After the animal is secured on the mounting board, the eye should be scraped with a scalpel to reduce the resistance of the cornea. As in the isolated eye, actually slicing off a thin layer appears to work well. Wald *et al.,* suggest that this is a painless procedure.

2. The board is mounted on a tilted stage so that the eye is oriented up toward the light. The stage must be positioned on the aluminum plate so that the eye will be in the light beam.

3. A small hole is made with a sharp pin somewhere on top of the animal for placement of the Ag/AgCl reference electrode, which just needs to make contact with the interior; it need not be pushed in deeply.

4. A wick electrode is brought in contact with the eye by adjusting the clamps holding it.

5. The photoelectric transducer is placed on top of the carapace so that it can collect adequate light.

6. The physioscribe cables are connected, the box is drawn forward over the preparation, and the experimentation can commence.

7. At the end of the experiment, the animal can be taken off the board and put back in the tank.

SAMPLE DATA

Figures 5–8 show representative ERGs and graphs of data for 1) response amplitude and latency as a function of illumination, 2) response amplitude as a function of duration, and 3) the time course of dark adaptation. These are the key measurements one makes during the course of the laboratory period. The data in Figures 5, 7, and 8 were obtained by students using techniques des-

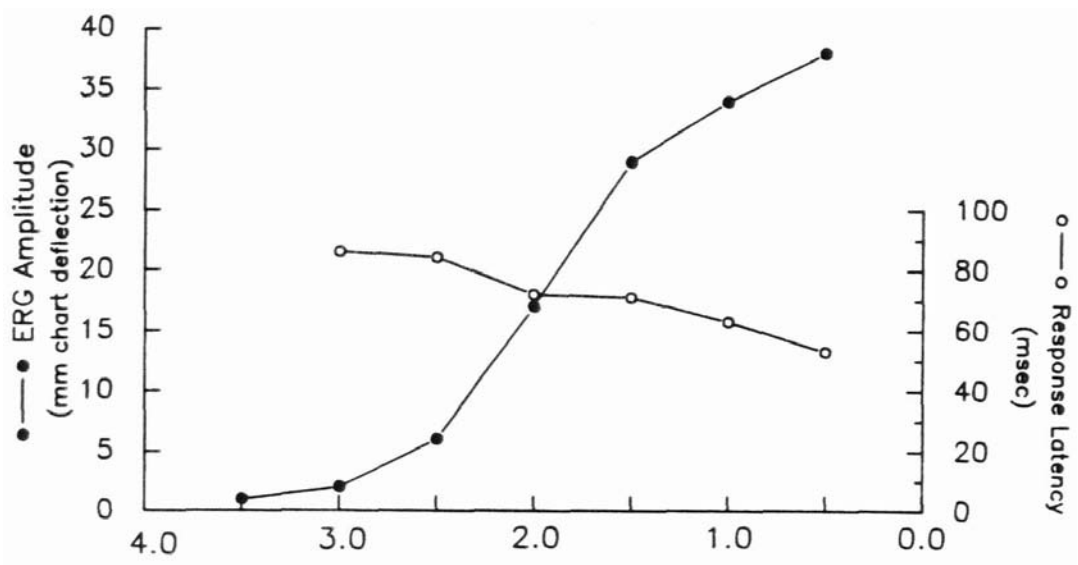

Figure 5. Response amplitude and latency vs. illumination. Measurements were made in one dark–adapted isolated eye preparation. An interval of 45 sec was allowed between flashes, which were each 40 msec in duration.

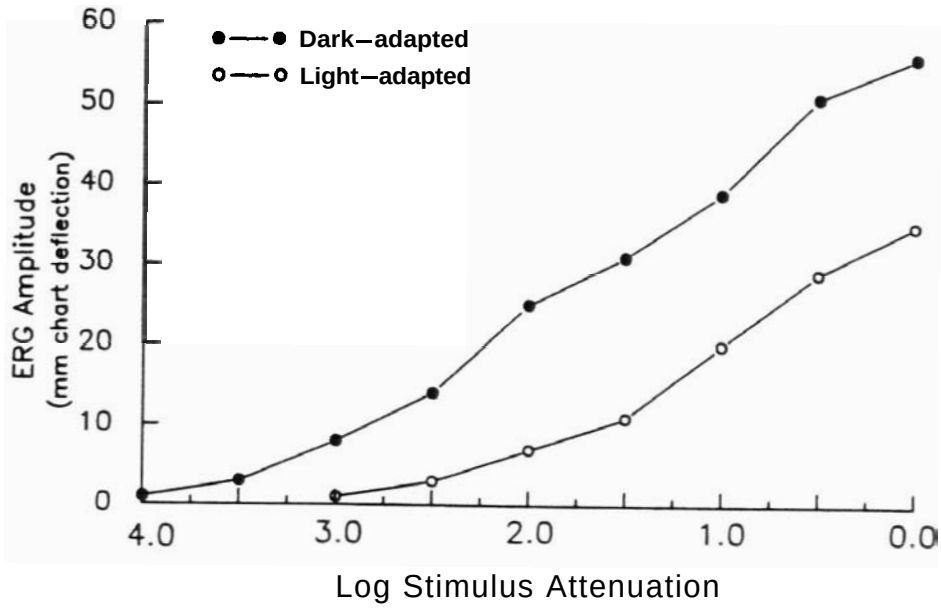

Figure 6. **Response amplitude *vs.* illumination for the intact eye preparation under dark–adapted (filled circles) and light–adapted (open circles) conditions.**

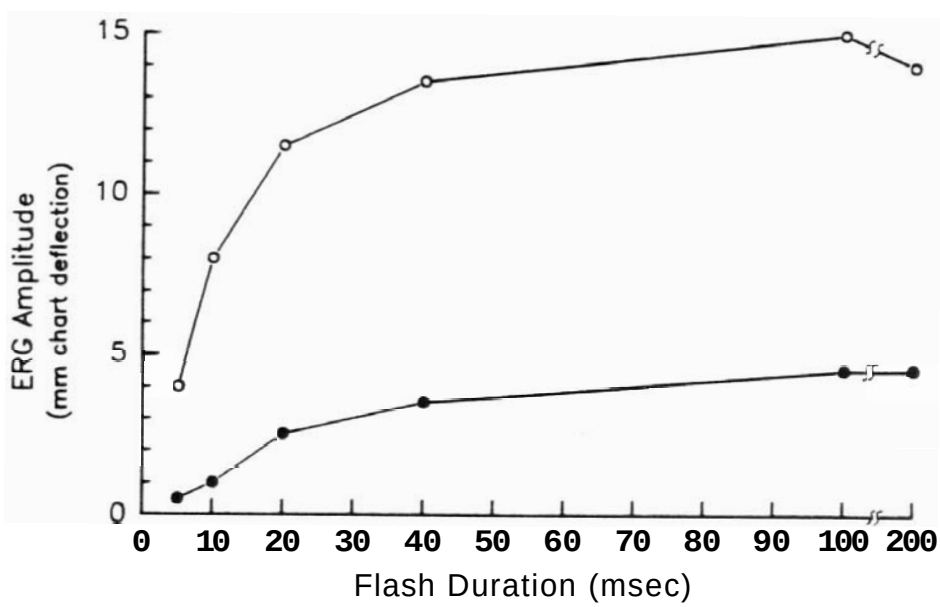

Figure 7. **Response amplitude *vs.* flash duration in two dark–adapted isolated eyes. Stimulus attenuation was 3.0 log units.**

cribed in the Appendix. The data in Figure 6 were obtained by the authors using an intact-eye preparation. Since the Physioscribe sensitivity is uncalibrated, response amplitude is taken to be simply pen deflection in mm. The absolute size of the response cannot easily be compared among lab groups, but there would be reasonably good agreement if one plotted response as a percentage of maximum. The actual maximum amplitude of the ERG is generally between 100 and 500μV, as determined using the apparatus shown in Figure 3.

Some of these data can be fitted to equations if desired. A relatively large range of the intensity-response function is usually well described by the simple relation $R = K_1 \log I + K_2$, where K_1 and K_2 are constants. R is ERG amplitude, and I is illumination or intensity. This shows that a large range of stimulus intensities are encoded into a relatively small range of responses. Sometimes it has been possible to fit intensity-response curves more completely with the sigmoidal function familiar as the Michaelis-Menten equation, $R = R_{max} I/(I + s)$, where R_{max} is the response at saturation, and s is the "half-saturation" corresponding to the value of I where $R = R_{max/2}$.

An interesting difference can be observed between the isolated eye and intact eye stimulus response functions in Figures 5 and 6. The ERG from the iso-

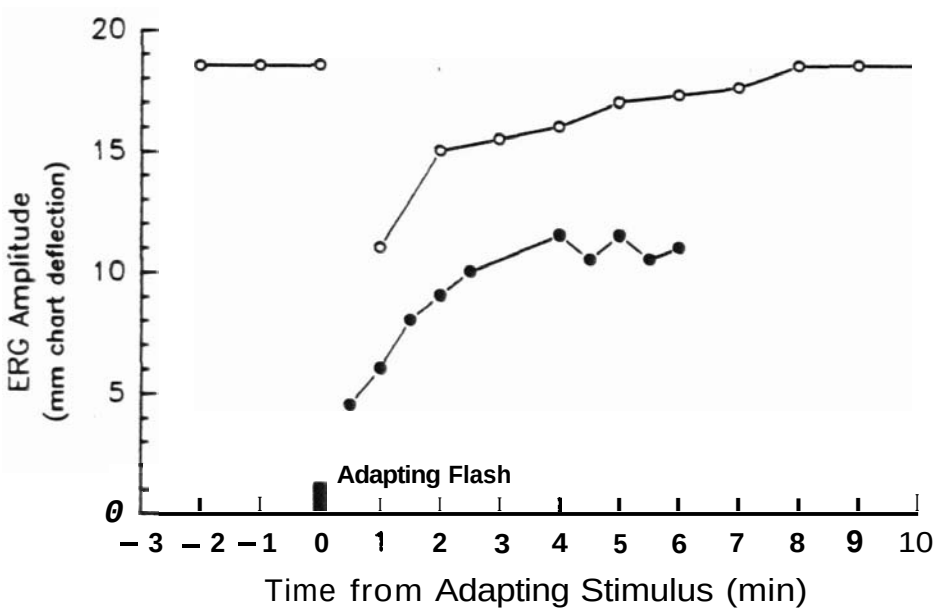

Figure 8. Time course of dark adaptation following a 5 second flash of unattenuated light for two isolated eyes. For one eye (open circles), the control responses are plotted at negative times. The test flashes were at 1.5 log units attenuation. For the other eye (filled circles), the student failed to record the control flashes in the lab report. The test flash illumination in this case was 3.0 log units attenuation.

lated eye tends to saturate at higher stimulus intensities, showing the entire form of the curve. whereas the response from the intact eye does not. This appears to be a consistent difference, and it is surprising since the microscope illuminator is rather bright. It may be possible to saturate the response of the intact eye by using a photoflash unit (D. Mayette, personal communication), but then one would not be able to study the effect of stimulus duration.

The dark adaptation curves can usually be fitted very well by a single exponential of the form $R(t) = R_{final}(1 - e^{-t/\tau})$, or if the response just after extinguishing the adapting light is greater than zero,

$$R(t) = (R_{final} - R_{min})\ (1-e^{-t/\tau}) + R_{min}.$$

where R_{min} is the response at the beginning of dark adaptation, R_{final} is the dark-adapted response, and τ is the time constant. To fit these functions to the data, it is best to transform the equations to a form where some function of R is linear with time. For the first of these, this would be

$$-ln[(R(t) - R_{final})/R_{final}] = t/\tau.$$

REFERENCES

1. Armington, J. C. (1974) The Electroretinogram. Academic Press, New York.
2. Barlow. R. B. (1984) Circadian rhythms in the *Limulus* visual system. J. Neurosci. 3:856-870.
3. Barlow, R. B., Kaplan, E., Renninger, G. H., and Saito, T. (1987) Circadian rhythms in *Limulus* photoreceptors. J. Gen. Physiol. 89:353-378.
4. Barlow, R. B., Powers, M.-K., and Kass, L. (1987) Vision and mating behavior in *Limulus.* In Symposium on Sensory Biology of Aquatic Animals (A. N. Popper, R. Fay, T. Atema, and W. Travolga, Editors), Springer-Verlag, in press.
5. Chapman, R. M. and Lall, A. B. (1967) Electroretinogram characteristics and the spectral mechanisms of the median ocellus and the lateral eye in *Limulus polyphemus.* J. Gen. Physiol. 50:2267-2287.
6. Ferris, C. D. (1974) Introduction to Bioelectrodes. Plenum Press, New York.
7. Hanna, W. J. B., Horne. J. A., Renninger, G. H., Kaplan, E., and Barlow, R. B. (1985) The tail of *Limulus*: an extraocular photoreceptor organ for a circadian clock. Invest. Ophthalmol. Visual Sci. Suppl. 26:113 (abstract).
8. Hartline. H. K. (1927) A auantitative and descriptive study of the electric response to illumination of the arthropod eye. Am. J. Physiol. 83:466-483.
9. Hartline. H. K. and McDonald, P. R. (1947) Light and dark adaptation of single photoreceptor elements in the eye of *Limulus.* J. Cell. Comp. Physiol. 30:225-253.
10. Kaplan, E. and Barlow, R. B. (1976) Energy. quanta, and *Limulus* vision. Vision Res. 16:745-751.
11. Millecchia, R.. Bradbury, J., and Mauro, A. (1966) Simple photoreceptors in *Limulus polyphemus.* Science 154: 1199-1201.

12. Miller, W. H., Ratliff, F., and Hartline, 9. K. (1961) How cells receive stimuli. Sci. Amer. 205(3):223-238 (Reprint #99).

13. Oakley, B. and Schafer, R. (1978) Visual reception: frog electroretinogram. In Experimental Neurobiology, The University of Michigan Press, Ann Arbor, MI pp. 166-173.

14. Packer, L. (1967) Bioelectric Potentials II. Electrical activity of the *Limulus* eye. In Experiments in Cell Physiology, Academic Press, New York, NY pp. 269–276.

15. Wald, G.. Hopkins, J., Albersheim, P., Dowling. J., and Denhardt, D. (1968) Electrical activity of a sense organ: the *Limulus* eye. In Twenty-six Afternoons of Biology (Second Edition), Addison–Wesley Publishing Company, Inc., Reading, MA pp. 112–116.

ACKNOWLEDGEMENTS

We thank Mr. Ed McGowan of E. J. McGowan and Associates, Elmhurst, Illinois, for making a Physioscribe of the latest model available for us to demonstrate. We also thank Dr. J. S. Bjerke for assistance with the manuscript.

APPENDIX

SENSORY PHYSIOLOGY: THE ELECTROETINOGRAM OF THE HORSESHOE CRAB

Introduction

One of the fundamental characteristics of all cells, and consequently, of all living organisms as well, is their ability to monitor external conditions and to detect changes in these parameters. This characteristic – the sensory capability of living organisms – may in general be referred to as "sensitivity". Even the simplest single–celled prokaryotes exhibit sensitivity; bacteria can detect food sources as indicated by their preferential movement up a concentration gradient of nutrient molecules. Photosynthetic organisms from single–celled algae to the most complex plants are exquisitely sensitive to light. It is among the animals, however, that the characteristic of sensitivity is most conspicuously developed.

During the long course of animal evolution, certain cells have become specialized in a variety of ways for carrying out different sensory functions in a highly efficient way. These receptor cells may occur singly, scattered throughout the body, or they may be aggregated into large and complex sense organs which function in remarkable ways. For example, sensory organs often operate reliably over an enormous range of stimulus energies – 10^9–fold in the case of the human auditory system. Others can discriminate minuscule differences in stimulus intensity – 0.002°C in the case of the reptilian pit organ which detects infrared radiation.

Receptor cells are typically specialized for detecting a single specific

form of energy. On this basis, they can be categorized into chemoreceptors, mechanoreceptors, photoreceptors, electroreceptors, and thermoreceptors. The receptor cells responsible for sensing all of these different parameters in the external world are quite different in their structural and functional properties. These differences are in large part due to the very different types of stimulus energy to which they are sensitive. At the same time, there are also important similarities between different types of receptor cells. This is because they all function as transducers, converting energy of various sorts into membrane potentials. These receptor potentials then trigger the generation of trains of action potentials, either directly in the axon of the receptor cell or, by causing the release of a transmitter substance which ultimately leads to activity, in the axon of an afferent nerve. Consequently, the neural outputs of all different sensory systems are essentially identical. Thus, by studying a specific sense organ, one can learn principles that apply broadly to sense organs in general, as well as features specific to the organ under study. In addition, since there has been evolutionary conservation of the principles employed, one may study sensation in a specific preparation - even from an invertebrate - and elucidate principles that are applicable to sensory systems in all animals.

Some of the principles that can be illustrated are 1) gradation of response as a function both of stimulus intensity and of stimulus duration, 2) gradation of response latency (*i.e.*, the time lag between receipt of the stimulus and onset of the response) as a function of stimulus intensity, and 3) adaptation under conditions of continuous stimulation. These properties could be investigated with intracellular recording from individual receptor cells, or with extracellular recording of action potential discharge at some other cell in the system. Often, however, they can more easily be studied by recording massed potentials or field potentials. These represent the sum of extracellular current flow from a group of neurons or other cells.

In this exercise, you will record the electroretinogram (ERG) from the lateral eye of the horseshoe crab, Limulus polyphemus. The ERG is a field potential from the eye in response to light; it is analogous to the electrocardiogram (ECG), which represents the sum of current from depolarization of cardiac muscle fibers. ERGs can be recorded from the eyes of vertebrates and invertebrates by placing a recording electrode in contact with the cornea or external surface of the eye and a reference electrode at some remote point on the animal. The vertebrate ERG is a complex series of potential changes having components that arise from several of the cell types in the retina. Invertebrate ERGs are much simpler, reflecting the simpler structure of the eye. There are only two components to the Limulus ERG; a transient, corneal-negative potential and a smaller, steady component that is maintained for the duration of the stimulus. Because of the limitations of your recording instrument (the Physioscribe), you will see only the transient component. You will study variations in the amplitude of the response as a function of changes in stimulus conditions. Before continuing with the details of your experiment, however, a digression about the biology of Limulus is in order.

Limulus polyphemus is a marine arthropod found in tidal regions along the Atlantic coast. While they are called crabs, they are not crustaceans as are the edible crabs and other "true" crabs. Rather, they are classified in the class, Merostomata, in which they are the only Living species. They are distantly related to the ticks, mites, and spiders - members of the class Arach-

nida. The fossil record documents that as a species, *Limulus polyphemus* has existed for at least 300 million years. All of its close relatives, phylogenetically speaking, are extinct. Like other arthropods, horseshoe crabs must molt in order to grow, and the lenses of the eyes are shed with the rest of the exoskeleton. Over at least a few years, the age of a horseshoe crab is approximately equal to the diameter of its carapace in inches. The maximum size is about thirty inches.

Limulus adults have two large compound lateral eyes and two small simple median eyes. Both have been useful in understanding certain aspects of visual physiology, and the importance of this animal for research in vision cannot be overestimated. This is largely because the recordings of the activity of single optic nerve fibers and of membrane potentials in photoreceptors were feasible in *Limulus* many years before such recordings could be obtained from vertebrates. The experiments which you will do were pioneered in the 1930s and 1940s by Professor H. Keffer Hartline who worked at the University of Pennsylvania, the Marine Biological Laboratory in Woods Hole, Massachusetts, and at Rockefeller University. In 1964, Professor Hartline was awarded the Nobel Prize in Physiology and Medicine for his work on the visual system of *Limulus*.

The compound lateral eyes of the horseshoe crab each consist of approximately 300 individual, identical units known as ommatidia. For an illustration of ommatidial structure, a description of the optical properties of compound eyes, and a discussion of some aspects of the visual physiology of *Limulus*, you are referred to pages 262-265 and 277–279 of Eckert and Randall ANIMAL PHYSIOLOGY (Second edition).

You will begin the experimental portion of this exercise by excision of one of the lateral eyes from a horseshoe crab, leaving it attached to a section of the carapace. Remarkably, this eye will continue to generate responses for hours with no special precautions concerning oxygenation, buffering, provision of substrates, *etc*. You will mount your preparation over sea water in a small dish and place electrodes on the surface of the eye and beneath it in the sea water which contacts the back of the eye. The preparation will be housed in a box that serves two functions: First, it provides electrical shielding, and second, it ensures that room light does not reach the preparation. The only light the eye will receive will be provided by a simple optical system. Responses from the eye will be recorded on the biopotential channel of the Physioscribe. A photoelectric transducer located inside the box will be connected to a transducer channel of the Physioscribe in order to record the timing of the light flashes.

After your preparation has been assembled, you will first need to allow the eye to adjust to darkness in the box so that it will be maximally sensitive to the flashes you deliver. You will be able to tell when the eye has attained its maximal dark–adapted sensitivity by looking at its response to weak test flashes. You will then be able to study stimulus–response relationships in the steady–state, dark–adapted eye. In doing this, and throughout the exercise, it is essential that you realize that a single bright flash or too many dim ones spaced too closely together will light–adapt the eye and change its sensitivity.

Once you have determined some aspects of the physiology of the dark–adapted eye, you will proceed to study the time–course of the dark–adaptation

process, *i.e.*, how the eye's responses change after exposure to a steady light. Finally, you will determine how the eye's responses change during continuous exposure to a low level of light, *i.e.*, during the process of light–adaptation. You will then have the opportunity to devise your own additional experiments to investigate further aspects of the physiology of vision in the *Limulus* eye. Questions which can be answered using your experimental preparation will be posed for you to consider.

Objectives

When you have completed this laboratory exercise, you should be able to:

1. describe in general how to use a simple optical system to experimentally stimulate a visual system preparation
2. describe the waveform of the *Limulus* ERG, and compare it to the vertebrate ERG as illustrated on page *267* of Eckert and Randall ANIMAL PHYSIOLOGY (Second edition)
3. describe the essential structural features of ommatidia and of compound eyes; explain what is meant by dark adaptation, light adaptation, and response latency
4. tell how response amplitude depends on stimulus intensity and stimulus duration in the dark–adapted *Limulus* eye; interpret data which illustrate these relationships
5. tell how response latency depends on stimulus intensity; interpret data which illustrate this relationship; explain why an experiment to show the effect of stimulus duration on response latency was not suggested
6. describe the time–course of dark adaptation; interpret data which illustrate this phenomenon
7. describe the effect of maintained illumination on the stimulus–response relationship

Laboratory Procedures

1. Instrumentation.

   a. Optical system. To stimulate the eye, you will use a simple but effective optical system consisting of three parts – light source, neutral density filters, and a shutter. The shutter is built into the box top; the filters and the light source are positioned above so that they will be accessible. The source provides a large continuous amount of light, the filters attenuate the light to different degrees, and the shutter controls the timing of flashes that reach the eye. This system is preferable to simply switching the light on and off because 1) the duration of the flashes can be more accurately controlled, 2) the intensity changes instantly when the shutter is opened. and 3) the light switch could introduce electrical artifacts. One could change the light intensity by simply changing the voltage to the illuminator, but filters are preferable because intensities can be 1) adjusted more reproducibly, and 2) varied over a wider range.

1. Light source. A Nicholas microscope illuminator will be used as the light source.

2. Shutter. The shutter is operated by a push-button cable release. Because it is a mechanical shutter, one must load the internal spring with the small cocking lever before each use. You will also need to set the duration for which the shutter is open. This is done by rotating the toothed wheel at the outer edge of the shutter. It can be set for durations as short as 1/200 of a second (shown as 200 on the shutter) and as long as 1 second (shown as 1). In addition, there is a setting labelled T. This is the timed position, and one trip of the release will open the shutter and leave it open. The shutter will not close until the cable release is pressed a second time. You will use this setting when lining up your preparation with the light beam, and later, when studying light adaptation.

3. Filters. The filters used in this experiment attenuate all wavelengths approximately equally, and are therefore called neutral density filters. Optical density (OD) is specified in log units of attenuation. A 1 log unit filter (OD = 1.0) transmits only $1/10^1$ or 1/10 or 10% of the incident light. A 2 log unit filter (OD = 2.0) transmits $1/10^2$ or 1/100 or 1% of the incident light, and a 0.3 log unit filter (OD = 0.3) transmits $1/10^{0.3}$ or 1/2 or 50% of the incident light. The advantage of using optical density is that the units are additive; a 2 log unit filter and a 1 log unit filter together attenuate the light by 3 log units. In your system,' the filters will simply be stacked on top of the shutter. You will have three 1.0 log unit filters and one 0.5 unit filter to work with in this exercise. Used in various combinations. these will enable you to stimulate at seven different attenuated light intensities (plus, of course, the intensity of unattenuated light).

b. Physioscribe. You will be using the Time and Event channel, the Biopotential channel, and one Transducer channel for this exercise.

1. Time and Event Channel: use to set chart speeds and to gauge short time intervals.

2. Biopotential channel: displays the ERG. A signal cable is plugged into this channel; the two banana plugs at the other end of the cable are seated in the binding posts mounted in the lucite stage.

3. Transducer channel: displays the timing of the light flashes. The cable from the photoelectric transducer is plugged into this channel. The transducer itself will be positioned beneath your preparation on the lucite stage inside the Light-tight box.

4. Grounding: the Faraday cage (mounted inside the light-tight box), the heavy aluminum plate, and the Physioscribe will all be grounded.

2. Preliminary testing.

a. Position the bottom of a small glass petri dish close to the binding posts on the lucite stage.

1. Arch the wire electrodes up over the edge and down into the dish to touch its bottom.

2. These are silver–silver chloride electrodes, so take care not to scrape the silver chloride off the silver wire core.

b. Fill the dish approximately half full of sea water, making sure that the electrodes are immersed.

c. Position the lucite stage in the center of the heavy aluminum plate, with the binding posts toward the left end.

d. Secure the photoelectric transducer to the lucite stage on the lower level beneath the petri dish if it is not already in place.

e. Slide the box forward over the aluminum plate.

1. With the curtain open and the shutter open (use the T setting), position the illuminator so that its light beam shines down through the center of the shutter and into the center of the petri dish.

2. Lower the curtain and secure its edges; close the shutter.

f. Turn on the Physioscribe; fill the pens; switch on the illuminator (use the 3 setting).

g. Test the biopotential channel as follows:

1. *Slowly* and *carefully* turn up the sensitivity knob of the biopotential channel. You should reach the maximum sensitivity level without detecting 60–cycle noise.

2. If you pick up noise below a setting of 70, check the grounding connections or summon your teaching assistant.

h. Adjust the sensitivity of the transducer channel and test it out as follows:

1. Using 1/25 second flashes with the light attenuated by 3 log units, set the sensitivity of the transducer channel so that the deflection of the pen is minimal. Begin with a setting of 10, and increase by increments of 5.

2. At this sensitivity, unattenuated light flashed with a duration of 1/25 second would give a large but not maximal deflection; does it?

3. With an attenuation of 1 log unit, observe the response of the transducer channel when flashes of different duration (1/50, 1/25, 1/10 1/5, 1/2, and 1 sec) are given. Note that there is a deflection at the onset of light and, for longer flashes, at the offset, but that there is no plateau while the light is on. This is a limitation of the Physioscribe, but not a serious one, since you really only need to keep track of when the flash began. (You may also note that the timing mechanism in the shutter is not reliable; it tends to stick at longer flash durations. Fortunately, you will be using short flashes for the most part.)

When you have completed checking out your system, return the sensitivity setting of the biopotential channel to its minimal level, but leave the sensitivity of the transducer channel as you have adjusted it.

1. Turn the Physioscribe and the illuminator off.

2. Open the box and slide it back to expose the aluminum plate and your experimental set–up.

3. Empty the sea water from the petri dish and dry it with a Kimwipe.

4. Construct two or three small clay pyramids in the middle of the dry glass petri dish. These should be about 0.5 cm across and 0.5 cm high. Your teaching assistant will have a sample illustrating the appropriate dimensions at the front desk.

3. Dissection of the lateral eye; mounting the preparation.

a. Pick up a dissecting kit and a horseshoe crab which has been secured to a dissecting board from the preparatory lab.

b. First, you will have to collect a small sample of blood in a hypodermic syringe using the method of heart puncture. The heart runs antero-posteriorly in the dorsal midline of the body.

1. Locate the hinge which joins the horseshoe-shaped carapace with the angular posterior section (abdomen) of the body.

2. Aim the needle anteriorly and push it through the elastic hinge cuticle in the midline of the animal's body (see Figure 1) to a depth of approximately 0.5 cm.

3. Pull the plunger slightly and adjust the depth of the needle until the syringe begins to fill with blood. Collect approximately 0.5 ml. The blood will be almost clear or slightly bluish; this is due to the presence of the blue respiratory pigment, hemocyanin, which becomes bluer as it is oxygenated.

4. Lay the syringe with its contained blood aside for future use.

c. Examine the lateral eye under the binocular dissecting microscope. Note the facets, each of which represents the lens over one ommatidium.

1. While holding your animal securely, slice off a section of the waxy epicuticle over one of the lateral eyes with a sharp, single-edge razor blade. You should slice deeply enough so that the surface ends of some of the ommatidia are removed with the waxy layer, but not so deeply that you remove most of the eye.

2. With the corner of the razor blade, make a rectangular incision approximately 1.5 cm square through the carapace around the eye (see Figure 2). You need cut only through the cuticle (approximately the thickness of a fingernail); not deeply into the animal, which is only a few millimeters thick in this area.

3. With forceps, lift up the section of excised carapace, separating it from underlying tissues with the tip of a sharp scalpel. Do not, however, poke your scalpel in so far that you damage the soft, bottom side of the eye.

d. Pick up the section and, holding it securely along its edges between your fingers, dry the surface of the cuticle carefully with a Kimwipe. The dryness of the cuticle around the eye is a critical factor.

e. Take the dry-surfaced section securely in the forceps again, and with the narrow end of a stainless steel spatula, apply a wall (2 mm high) of silicon grease around the edge on all four sides. The integrity of the wall is a second critical factor.

1. Why are a dry surface and an intact wall so important? Shortly, you will place one recording electrode in the liquid within the wall where it will contact the surface of the eye. Your reference electrode will be in the sea water bath which will be in contact with the back of the eye. It is essential that the electrical resistance between these two compartments be very high, and this requires 1) making a good wall with no Leaks and no gaps, and *2)* not allowing fluid to spill over from one compartment to the other.

2. Electrical short-circuiting across or beneath a faulty wall is a comnon cause of trouble in this exercise.

f. Mount the rectangle between your clay pyramids, securing its edges firmly in the clay so that it won't later float off.

g. With a pasteur pipette, add sea water to the petri dish to a depth such that its surface is level with the top of the mounted section of carapace. Tilt the dish appropriately so that trapped air bubbles escape from beneath the section.

h. Expel a couple drops of blood from the syringe onto the cut surface of the eye.

i. Place the petri dish containing your preparation in its place on the lucite stage.

j. Position one wire electrode (black) in the sea water, and the other (red) in the drop of blood over the surface of the eye.

k. Draw the box forward over the preparation.

1. Stack 2 log units of filter on the open shutter, switch the illuminator on (use the 1 setting), and quickly check to make sure that the light beam illuminates our preparation; then close the shutter promptly.

m. Position a second Nicholas illuminator to serve as an adapting light source for Experiment IV.

1. Remove the patch covering the porthole on the right side of the box.

2. Aim the light beam of the second illuminator (use the 1 setting) into the box somewhat to the side of your preparation. Then turn off this illuminator and cover the porthole again.

n. Secure the curtain and wait 5 minutes for the eye to dark-adapt.

o. *Nota bene!* Take care not to knock or jar your preparation, pull on the electrical cable, or move the second illuminator during your subsequent experiments. If the alignments are disturbed and you have to open the light-tight box again, valuable time will be lost in waiting for the eye to dark-adapt a second time.

4. Final testing.

a. Set the chart speed of the Physioscribe at a slow speed (approximately 1 mm/sec).

b. *Slowly* and *carefully* turn up the sensitivity of the biopotential channel to maximal. You should observe no greater 60-cycle noise than you did in your preliminary test.

1. If this is not the case, summon your teaching assistant.

2. Set the sensitivity of the biopotential channel at approximately 40 to begin your final testing of the system.

c. Turn the top illuminator on; use the 3 setting for all experiments.

d. Set up and administer a test flash at 1/25 sec of light attenuated at 1 log unit.

e. If there is a response, wait 5 minutes and repeat with another identical flash.

1. Continue with repeated identical flashes after 5 minute intervals until the response amplitude no longer changes; at this point, your preparation has reached the stable, dark-adapted state.

2. Proceed to Experiment I.

3. During the final testing, and throughout all experiments, you will need to keep track of the following: Write them on the chart for each flash!

a. Absolute time - clock time.

b. Attenuation of the light beam - number of log units.

c. Flash duration - shutter speed.

d. Sensitivity setting of the biopotential channel.

f. If there is no response, summon your teaching assistant. You will need to re-check your electrical connections, the alignment of your light beam, the intensity and attenuation of the light, the placement of your electrodes, the quality of your dissection, and the status of your mount. If these all appear to be in order, close the dark box again, wait 5 minutes, and try part d again.

1. If there is still no response, take the eye out of the dish, dry the carapace and re-grease it, re-mount it as described in the previous section, and proceed with final testing once again.

2. As a last resort, you will have to do a second dissection.

5. Experiment I. Response amplitude and latency of response as functions of stimulus intensity.

a. Set the Physioscribe speed at maximum.

1. Run the chart mover only at the times when you will actually be delivering flashes; you will waste enormous amounts of costly paper if you run it continuously.

b. The sensitivity settings of both channels should remain as previously set.

c. Use 1/25 second flashes for this experiment.

d. Begin with the dimmest flash possible (3.5 log units of attenuation); stimulate the eye and record the response. It is important that you get good traces on both recording channels, although the responses will be very small. Readjust the sensitivities of the channels if necessary, and repeat the flash until you get a good trace.

e. Continue to stimulate the eye with progressively brighter flashes of light. By stacking different combinations of filters, six additional intensity levels are possible.

f. How long should you wait between stimuli so that the response to a given flash is not influenced by the previous stimulus? Your experimental protocol will need to include controls which show that your stimuli were not given too close together. There are several possible controls that could be done. You will have to think about the problem; to devise a suitable control, and include it during the course of the experiment.

6. Experiment II. Response amplitude as a function of stimulus duration.

a. Continue with the previous Physioscribe settings.

b. Choose an intensity for which the shortest possible flash (1/200 sec) gives a minimal response; stimulate the eye, and record the response.

c. Continue to stimulate the eye with progressively longer flashes of light at the same intensity; there are seven additional settings up through 1 second on your shutter.

d. Use the data obtained in your controls for Experiment I to determine the approximate delay time between successive stimuli.

7. Experiment III. Time course of dark adaptation.

a. Slow the chart speed to approximately 1 mm/sec, and allow the chart to run continuously during this experiment.

b. Choose a stimulus duration and intensity that will give a half-maximal response.

1. Stimulate repeatedly at suitable intervals until you obtain at least two equal responses.

2. Note the minimal time that must elapse between these flashes to obtain equal responses. This is your dark-adapted control. Make sure that you do not underestimate this time.

c. Light-adapt the eye by giving unattenuated light for 10-15 seconds; the T setting of the shutter is useful here.

d. Return immediately to the stimulus conditions used for the dark-adapted control flashes.

1. Deliver flashed regularly at the interval previously determined until the dark-adapted control response is once again obtained.

8. Experiment IV. Sensitivity during light adaptation.

a. This experiment is in part a variation on the previous experiments. Here you will measure responses while a second dim light enters the box; this light will serve to partially light-adapt your preparation. As an adapting Light source, you will use a second Nicholas illuminator which you have already positioned to shine through the side porthole.

b. Continue with the previous Physioscribe settings.

c. To begin the experiment, you should repeat a mini-Experiment I using only three different intensities of light. This will give you data for a second dark-adapted intensity-response curve (Experiment IV-A).

1. Note that since you are running the Physioscribe at a slow speed, you will not have data for a second intensity-latency curve as you had in Experiment I.

d. Next, proceed to repeat the dark-adapted control of Experiment III, giving flashes at an intensity which produces an intermediate response repeated at the standard interval (section 7.b. *1.&2.*). This will give you data for a second dark-adapted control (Experiment IV-B).

e. After 2 or 3 flashes, open the porthole. This should produce a response, the magnitude of which will tell you the intensity of the adapting light from the second illuminator.

f. Continue to administer standard test flashes at the standard interval.

1. The response to your first stimulus during light adaptation should be reduced. If this is not the case, you should reposition the light beam of the second illuminator.

2. Administer test flashes with the porthole open until the response has stabilized. This will give you data for a light-adaptation curve (Experiment IV-C).

3. Once the response has stabilized, take data for a light-adapted intensity-response curve (Experiment IV-D). Use at least the same three light intensities that you used in 8.c. above.

g. Conclude by closing the porthole and continuing with the standard test flashes until the dark-adapted control response is once again attained. These data will enable you to plot a second dark-adaptation curve (Experiment IV-E).

9. Experiment V (Optional). Latency of response as a function of response amplitude under conditions of light adaptation.

a. Design and carry out an experiment which will provide data to answer the question: Is the latency of equal-sized dark- and light-adapted responses the same?

10. Experiment VI (Optional). Time course of dark adaptation under different stimulus conditions.

a. Repeat Experiment III using stimuli of different durations or of different intensities. Your data should enable you to answer two questions:

1. In what respect(s) is(are) all dark-adaptation curves similar?

2. In what respect(s) is(are) all dark-adaptation curves different?

11. Experiment VII (optional). Intensity-response curves as a function of response duration under conditions of dark adaptation.

a. Repeat Experiment I using stimuli of different durations.

b. Your data should enable you to answer the question: How is response

amplitude related to the total amount of light (*i.e.*, intensity x duration) administered?

1. Does an increase in intensity by a factor of 2 exactly compensate for a decrease in duration by a factor of 2?
2. Over what range can intensity and time be traded off? (In humans, the sensitivity to flashes of equal total energy is constant for durations less than 100 msec).

12. Termination of the exercise.

a. Return the dissecting equipment and the dish containing your preparation to the preparatory lab.

b. Wipe the grease off the spatula; return it and the container of silicon grease to the front lab bench.

c. Clean the salt from the stage of the binocular dissecting microscope.

d. Turn the Physioscribe off; empty the pens; return all sensitivity knobs to their minimal settings.

e. Check out your data with your teaching assistant

Data Analysis and Report

1. Preliminary testing.

a. Strip chart – in Appendix.

2. Final testing.

a. Strip chart – in Appendix.

3. Experiment I.

a. Summarize the data in tabular form.

1. Stimulus intensity in log units of attenuation.
2. Response amplitude in mm
3. Latency of response in mm, convert to seconds.

b. Plot response amplitude as a function of stimulus intensity. This will be your dark–adapted intensity–response curve.

c. Plot latency of response as a function of stimulus intensity.

d. State conclusion(s) which can be drawn from these data.

e. Strip chart – in Appendix.

4. Experiment II.

a. Summarize the data in tabular form.

1. Stimulus duration in seconds (from shutter).
2. Response amplitude in mm.

3. Latency of response in mm, convert to seconds.

b. Plot response amplitude as a function of stimulus duration.

c. State conclusion(s) which can be drawn from these data.

d. Strip chart – in Appendix.

5. Experiment **III.**

a. Summarize the data in tabular form.

1. Time in seconds after previous flash for dark–adapted controls.

2 Time in minutes after the end of light–adaptation for experimentals.

3. Response amplitude in mm.

b. Plot response amplitude as a function of time following the end of light–adaptation. This will be your dark–adaptation curve.

c. State conclusion(s) which can be drawn from these data.

d. Strip chart – in Appendix.

6. Experiment IV.

a. Summarize the data in tabular form.

b. Plot the data for Experiment IV–A on the graph which you made for Experiment **I** as described in 3.b. above.

c. What was the intensity of the adapting light from the second Nicholas illuminator. How did you determine this?

d. For Experiment IV–C, plot response amplitude as a function of time following the beginning of light adaptation. This will be your light-adaptation curve.

e. Plot the data for Experiment IV–D on the graph which you made for Experiment **I** as described in 3.b. above. Your light–adapted intensity-response curve will thus be directly comparable with your two dark–adapted intensity–response curves.

f. Plot the data for Experiment IV–E on the graph which you made for Experiment **III** as described in 5.b. above.

g. State conclusion(s) which can be drawn from these data.

h. Strip chart – in Appendix.

7. Optional Experiments.

a. Outline your experimental protocol so that your experiment can be repeated by someone else with the same results.

b. Tabulate the data in an appropriate way.

c. Plot data on graphs if appropriate.

d. State conclusion(s) which can be drawn from these data.

e. Strip chart – in Appendix.