Tested Studies for Laboratory Teaching

Proceedings of the First Workshop/Conference of the Association for Biology Laboratory Education (ABLE)

Jon C. Glase

TESTED STUDIES FOR LABORATORY TEACHING

Proceedings of the First Workshop/Conference of the Association for Biology Laboratory Education (ABLE)

Edited by

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We dedicate this volume to hardworking, effective laboratory biology teachers everywhere.

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Foreword

Don Igelsrud

Another biology organization and publication! In 1902 the editor of *The New Phytologist* suggested, in concert with the current interest in evolution, that if the publication survived it was needed. More recently rapid publication has become a major justification for a new publication. Laboratory instruction at the undergraduate level has placed strenuous demands on biologists because of lack of communication. In the past most laboratory positions have been temporary in nature so that by the time the instructor understood the problems that needed to be solved they were no longer of personal concern. The Association for Biology Laboratory Education is the result of cooperation among biology laboratory instructors who have found more permanent positions.

My growing involvement with laboratory instruction probably parallels that of others. An early interest in biology was initiated by an excellent elementary school teacher (Elizabeth Long) and developed by a charismatic scientist (W.J. Breckenridge). An appreciation for the necessity of laboratory experience beginning with B.S.C.S. was developed with the support of a strong administrator (L.M. Adelson, Delaware Valley College) who allowed me to discover the biological problems of laboratory instruction. A position at Northwestern University helped me develop an appreciation for the administrative problems of laboratory instruction involving teaching assistants, exceptional students, and a research-oriented department. And I gained an appreciation for the high quality of laboratory instruction possible when a department and institution give it strong support (The University of Calgary). I spent a considerable amount of time trying to get laboratory exercises to work and realized that most of the solutions to the biological problems of laboratory instruction were well known to the research community. New laboratory materials consisted mainly of new methods of instruction using traditional materials. An organization concerned with improving the biological materials used in instruction was needed. For several years I felt biology laboratory instructors with similar experiences needed to get together and form an organization which involved those research scientists who had developed exceptional teaching materials so a formal association could be established.

When I came to the University of Calgary in 1976 I decided I would begin trying to form an organization before I got too involved with my work, as I had at Northwestern University. I talked over the idea with Jon Glase of Cornell University and Ruth Von Blum of the University of California, Berkeley, indicating I felt we needed to find a way to identify the most creative

and cooperative biologists. We decided to send out a letter indicating we wanted to establish a library and organization. John Williamson, the new department chairman at the University of Calgary, indicated he would help support the development of the library and a request for special projects funds to the university. Jon Glase asked his section chairman, Kraig Adler, for support. The cost of sending a letter to all the department chairmen on the A.I.B.S. mailing list was shared by Cornell University and the University of Calgary. I asked Patricia Paulus of Texas Christian University to join us so we would have a geographically representative committee. The four of us drafted the letter and became the "Committee to Establish a Laboratory Biology Teaching Organization and Library."

It took a year to draft and send out the letter and news releases, and another year to collect and examine the material submitted to the library. In May of 1978 the committee met in Calgary to decide what to do next. After a week's discussion we decided to hold a workshop and organizational meeting in Calgary about the same time the following year.

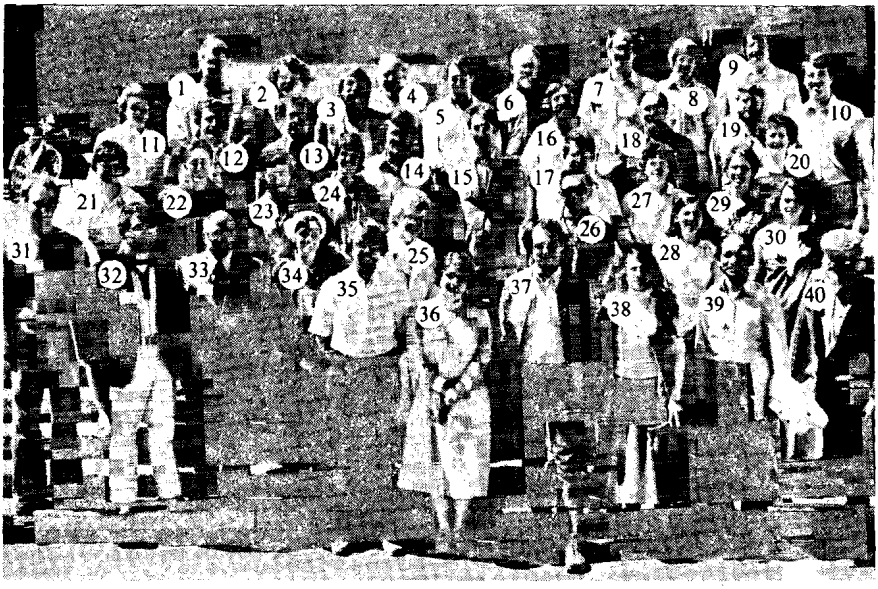
Initially we felt we could obtain a grant to support the cost of organizing the workshop but discovered funds were hard to obtain, particularly for conferences by unestablished organizations. Finally, I was able to get the special projects grant for the development of the library modified so we could pay for an ad in *Science* and many long distance phone calls. The workshop would not have been possible if we had not received strong support from the department, now under the chairmanship of Dennis Parkinson, the Dean of the Faculty of Science, Thomas Oliver, and the Instructional Methodology and Development Committee of the university.

Our strategy of trying to find creative and cooperative biologists worked well. By requiring the submission of sample teaching materials we assembled an exceptionally cooperative and constructive cadre. The group attended workshops during the day and business meetings at night. In four hectic days we established an organization, transferred a lot of biological information useful in laboratory instruction, and made many new friends.

Loy Crowder described the experience in a letter following the workshop as follows:

> Here's to ABLE! May it be long-lived, motivating and stimulating, and bring together biology laboratory educators with the interest, enthusiasm and congeniality as was experienced by the charter members.
>
> Honestly, I have never attended a conference or workshop with such delightful people. The spirit of camaraderie which developed so quickly was incredible.

Copies of the initial letter and news release, the ad in *Science*, and the second news release are found in an appendix at the end of this volume. They summarize the goals and aspirations of A.B.L.E.



THE ASSOCIATION FOR BIOLOGY LABORATORY EDUCATION [A.B.L.E.]

First Annual Workshop The University of Calgary June 1979

*1. Richard Montgomery—Hagerstown Jr. Col.
2. Iain Taylor—U. of British Columbia
3. Donald Mansfield—U. of Calif. at Davis
4. Werner Kundig—Hood College
5. Charles Curry—University of Calgary
6. Norman Davis—Univ. of Connecticut
*7. Carl Gilbert—University of Illinois
8. Donald Fritsch—Virg. Commonwealth Univ.
*9. Daniel Burke—Mercer University
10. Michael Ernest—Yale University
11. Eloise Carter—Agnes Scott College
*12. William Elliott—Hagerstown Jr. College
13. John Gapter—Northern Colorado Univ.
14. Dennis Brown—University of Winnipeg
*15. Bruce Virgo—University of Windsor
*16. Lester Eddington—Biola College
17. Hans Boerger—University of Calgary
*18. Eugene Kaplan—Hofstra University
19. James Waddell—University of Maine
20. Cairine Milner—University of Alberta
21. Ruthanne Pitkin—Smith College
*22. Susan Boutros—University of Pittsburgh
23. Rosalie Talbert—Nassau Community College
*24. Marcia Allen—Stanford University
25. Sister Dunstan Plantenberg—St. John's
*26. Charles Barr—SUNY at Brockport
27. Cynthia Penney—Memorial U.Nfld.
28. Sylvia Kao—Memorial U. of Nfld.
29. Anna Wilson—Purdue University
30. Mary Jane Turtle—U. of Alberta
31. William Clark—Univ. of Alberta
32. William Ogata—Fungal Genetics
*33. Osiris Boutros—Univ. of Pitt.
34. Janet Emerson—Emory University
*35. Donald Igelsrud—U. of Calgary
36. Ruth Von Blum—Nat. Sci. Found.
*37. Jon Glase—Cornell University
38. Jenny Xanthos—McGill Univ.
39. Adeel Siddiqi—Univ. of Toronto
40. Douglas Fraser—Siena College

Additional Participants

41. Terry Crawford—U. of Brit. Col.
*42. Loy Crowder—Cornell University
*43. C. Leon Harris—SUNY Plattsburgh
44. Jaume Josa—Univ. of Barcelona
45. Kenneth Laser—SUNY Stony Brook
46. Dixie Morrow—U. of Calgary
47. C.O. Patterson—U. of Missouri
48. Helen Ross—Univ. of Calgary
49. Dow Woodward—Stanford Univ.

*Author of a chapter in this proceedings volume.

Preface

ABLE was created to serve as an organization to promote information exchange among teachers actively concerned with teaching biological subjects in a laboratory setting. ABLE's annual workshop/conferences bring together two groups of people: a group of about one dozen selected demonstrators (individuals who have developed interesting, innovative, and reliable exercises, approaches, or systems in laboratory biology teaching) and a larger group of participants (individuals desiring hands-on experience with these exercises, approaches, or systems in order to evaluate and potentially adapt them to their own programs). In three very full days, participants are involved in six laboratory sessions. The demonstrators are asked to provide all of the essential information and experiences that the potential user of the laboratory would require in order to "take it home" and use it at their own institution. This essential feature of the meeting is retained in this volume of the proceedings of the first ABLE workshop/conference. The following **14** chapters were written by the demonstrators at the first meeting last 2–5 June **1979** at the University of Calgary as a further effort to make laboratory materials and approaches that they have successfully used available to a wider audience.

Each of the chapters in this volume is written for the potential user and seeks to clearly present the essential features of the laboratory so it can be evaluated, adapted, and used by the reader. Most of the authors have expanded and clarified their presentations as a result of the experiences and suggestions they gained during the laboratory sessions at the workshop. In some cases, authors have included significant student material as an example of the background information that students will require to successfully understand the laboratory or, as in Kaplan's chapter, as an example of a specific approach to laboratory teaching. Extensive preparatory information is included in all chapters to make each laboratory as easily adapted as possible. Suggestions for the best sources of required materials, specific information on quantities of materials needed, and alternative uses of the laboratory at different instructional levels are included in most chapters. An annotated reference list is provided to direct the reader to sources of supplemental information. Also, the authors invite correspondence with readers who have further questions about their laboratory presentation or who have comments based on their own experiences with and use of the material. The Table of Contents of this book

includes, for each chapter, a list of key words and an abstract so one can easily find chapters of interest.

The editor wishes to acknowledge the extensive and invaluable help of Kate Bloodgood, who has served as an assistant editor in the production of this volume. All of the authors have benefited from her efforts, which have contributed to improving the uniformity and clarity of the written materials included in this book.

Ithaca, New York Jon C. Glase

Chapter 1

A Review of Sources of Living and Supplementary Materials for Laboratory Instruction with a Note about Vivisection

Don Igelsrud
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Don Igelsrud received his B.S. from the University of Kansas in 1964 and an M.A. from Washington University in 1965. From 1966 to 1973 he taught at Delaware Valley College, where he developed a strong interest in laboratory teaching. In 1973 he became Biology Laboratory Director at Northwestern University. He took his current position at The University of Calgary as instructor in charge of the introductory zoology laboratories in 1976. His main interest is in increasing awareness and understanding of living phenomena and in finding living organisms that survive well in the laboratory with a minimum of maintenance.

The materials necessary for teaching biology laboratories can be obtained from many sources and it would be impossible for one person to be familiar with all of them. A comprehensive list would be easy to compile from publications such as *Techniques and Materials in Biology* by Marjorie Behringer, *Animals for Research* published by the National Academy of Sciences, the buying guides published by *Pets/Supplies/Marketing* and *Science,* or the exhibit guides for the annual meetings of the Federation of American Societies for Experimental Biology and the National Association of Biology Teachers. As anyone who has used these lists knows, many of the companies that supply living materials are often out of business by the time the lists are published.

This paper will summarize more than a dozen years' experience trying to find reliable sources of material. *The list of sources in no way implies that those not listed are of lesser quality;* undoubtedly there are many of equal or better quality I have yet to experience. Many of the sources have been discovered by talking with persons doing research in the specific area or by talking with purchasing agents, but most have been the result of my own inquiry.

I have assumed those reading this paper have some experience using living organisms and have had the same problems I have had in finding reliable sources and information. Consequently, the discussion is relatively brief and is mainly intended for identification rather than comprehensive explanation. Detailed information on the methods indicated is readily available. I have also limited myself to those methods and sources which have worked well for several years and have been the basis for the laboratory courses I have taught. I have decided not to list the methods other biologists have told me are successful. I have avoided talking about organisms which are difficult to maintain such as *Hydra* or *Daphnia.*

Living Organisms

Protozoa

"A Catalogue of Laboratory Strains of Free-Living and Parasitic Protozoa (with sources from which they may be obtained and directions for their maintenance)," published by the Committee on Cultures, Society of Protozoologists, in *The Journal of Protozoology* 5(1): 1–38 (1958) is one of the most complete lists of culture media for protozoa and algae. The American Type Culture Collection catalogues also contain media information.

I have cultured *Paramecium, Amoeba proteus,* and *Paranema* for many years without much difficulty using the media sold by Connecticut Valley Biological Supply Company. Four grains of preheated wheat (for *Paramecium,* preheated rice for *Amoeba* and *Paranema*) are added to about 200 ml of *Paramecium* media (or *Amoeba* media for *Amoeba* or *Paranema*). The

Paramecium medium works with *Stentor* and some other ciliates as well. The medium is placed in four-inch culture dishes (Carolina Biological Supply Company sells culture dishes that stack well). Since the medium comes in one liter containers and several subcultures are needed to insure success, I usually make five subcultures. If about 2 ml (a pasteur pipetful) of dense culture (taken from parts of the culture where the animals congregate) is added to 200 ml of media, a good culture should develop. A commercial culture for a class of 25 should contain enough protozoa to start five subcultures.

Enough food material is usually transferred with the animals to start the subculture. *Chilomonas* (a very small flagellate) or bacteria are the main source of food for the protozoa. As populations of bacteria, *Chilomonas* and the protozoan you are culturing build up the fate of the culture is determined. The subcultures are competition experiments among the three types of organisms. If the subculture has been made from a dense part of the culture where the protozoan is successful and not limited by *Chilomonas* a good culture should develop. Eventually cultures become very dense and cloudy with bacteria. If subcultures are made at this stage they are usually not successful. The advantage to this culture method is that growth is relatively slow so that subcultures can be made when the medium is clear and, yet, heavily populated with protozoa. If subcultures are made at this time (during the log phase of the protozoan's growth) good cultures can be maintained. Occasionally cultures will be left too long and you may have to start over again to develop a good culture. Sometimes, particularly with *Amoeba*, rotifers will be found in the cultures. It seems to happen most often when the biological supply companies have a high demand for protozoa at the beginning of term. Sometimes *Amoeba* will return to the culture if it is left for a few months. Usually, however, you will need to establish a new rotifer free culture.

I do not know what is in the Connecticut Valley media; however, I have concluded that this method works with other liquid media. Pasteurized Carolina springwater works well. Aged (aerated with an airstone for a week) Chicago city water also works. Turtox cultured their protozoa on aged Chicago water for many years. I suspect that if good cultures are established with Connecticut Valley media they could be selected against any water supply. An artificial springwater like Chalkey's medium should also work. Currently I am using water from a local rainbow trout hatchery.

Glassware should *not* be washed with soap, instead it should be thoroughly rinsed with hot water. Plain steel wool can be used to remove salt deposits. It is the best cleaning agent for all glassware, even when soap is used!

This method of protozoan culture works well because it is simple, not subject to drastic change, not time consuming, and inexpensive. The cultures can stay in useful condition between subculturing for from a few weeks to several months and as one becomes more experienced it is possible to examine the cultures quickly, without a microscope.

The main advantage of culturing protozoa is that dense cultures are available for student use. It is easier to study protozoa if many are on the slide. Protozoa congregate around food, and under the dissecting microscope it is easy to obtain a drop containing many animals. If a small amount of food is also taken in, the protozoa will often congregate around it, making observation of the protozoa easy. Carolina's Protoslo works well for slowing down protozoa.

Tetrahymena grow well axenically on a medium made of 5 g proteose-peptone, 5 g tryptone, 0.2 g K_2HPO_4 in 1 liter of distilled water and adjusted to pH 7.2 before autoclaving. Some people use *Tetrahymena* to feed monoxenic cultures of *Amoeba* and other protozoa. This method works well but requires more time.

Much more could be said about protozoan culture. However, if one is interested in maintaining a few major types of protozoa, the above method works well.

Marine Aquaria

Many animals, particularly invertebrates, are best represented by marine (saltwater) forms. The maintenance of marine aquaria is not difficult or expensive. For classroom use there are many forms that survive well at room temperature. Many biologists have used cold-water forms and refrigerated aquaria. Recently it has become possible to obtain more colorful warm-water forms because of the increased interest shown by hobbyists. These animals survive well for several hours when placed in small containers of seawater. This makes it easy to set up many small, inexpensive, plastic aquaria (such as those sold by Nasco) so a survey of major living forms can be studied by students. I return the animals to the main tank at the end of the day and then put them in freshly poured seawater the next day. The old water should be returned to the main tank each day.

A major scientific supplier of warm water marine animals is Gulf Specimen Company in Panacea, Florida. Animals from this locality seem to tolerate fluctuations in temperature and salinity well since these changes occur in their natural environment. Gulf Specimen Company is the only company I know of that can regularly supply ctenophores, jellyfish, and amphioxus.

Many tropical forms also survive well at room temperature, and the most colorful forms are available from pet suppliers. Most cities have clubs interested in marine aquaria and their experience can be invaluable. Often very synergistic relationships can develop between biologists and hobbyists. If a city has a large wholesaler animals can sometimes be rented or loaned. I once provided a survey of animal phyla for my students by renting whatever animals a wholesaler had in stock that week at 10% of wholesale cost.

The simplest marine aquarium consists of an undergravel filter and a dolomite substrate. Dolomite can often be purchased locally. The calcite sold by feed stores for chickens works well. Local hobbyists usually know the best local source. If the aquarium is maintained properly, other accessories are not necessary. Nektonics undergravel filters have a simple protein skimmer attachment which is placed on top of the airlift tubes and is useful in maintaining water quality. An outside filter such as a Diatom Filter or an Eheim filter can also be useful in cleaning the aquarium to remove algae or decaying material but these are not necessary. An inexpensive and excellent introduction to marine aquaria is the *Marine Aquarium in the Research Laboratory* by John M. King and Stephen Spotte (1974 Aquarium Systems, Inc.). It costs two dollars and is often available in local aquarium stores.

If your laboratory has compressed air it can be used to run the aquarium. A trap can be set up to filter the air, or a commercial filter such as those produced by Balston, Inc. can be used. Commercial plastic laundry carts are useful for transporting and mixing sea water.

Many colorful tropical forms come from the Pacific and can be ordered from the large wholesalers in Los Angeles. The largest is Salt and Sea Enterprises, Inc. Two other companies are Marine Fish Imports and Pacific Marine Imports. Walter Smith and Dennis Reynolds can supply specimens from all the Los Angeles wholesalers and Reynolds will also collect local marine organisms. *Marine Invertebrates* by U. Erich Friese (T.F.H. Publications, Inc., New Jersey. 1973 240 p.) or *The Lower Animals: Living Invertebrates of the World* by Ralph Buchsbaum *et al.* (Hamish Hamilton Ltd., London. 1960 304 p.), are useful in identifying animals. Less colorful warm water animals not likely to be of interest to hobbyists still need to be ordered from companies like Gulf Specimen Company or the companies listed under sea urchins.

Verilux, Inc. sells fluorescent bulbs that have a spectrum similar to daylight which makes the animals look more colorful and allows accurate color photography.

Clams

In many cities with fresh seafood dealers large living clams, *Mercenaria* (Quahog), and sometimes other species, are available at low cost. These can be stored in a 4°C refrigerator for several days or placed in a marine aquarium. These animals usually come from New England and should be kept in refrigerated marine aquaria at about 10°C but will survive at room temperature.

Sea Urchins

The short-spined sea urchin, *Lytechinus variegatus,* is excellent for studying development because it has extremely clear cells. Specimens from warm-water localities allow students to study development in Syracuse watch glasses under the 10x objective at room temperature. Marine Specimens Unlimited can supply fertile *Lytechinus* anytime of the year. John C. Noyes and Marine Specimens Unlimited can also supply many forms of invertebrates from south Florida. Cistron Corporation sells a 500-mesh sieve (catalog number 676–500) for about $10 which is useful in concentrating embryos and protozoa.

Nematodes

Turbatrix aceti is easy to subculture in preservative-free apple cider vinegar. I have maintained cultures for many years and they only have to be subcultured 3 or 4 times a year if a small amount (1 ml) of inoculum is used. Because labels do not always indicate if a preservative has been added you may have to try several brands to see which works best.

Rotifers

Rotifers can be maintained on the remains of protozoan cultures after they have been subcultured.

Earthworms

Despite the many claims about the ease of worm culture, I know of no one who maintains a successful large culture of *Lumbricus terrestris.* They can be maintained for several months in the refrigerator at 4°C in the *soil* in which they are shipped. The problem most often encountered is mold infections which happen easily at warmer temperatures. I have had good luck with nightcrawlers from Robert Conroy Live Bait. They collect nightcrawlers on golf courses at night when they are watered. You could probably collect your own in the same way and maintain them on a good garden soil or Buss worm bedding available from bait dealers.

Insects

Blaberus cranifer, a large tropical cockroach, is excellent for teaching because it is large, relatively slow, and does not crawl up the sides of smooth glass or plastic containers. It is easy to culture but grows best at about 30°C and 80% humidity. At room temperature growth is slow and it is difficult to enlarge the colony. They grow well on high protein puppy chow. Selph's Cricket Ranch, Inc. sells inexpensive equipment for culturing crickets which can be used for *Blaberus.* The heater, thermostat, plastic fountain bases, rubber donuts, and plastic cricket buckets are useful. If the rubber donut is covered with a ring of polyester fleece (felt) that is cut so a wick leads inside

the mason jar, a more effective watering device is produced. Foam rubber does not conduct water very well. The plastic cricket buckets are useful for putting the cockroaches out for students' use.

The sunflower seed strain of *Oncopeltus faciatus,* the milkweed bug, is also easy to culture. H.T. Gordon of the University of California, Berkeley, has developed a very successful method for rearing and storing the insect. See *Annals of the Entomological Society of America,* Volume 67: 976–980, November 1974.

Crayfish

Monterey Bay Hydroculture Farms can supply *Procambarus clarkii* at any life stage desired and in any volume throughout the year. They can also supply *Pacifastacus* and *Orconectes* as well as *Corbicula, Gambusia* and crayfish food.

Ammocoetes Larvae

Living ammocoetes larvae are available from College Biological Supply Company for about twenty-five dollars a dozen. They survive for several months in their shipping container with a loose lid. They can be anesthetized with Tricaine Methane Sulfonate and examined with a dissecting microscope. They exhibit very strong negative phototaxis and burrow into the substrate.

Trout

If a trout farm is near your department, living trout can be obtained at low cost.

Frogs

The availability, cost, and health of *Rana* is causing many biologists to use *Xenopus* and it appears to be equally useful. Currently the cost of *Xenopus* is about equal to *Rana* but it will, most likely, become less expensive. Nasco appears to have the most experience and best prices for *Xenopus.* Many of the problems associated with *Rana pipiens* are related to the method and places of collection. After discussions with frog suppliers and users it appears to me that several conclusions can be drawn about *Rana pipiens*:

1. The southern form is not the same species and is physiologically different.
2. The high use of nitrogen fertilizers in Mexico may be the cause of the exceptionally high mortality rate in southern frogs.
3. Northern frogs are held in hibernation by suppliers and often are in poor health during the spring.

4. The shipping of frogs from wholesalers to biological supply companies probably causes stress and increases mortality. Northern frogs appear to hold up better during shipping than southern frogs. Consequently, healthier frogs can probably be obtained from wholesalers, particularly in the spring.

I have been able to obtain healthy frogs most of the year from the Wm. A. Lemberger Associates Ltd. This company has considerable experience with frogs and is one of several companies in Wisconsin that supply frogs to other dealers. Because northern frogs are in short supply (populations seem to be growing, however) it is best to place orders early (July or August) with any of the wholesalers so you can obtain frogs when they are scarce in the spring.

Rats

Rats can be obtained from many places. Because of the high cost of shipping local sources should be investigated. Retired breeders can be purchased at lower cost than other rats from most dealers. Animal care facilities usually charge a few cents per day per animal for maintenance. Because these charges add up rapidly one should get a fixed price from animal care facilities or, if the animals are obtained elsewhere, be sure they are delivered as close to the date of use as possible.

The ease with which the animals are handled depends on the frequency and method of handling to which they are accustomed and their strain. Ask the dealer how docile the animals are and whether they can be used with inexperienced students. I have used rats with first-year students for several years and only three students out of more than a thousand have been bitten (mainly because they frighten the animal). None of the students complained about the experience. Students can handle the animals without much difficulty if they are shown how. The best way to learn how to handle the animals is to be shown how by someone who is experienced and enjoys working with rats. Inexperienced students will usually be frightened by the animals so they need to let the animal explore their hand and to stroke the rat before picking it up. They should feel comfortable with the rat before picking it up so they do not suddenly jerk their hand away and frighten the animal. If the rat struggles, tries to get away, or squeals, they should put it back in the cage and find a more docile animal. I have been amazed at how nonaggressive the rats are toward the students. Brief discussions on how to handle rats can be found in *Animal Care from Protozoa to Small Mammals* by F. Barbara Orlans (1977 Addison-Wesley pp. 266–268) or in The Universities Federation for Animal Welfare information leaflet: The Laboratory Rat and Mouse (UFAW, 230 High Street, Potters Bar, Hertfordshire, England).

I have students handle the rat and then place it in a large plastic garbage can filled with carbon dioxide. They observe the animal's response to anesthesia (the carbon dioxide) noting the major stages of anesthesia. When the rat is relaxed the rat is given an intraperitoneal injection of sodium pentobarbital. Usually about 1 cc is an effective overdose but if the animal shows signs of recovery more is injected. If the animal's abdominal cavity is opened as soon as it is insensitive to pain the movements of the lungs can be seen through the relatively transparent diaphragm. I have the students make vaginal smears, examine sperm from the epididymus, study the heartbeat and review the anatomy they have learned from demonstration fetal pigs studied in earlier laboratories, paying particular attention to the differences between a preserved fetal animal and a living adult. Students generally are anxious at the beginning of the lab about handling and killing the rat but come away from the lab feeling it was one of the most worthwhile laboratory experiences.

Anesthesia

Many anesthetics for invertebrates require long induction times and are consequently difficult to use in the classroom. This is well illustrated with clams. I have used many anesthetics but have been unable to find one that causes the clam to relax and open its valves. Striking one of the valves with a pliers to crack the valve and then removing the valve carefully while the clam is in water seems to work as well as anything. The clam relaxes again and does not seem to behave any differently than when it is anesthetized.

It appears that many so-called anesthetics are really immobilizing agents rather than anesthetics. The mode of action of many of these substances is not known or at least difficult to find in the literature. MS 222 (or Tricaine Methane Sulfonate or Ethyl m-Amino Benzoate Methane Sulfonate) appears to be an excellent anesthetic for fish, amphibia, and several other organisms but its mode of action seems impossible to find. I use pithing for frogs because I am unsure of the effects of MS 222 and the level of recovery the animals experience. MS 222 is excellent for studying phenomena where the animals can recover. It is the best way to study capillary activity, far superior to wrapping the frog in a cloth and tying it to a board. An inexpensive source of the drug is Kent Laboratories Ltd.

I also question whether carbon dioxide is as painless and pleasant as claimed. I have tried inhaling it in various concentrations and would describe the experience like breathing soda water.

Organs

Fresh organs can often be obtained from local slaughter houses; however, sometimes it is difficult to consistently obtain organs in good condition, particularly hearts and lungs. Nebraska Scientific is a major wholesaler of slaughter house materials and can supply most organs at reasonable cost in fresh

condition by shipping them on ice via air. I regularly use hearts with vessels and pericardium intact, lungs, and pig uteri with small embryos. Water can be used to demonstrate heart valve function and coronary circulation; lungs can be inflated to demonstrate their remarkable qualities. The extraembryonic membranes of the fetal pig can be studied in their natural, nearly odorless condition. The *Instructor's Manual to Accompany Laboratory Guide for Biological Science* by Keeton *et al.* contains some good information on the use of fresh organs.

Algae

The soil used for the successful culture of algae is more specific than the description given by E.G. Pringsheim. It is probably more efficient to find someone who has had good success and has an abundant source of soil than to find one by trial and error. The following experience illustrates the problem. In 1969 Indiana University announced it was no longer going to supply algae cultures for teaching. Instead, it was going to give cultures to Carolina Biological Supply Company and Ward's Natural Science Establishment so they could supply them. I was teaching at an agriculturally oriented college and asked a colleague in the agronomy department for a soil that met Pringsheim's requirements so we could establish our own collection for teaching. We were surprised when our subcultures did not grow well. I wrote to H. Wayne Nichols at Washington University in St. Louis where I had taken phycology and he replied as follows:

"During our past history, we have tried many, many soils from many, many peoples and areas in the country and have found few that are adequate for the cultivation of algae. One of the soil types can generally be obtained from Dr. Philip Cook of the Department of Botany at the University of Vermont, and has proved to be very successful. One of the most important factors to consider when obtaining soil is the clay content. If indeed there is excess clay content in the medium, it will appear cloudy, with a great deal of particulate matter in the liquid medium after regular autoclaving. I would suggest one add no more than ¼″ or less of soil to each culture type, plus a pinch, or very, very small amount of calcium carbonate. The tubes or milk bottles can be plugged with cotton and regular autoclaving employed. Additionally, steaming or active boiling with a cover for 1 hr. on two successive days has proved satisfactory."

In 1973 I visited Daniel E. James at Carolina Biological Supply Company. He worked with the Indiana University collection as a graduate student and took a position with Carolina. I told him of the problem I had in 1969 and was surprised when he told me he used recycled soil from Carolina's greenhouses.

The cost of algae cultures is not significantly higher than they were from Indiana University. A detailed description of the Indiana collection and how it is maintained is found in "The Culture of Algae at Indiana University" by Richard C. Starr, the *American Journal of Botany,* 51: 1013–1044, October 1964.

Fungi

Sordaria brevicollis is a heterothallic species which can be used like *Neurospora* but does not contaminate other microbial and fungal cultures with microspores as *Neurospora* does. Because it is heterothallic it is more useful than *Sordaria fimicola* which is widely used but homothallic. During meiosis *Sordaria brevicollis* exhibits spindle overlap but this is of little consequence since only one ascospore pattern (symmetrical MII asci) needs to be counted to obtain data to calculate crossover frequency and map distance. I have found spindle overlap useful in determining if students understand meiosis. I used *Sordaria brevicollis* strains for several years which I obtained from William G. Fields of Michigan State University. He sent cultures which were started by ascospore isolation and said this was necessary to maintain the strain's viability. Dr. Fields died in an accident in 1975 and as far as I know the strains are no longer available. If anyone has viable heterothallic strains of *Sordaria* and is using them successfully in teaching I am sure many laboratory teachers would be interested in obtaining cultures. The strain of *Sordaria fimicola* described by Cassell and Mertens in *The American Biology Teacher,* May 1968, pages 367–372 is not viable anymore and apparently unavailable. Their paper, "A Laboratory Exercise on the Genetics of Ascospore Color in *Sordaria fimicola,*" however, is a good summary of how to use *Sordaria.*

Physarum

Physarum polycephalum is exceptionally easy to culture on wet filter paper in petri dishes as described in many places. Once you have obtained some dry sclerotium you can grow as much as you need. Each student can have his own culture and study the life cycle. The only stage I have had trouble seeing is the amoeboid stage. Flagellated swarm cells can be seen without much difficulty. The only precaution is to use Quaker Oats that need to be cooked rather than the instant variety. The investigative exercise in the *Laboratory Guide for Biological Science* by Keeton *et al.* (1968. W.W. Norton & Co., Inc. pp. 24–27) contains some interesting ideas for student use.

Other Plants

Many plants can be obtained locally from wholesalers. In larger cities cycads, *Metasequoia,* and bonsai *Ginkgo* can be purchased. Carnivorous plants are usually easy to find. Cycads can be obtained from some nurseries. Walter J. Harmar of Louisiana State University suggested Singers Growing Things and California Jungle Gardens as suppliers of cycads in 1973. He said *Zamia floridana* can be obtained from Robert E. Brown and reminded me that the trunk is subterranean. His experience with Plant Buyer's Guide is similar to other lists of suppliers; the ones listing cycads either were out of business or out of stock. Pine seeds (pinon or pignolia nuts) can easily be found in local nut shops and are excellent for dissection.

Often biological supply companies are the least expensive source of materials. They are also the only source for many items. Carolina and perhaps a few other companies can supply living fern prothallia. Ward's is the only supplier of living *Psilotum* I know of.

Gro-Lux wide-spectrum fluorescent bulbs made by Sylvania are excellent for growing plants indoors. They have a wide spectrum so additional incandescent illumination is not needed as with other types of Gro-Lux bulbs.

The instructor's manual for *Botany Laboratory Manual* by Steiner *et al.* (1965 Holt, Rinehart and Winston, Inc.) contains many good ideas for utilizing living or fresh plant material.

Chromosomes

The large chromosomes of the broad bean, *Vicia faba,* are among the very best for study. If seeds are grown and the secondary root tips used, excellent preparations can be made at little cost. After the root tip is removed from Feulgen's stain about 1.0 mm of the meristematic zone (the part which takes up most of the stain and is just behind the root cap which does not take up much stain but is often lost in staining) should be excised and placed in a drop of 45% acetic acid. It should then be macerated (ground) with the ground edge of a #11 scalpel blade until it is broken into individual cells. A coverslip should be added and the preparation placed on a piece of filter paper. Any clumps of cells should be broken up by gently tapping with the end of the scalpel handle. This is important because the chromosomes need to be well flattened to see details. Most techniques use too much material. This produces clumps of cells and prevents flattening. Excellent results have been obtained with the above method.

Local Resources

In many cases local researchers or groups can supply very usable materials for free. Hospitals routinely take blood samples from patients who do not have infectious diseases, and this blood can be obtained the next day if it is not used for additional tests. It is one of the best sources for red blood cell

permeability studies. Examples of plaques to show viruses are usually easy to find. If glassware is a problem many disposable items can be obtained from hospitals. For several years I have obtained tissue cultures of rainbow trout gonad cells used by a colleague to demonstrate mitosis in living cells. Fresh yellow elastic connective tissue (back strap), white connective tissue (tendon), hyaline and fibro-cartilage (knee) and other tissues can easily be obtained from supermarkets. A little investigation of local resources can pay big dividends. Often many organisms can be collected locally.

Dissecting Kits

The basic dissecting kit used in introductory biology courses is often of poor quality and almost useless with delicate living material. Large college or university bookstores can purchase high-quality kits in quantity from wholesalers and will often mark them up 20%, as they do textbooks, instead of 50% or more. By having your bookstore solicit bids, an inexpensive kit can be made available to students. They don't think 15 to 20 dollars is inexpensive at first, but they soon discover the instruments are very useful. The stainless steel instruments made in Pakistan vary considerably in quality but good instruments can be obtained at relatively low cost. A new Dumont Style No. 5 watchmaker's forcep which is dipped in epoxy is now available from Germany at low cost and has high-quality tips. We have sold a completely stainless steel kit with a No. 3 scalpel handle, 4 each of #10 and #11 scalpel blades, sharp and blunt 5″ scissors, straight iris scissors, tissue forceps, #5 watchmaker's forceps, 4½″ curved forcep, Huber probe, 6″ ruler, 2 teasing needles, 5½″ artery forceps and a hard plastic case to students for about 15 U.S. dollars. The companies I have used in the past half dozen years are E.A. Instruments, Inc., Fine Science Tools, Ltd., Hamilton Bell Co., Inc., Indigo Instruments Ltd./LTEE., The Industrial & Scientific Instrument Company, and Ward's Natural Science Establishment, Inc.

Audiovisual Materials

Films

Generally time is too precious to use films in the laboratory, largely because so many films are inefficient. Often films show phenomena which can easily be seen in the laboratory, usually much more clearly. There are, however, some kinds of phenomena that can only be demonstrated with film. Because such a small portion of films produced are of high quality and useful at the undergraduate level the following annotated list may be of interest. These are films I use in my introductory zoology laboratories and represent the highest quality films of which I know. I have only looked at about 150

films, many on behavior, but for the areas involved these must be among the very best. If you have seen these films and know of better ones I would like to know about them.

The Behavior of the 3-Spined Stickleback—15 minutes, 16 mm, color, sound. Oxford Scientific Films Limited. About $300.00.

The best animal behavior film available. Shows normal behavior and experiments with releaser stimuli.

The Uganda Kob: Territoriality and Ceremonial Mating Behavior—20 minutes, 16 mm, color, sound. Helmit K. Buechner, National Zoological Park, Smithsonian Institution, Washington D.C. 20009. About $200.00.

An excellent film showing both the methods of study and behavior.

Secret in the Hive—30 minutes, 16 mm, color, sound. Eiji Murayama, Sakura Motion Picture Co., LTD., Tokyo. About $400.00.

Superb photography makes this the best film on the honey bee. Anthropomorphic narration and music make this a good contrast with the Uganda kob and stickleback films.

The Life Cycle of a Parasitic Flatworm—14 minutes, 16 mm, color, sound. Developmental Biology Film Program. About $200.00.

Demonstrates the complete life cycle of *Cyptocotyle lingua* with exquisite photography of living material.

Development and Metamorphosis of the Leopard Frog: Rana pipiens—25 minutes, 16 mm, color, sound. Developmental Biology Film Program. About $350.00.

The best film on frog development. Uses elegant photography and animation.

The Penetrating Eye—22 minutes, 16 mm, color, sound. Eli Lilly & Co., Indianapolis, Indiana 46206. About $60.00.

Explains the function and use of the scanning electron microscope in biology and compares it with the light and transmission electron microscopes. An excellent and enjoyable film.

Work of the Kidneys—20 minutes, 16 mm, color, sound. Encyclopaedia Britannica. About $350.00.

Using dyes living amphibian glomeruli and rat nephrons are studied. Animation is used to help explain how nephrons function. A dialysis patient talks about the purpose of the kidneys. The result is an excellent, relevant, human film.

The Physiology of Reproduction in the Rat—20 minutes, 16 mm, color, sound. Richard J. Blandau and A. Canedo, 1955, University of Washington. About $200.00.

This film is old but is the only film I can find that shows behavior, estrus cycle, vaginal smear, uterine peristalsis, egg transport and fertilization. Unfortunately the film does not show ovulation. It is shown beautifully in. . . .

Ovulation and Egg Transport in Mammals, Richard J. Blandau and R. Hayashi, 1973, 15 minutes, 16 mm, sound, color. About $150.00.

A film about human reproduction showing these events would be useful.

Film Loops

Like all other audiovisual materials, film loops should be viewed before they are purchased. The quality varies greatly, both in the production itself and in the print. You may have to return a poor print of a good presentation for a better print. All of the film loops listed below are silent, about 3 or 4 minutes in length, and super 8 in format. Unfortunately, loop films tend to fade and wear out with continuous use. If they are shown once at the beginning of a lab section they will last a few years. Often laboratories are not dark enough to show super 8 loop films on a large screen with good contrast and clarity. If the films are transferred to videotape the color can often be improved. A sound track can be added to fit the local context and sections of several films can be put together for a specific lab. Students can view the tape as many times as necessary. When the tape fades, a new copy can be made from the original film loop.

I have viewed several hundred film loops and the ones listed below are the ones I have found most useful. All the films listed use living materials. They are ones I have used in teaching introductory biology and are, consequently, quite general. Some areas, e.g., ecology, have not been examined at all. Film quality varies even when produced by the same individual. Distributors are listed in parentheses.

BSCS Single Topic Inquiry Films (Ward's, Hubbard):

The Feeding Mechanisms of Oyster Drills
Shows ingestion of food via proboscis after hole is drilled with radula.

Planarian Behavior
Demonstrates chemical transfer of learning.

The Behavior of a Purple Bacterium
Analysis of bacteria's response to light and dark.

Regeneration in Acetabularia
Repetition of Hammerling's classical experiments.

A Study of Frog Development
Complete development in four minutes.

Englemann's Inquiry into Photosynthesis
Repetition of classical experiment.

Mitosis
Demonstrates how mitosis is studied in *Haemanthus*

Flowering and Photoreception and Flowering
Hamner's classical experiments.

Phototropism
Classical experiments with auxins.

The Peppered Moth
Kettlewell's classical experiments on industrial melanism.

Oxford Scientific Films and Ealing Films (Ealing, BFA):

Plankton: Larval Forms
Shows living larva and adult forms of major animal phyla.

Hydra
Basic biology and response to glutathione.

Daphnia
Living anatomy and reproduction.

Courtship Behavior of the Stickleback
Same as 16 mm film but less complete.

Stickleback: Experiments with Models
Same as 16 mm film but less complete.

Fresh Water Sponge
Reproduction including planula larva and feeding.

Coelenterates
Basic marine forms and their biology.

Mollusks
Basic forms and their biology.

Segmented Worms
Basic forms and their biology.

Peripatus
Basic biology compared with annelids and arthropods.

Asterias
Basic functional anatomy.

Echinoderms
Basic forms and their biology.

Measuring Transpiration
Good substitute for potometer experiments.

Genetics of Sordaria
Good summary of methods and results.

Turtle Heart Neural Control: Experiment
Beautiful recordings of classical results.

DNA Transformation Experiment
Good summary of methods and results.

Mitosis: In Endosperm of Haemanthus katherinae
The best film of mitosis.

Fertilization and Cleavage
Sand dollar development to blastula.

Cytoplasmic Streaming in Plant Cells
Elodea, Nitella, Tradescantia and onion.

Fruit Ripening
Time-lapse of lemon, orange, pea, soybean and cotton.

Flowers and Trees: Bud to Blossom
Time-lapse photography.

Iowa State University Botany Films (Ealing, BFA, Ward's):

Gamete Transfer in the Bryophytes: The Splash Cup in a Moss
Moss life cycle includes gamete release.

Gamete Transfer in the Bryophytes: Splash Platform in Marchantia
Liverwort life cycle including gamete release.

Pollen Tube Growth
Demonstrates the tube nucleus and generative cell.

Algae Syngamy: Oogamy in Oedogonium
Antheridium, sperm release and fertilization in the oogonium.

Liberation of Zoospores: in the alga Basicladia
Dramatic release of zoospores.

Spore Dispersal in Equisetum
Hygroscopic movement of elaters.

Stomatal Opening and Closing
Osmotic experiments and model.

Harper and Row Films:

Mitosis in Animal Cells
Good for showing cytokinesis.

Gastrulation in the Sea Urchin
Blastula to dipleurula larva.

Reproduction in the Rat Part 2—Ovulation and Egg Transport
Living and animated views of ovulation plus living egg transport.

Brain Localization: the Motor Cortex
Electrical stimulation of the motor cortex including excision experiment.

Effects of Radiation on Chromosomes and Genes
Mainly good technique for preparing *Drosophila* salivary chromosomes.

Charts and Models

There are many charts and models available. They need to be examined before purchase. Often the terminology is out-of-date or too specific for class use. A chart listing 200 parts of the sheep brain doesn't help students find the midbrain.

Models are usually expensive and frequently quite different from the real thing. I prefer to use fresh material rather than a model, e.g., a fresh pig heart instead of a model. Models can be helpful in learning spatial relationships and the names of parts. Physicians receive models from pharmaceutical companies and will often give away extras.

Many charts contain drawings which are more artistic than real. The best example of excellence I know of is the Botany Series developed by Harriet B. Creighton for A.J. Nystrom and Company. These charts are large, clear and real. The new Johns Hopkins Human Anatomy Charts drawn by Leon Schlossberg and available from Carolina Biological appear to be among the best.

Hans Elias's drawings in the new 4th edition of *Histology and Human Microanatomy* (John Wiley and Sons, New York 1978) are examples of the finest biological illustration. These magnificent color stereograms should be published separately and in chart form.

A remarkable chart showing the relationship between organelles and biochemical pathways is available for free. The chart, entitled "Metabolic Pathways and Liver Organelles," as well as several other liver charts, can be obtained from Dr. Falk Gmb H & Co. and is in English.

Other Sources of Materials

Libraries often receive duplicate copies of journals. These are excellent sources of labeled electron micrographs and other illustrative materials. Sometimes outdated X-rays and X-ray motion pictures can be obtained from hospitals. Good X-ray motion pictures of swallowing, gastrointestinal motility, etc., would be very useful in teaching.

Several resources have been developed for medical education which may be useful to biologists, e.g., The Catalogue of Morphology Slides, Microfiche

and Teaching Sets (microfiche), American Society of Hematology, National Slide Bank, University of Washington, Seattle; or Medical Audiovisuals: A Comprehensive Catalog, compiled and edited by Robert S. Gresehover and Janice M. Goudreau, Baltimore, Md.: Johns Hopkins University. Members of the Health Sciences Communications Association (HeSCA) or the Biological Photographic Association probably have produced materials which would be useful to others. For example, there must be a near perfect set of photographs of rat anatomy somewhere. By improving communication and sharing these materials many of us would not have to try and find the time and money to duplicate others' efforts.

The Use of Living Materials and Vivisection

Many critics of the use of living materials feel students should understand basic biology before they use living materials. The majority of biology courses use preserved animals and no one complains. If it is ethical to kill a living organism so we can learn from it we must learn as much as we can, as efficiently as we can—involving as many students as possible without decreasing learning. Living phenomena are far more interesting and meaningful than studying the anatomy of a preserved organism. Certain kinds of analysis require preserved and prepared materials and these should be used when needed. When preserved materials are reusable they should be used instead of fresh preparations if the same amount of information can be learned from them. However, dissecting new preserved frogs every year is a poor way to learn. Students can learn so much more from a living or anesthetized frog. If a life is worth taking, students will develop much more respect for the organism if they understand its living qualities.

Often it is not the welfare of the animal that is the real concern of critics. They are frequently more concerned about their own discomfort during the killing of the animal. Much of this discomfort arises from their lack of understanding of physiology. If painless methods are used, particularly if students understand the process, they will treat the animal with more respect and learn more from it than if someone kills the animal for them.

By using demonstrations killing can be reduced to the minimum. Often organisms can be obtained from sources where they would be killed inhumanely and wasted, e.g., cockerels or unproductive hens killed by hatcheries.

There is no doubt that animals are treated inhumanely by students in classrooms. The solution, however, is not to have them use only preserved animals which are inhumanely killed by someone else. Rather as few animals as necessary should be sacrificed so that students can understand and appreciate the amazing qualities of life.

Addresses of Suppliers

American Type Culture Collection
12301 Parklawn Drive
Rockville, Maryland 20852
Catalog with media and organisms

Aquarium Systems, Inc.
33208 Lakeland Blvd.
Eastlake, Ohio 44094
Marine aquarium materials and literature

Balston, Inc.
P.O. Box C
703 Massachusetts Avenue
Lexington, Massachusetts 02173
Compressed air filters

BFA Educational Media
2211 Michigan Avenue
Santa Monica, California 90404
Film loops

Robert E. Brown
2008 Carr Street
Palatka, Florida 32077
Zamia floridana

Dr. Helmit K. Buechner
National Zoological Park
Smithsonian Institution
Washington, D.C. 20009
Uganda Kob film

California Jungle Gardens
11977 San Vicente Blvd.
Los Angeles, California 90049
Cycads

Carolina Biological Supply Company
Burlington, North Carolina 27215
Culture dishes, many other materials

Cistron Corporation
27th and Cumberland Streets
Lebanon, Pennsylvania 10742
500 Mesh filtering pans

College Biological Supply Company
21707 Bothwell Way
Bothwell, Washington 98011
Ammocoetes larvae

Connecticut Valley Biological Supply Co. Inc.
Southampton, Massachusetts 01073
Protozoa media

Robert Conroy Live Bait
37 Gladstone Avenue
Toronto, Ontario M6J 3K7
Earthworms

Developmental Biology Film Program
Education Development Center
55 Chapel Street
Newton, Massachusetts 02160

E.A. Instruments, Inc.
P.O. Box 673
Woodstock, Illinois 60098
Dissecting instruments

The Ealing Corporation
2225 Massachusetts Avenue
Cambridge, Massachusetts 02140
Film loops

Encyclopaedia Britannica
Educational Corporation
425 North Michigan Avenue
Chicago, Illinois 60611
Films

Dr. Falk Gmb H & Co.
Pharmaz. Präparte KG
D–78 Freiburg/Br.
Habsburgerstr. 79–81
W. Germany
Liver charts

Fine Science Tools Ltd.
1687 Arborlynn Drive
North Vancouver, B.C. V7J 2V7
Dissecting instruments

Gulf Specimen Company Inc.
P.O. Box 237
Panacea, Florida 32346
Marine specimens

Hamilton Bell Co., Inc.
30 Craig Road
Monvale, New Jersey 07645
Dissecting instruments

Harper & Row, Publishers, Inc.
2350 Virginia Avenue
Hagerstown, Maryland 21740
Film loops

Hubbard
P.O. Box 104
1946 Raymond Drive
Northbrook, Illinois 60062
Film loops

Indigo Instruments Ltd./LTEE
P.O. Box 425, Port Credit
Ontario, Canada L5G 4L9
Dissecting instruments

The Industrial & Scientific Inst. Co.
Div. of Educational Modules, Inc.
1665 Buffalo Rd.
Rochester, NY 14624

Kent Laboratories, Ltd.
1292 Franklin Street
Vancouver, British Columbia
Canada V6A 1K1
Tricaine methane sulfonate

The Wm. A. Lemberger Associates Ltd.
P.O. Box 222
Germantown, Wisconsin 53022
Frogs

Eli Lilly and Company
P.O. Box 618
Indianapolis, Indiana 46206
Scanning EM film

Marine Fish Imports
130½–132 W. Gardena Blvd.
Gardena, California 90248
Marine organisms

Marine Specimens Unlimited
Route 4, Box 879
Summerland Key, Florida 33302
Sea urchins

Monterey Bay Hydroculture Farms
844 17th Avenue
Santa Cruz, California 95062
Crayfish

Nasco
901 Hanesville Avenue
Fort Atkinson, Wisconsin 53538
Xenopus, plastic aquaria

Nebraska Scientific
3710 D Street
Omaha, Nebraska 68107
Fresh organs

Nektonics Research and Development Ltd.
1015 East 35th Street
Brooklyn, New York 11210
Marine aquarium equipment

John C. Noyes
P.O. Box 212
Fort Lauderdale, Florida 33302
Marine specimens

A.J. Nystrom & Co.
3333 Elston Avenue
Chicago, Illinois 60618
Botany charts

Oxford Scientific Films Ltd.
Botley Road
Oxford OX2 OHE England

Pacific Marine Imports
5420 West 104th Street
Los Angeles, California 90045
Marine organisms

Dennis Reynolds
221 28th Street
Hermosa Beach, California 90254
Marine specimens

Sakura Motion Picture Co., Ltd.
Tokyo, Japan
Honey bee film: Secret in the Hive

Salt and Sea Enterprises, Inc.
927 West Manchester Blvd.
Inglewood, California 90301
Marine organisms

Selph's Cricket Ranch, Inc.
Box 2123, Desoto Station
Memphis, Tennessee 38102
Insect culture equipment

Singer's Growing Things
P.O. Box 1482
Reseda, California 91335
Cycads

Walter Smith
615 30th Street
Hermosa Beach, California 90254

Instruction Media Services
Kane Hall
University of Washington
Seattle, Washington 98195

Verilux, Inc.
35 Mason Street
Greenwich, Connecticut 06830
Daylight fluorescent bulbs

Ward's Natural Science Establishment, Inc.
P.O. Box 1712
Rochester, New York 14603
Psilotum, film loops

Chapter 2

Use of *Nitella* in Biology Laboratory Classrooms

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Dr. Barr received his B.S. degree from Iowa State College and his Ph.D. degree from University of California at Berkeley in the area of plant physiology. He obtained postdoctoral training with electrophysiology of plant cells in the Biophysics Department at the University of Maryland Medical School. He was an assistant professor at Duquesne University from 1964–68. His current position is professor of biology and he has been on the faculty at the State University College at Brockport since 1968. Professor Barr teaches general biology and general and cell physiology. His research interests include studies of the membrane biology of *Nitella*.

Introduction

The internodal cells of the characean genera *Nitella* and *Chara* serve as excellent materials for simple demonstrations in biology laboratory classrooms. They can also be used for more detailed exercises in advanced biology courses. Because of their large size (several cm long and approaching one mm in diameter) these algal cells can be readily isolated from the intact plant, which resembles the common horsetail in its general form. Two of the striking features of internodal cells are (a) their ability to secrete acid and base in alternating bands along the cell length, and (b) the very smooth and rapid protoplasmic streaming. The basic procedures for setting up these demonstrations are described below, along with a method which has recently been developed for culturing *Nitella*. *Nitella* and *Chara* can also be collected from local ponds and lakes. In most species of *Chara* the internodal cells are corticated, i.e., have a jacket of small cylindrical cells surrounding them, and therefore are less suitable for these demonstrations.

If a reliable source of *Nitella* is desired, it is best to culture it in the laboratory since the material obtained from biological supply houses does not ship very well. The time invested in culturing will greatly exceed the one or two hours required to set up the above demonstrations. There are, however, some "fringe benefits" which derive from this effort: the open *Nitella* cultures often contain protozoans, small crustaceans, midge fly larvae, small flatworms, and snails. Harmony seems to prevail until the snails grow beyond a certain size and begin devouring the *Nitella*. These can be selectively removed.

Our original stock of *Nitella clavata* was collected in Llano Co., Texas by Dr. Vernon Proctor of Texas Technological College; we thank him for this.

The time required for a student to observe these demonstrations is very short, ordinarily not exceeding a few minutes. However, there is room for much flexibility here, depending on how intensively the phenomena are to be studied. Adequate discussions of acid-base formation and protoplasmic streaming are certainly not going to be easy for the instructor, but the simple beauty of these processes may entice the students into an interest in the underlying complexity. Some guidelines are provided in the annotated bibliography below.

Culturing of *Nitella clavata*

The plants can be propagated vegetatively in open cultures with aeration under cool-white fluorescent illumination of about 500 foot-candles (surface of culture about 50 cm below two 40-watt tubes). A 16-hour photoperiod is suitable. Growth occurs within the temperature range 10–30°C; above 30°C dying off may occur. Ordinary fish tanks can be used as containers. In nature *Nitella* and *Chara* are rooted at the base via rhizoids, but with our culturing

method there is no soil present and no rhizoids develop. Large ornate oospores typically are formed within two weeks if the medium is not renewed, presumably because of the depletion of one or more of the essential elements.

The culture solution, described below, should be renewed each week for dense cultures (average distance between strands less than 2 cm), less often for sparse ones. The plants can be exposed to air for a few minutes when the old culture solution is siphoned out during the renewal process. The solution is a modification of Forsberg's (1965) for *Chara*. Its suitability for other members of the *Characeae* has not been tested.

Culture Solution for *Nitella clavata*

Concentration	*Material*
0.4 mM	KNO_3
1.0 mM	$MgSO_4$
1.0 mM	$NaHCO_3$
1.0 mM	NaCl
1.5 mM	$CaCl_2$
10.0 μM	NaH_2PO_4
10.0 μM	Na_2MoO_4
3.7 μM	H_3BO_3
6.5 ppm	streptomycin sulfate (Sigma Chemical Co.)
1 ml per liter	1000-strength III 59 (see Appendix A)
1 ml per liter	1000-strength IV 76.5 (see Appendix B)

Notes:
1. Stock solutions of each of the materials to be added can be prepared by making them 1000 times the concentration present in the final culture and using 1 ml per liter. For further details see Appendix E.
2. The pH of the culture solution is adjusted to 7.5 ± 0.2 with 1 M HCl if necessary.
3. Faster growth occurs if 0.3 mM "Tris" buffer is present; a 0.3 M stock solution is prepared by neutralizing "Tris" base with 1 M HCl.

Harvesting and Maintenance of Isolated Cells

Nitella plants are laid horizontally in a large tray, immersed in the culture solution. An internodal cell is easily isolated from a strand by cutting away the two adjoining internodal cells and the branch cells at the nodes with a fine dissecting scissors. The isolated cells can then be transferred to a culture

dish containing 100 to 200 ml of a suitable solution. We use a minimal solution designated as Kb (see Appendix C) under the assumption that the cells will have a greater longevity if the rate of metabolism is low. The cells survive for more than a month in this solution when exposed continuously to 50 foot-candles of cool-white illumination. If the solution is renewed every 2 or 3 days, little contamination by microscopic algae occurs.

The transferring of internodal cells from one container to another should be done gently with a fine dissecting forceps, the tips of which have been covered with fine polyethylene tubing ("Intramedic," obtainable at Fisher Scientific and other companies); the tubing should extend slightly beyond the metal tips so that a soft grasp is possible.

Demonstration of External Acid and Base Formation

The formation of acid and base on the external surface of *Nitella* cells in alternating bands is promoted by light. The acid and base are readily detected through use of the pH indicator dye phenol red (Fig. 2.1). Two internodal cells are placed in a 60-mm petri dish top containing 25 ml of Kb solution plus 0.1 mM K salt of phenol red, pH 6.9 (see Appendix D). At pH 6.9 the solution color is orange; at lower pH it is yellow and at higher pH it is a deep red.

The recommended lighting is 150 foot-candles of cool-white; this is based on Lucas's (1975) finding that an intensity of 5 Wm^{-2} was saturating for OH^- efflux in *Chara corallina*. After 5 or 10 minutes of bright light the acid and alkaline banding pattern should be quite evident. The solution can then be gently stirred to once again render it uniformly orange, and the process may be repeated. A surge of acid formation generally occurs shortly after the cells are placed in darkness. White paper beneath the dish provides a suitable background.

The OH^- and H^+ effluxes observed in this way are large fluxes related to the general regulation of ionic traffic through the membrane (see the annotated bibliography). In nature calcium carbonate often precipitates on the cell surface in the alkaline zones; the $CaCO_3$ bands are called incrustations.

Demonstration of Protoplasmic Streaming

One or two internodal cells are placed in a 60-cm petri dish bottom with enough Kb solution to cover them. The dish is placed on the stage of a monocular microscope and the cells observed with the 10X objective. An alternative is to use a greater depth of solution and immerse the objective (a 20X objective is also suitable). The microscope should be focussed on the

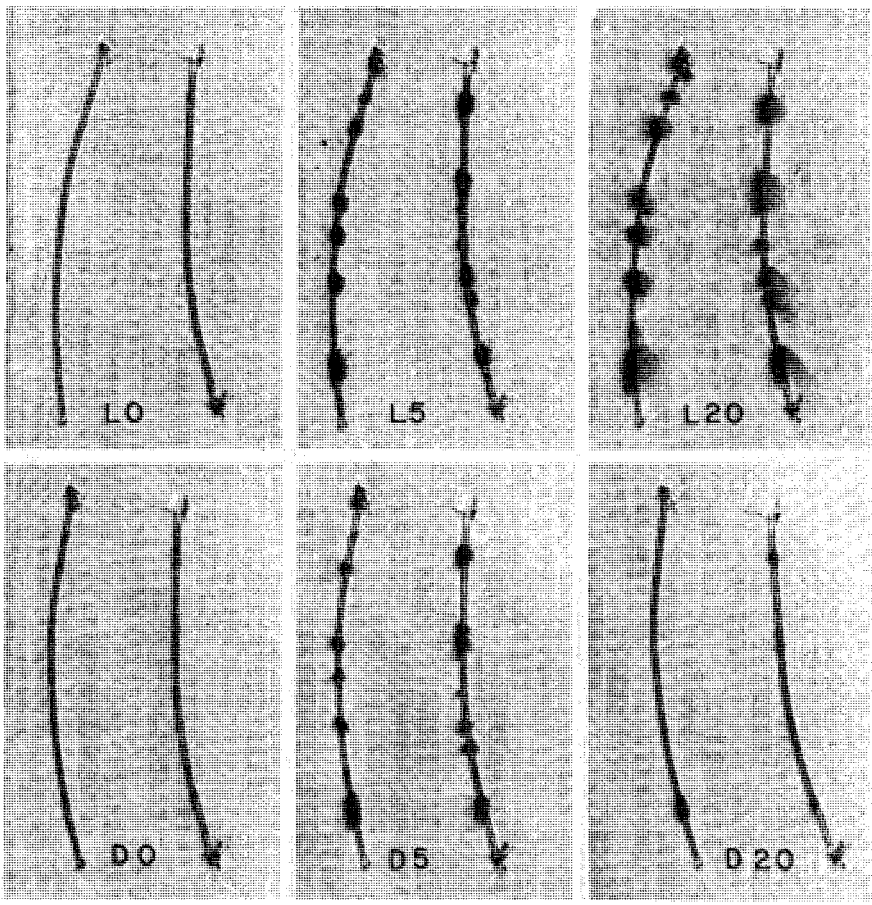

Figure 2.1. Accumulation of base (dark areas) and acid (light areas) on the external surface of two internodal cells of *Nitella clavata*. Upper sequence: cumulative amounts of base and acid produced in light after zero, 5, and 20 minutes following 20 minutes of dark pretreatment. Lower sequence: decreasing rates of base and acid formation in darkness immediately following the 20-minute exposure to light above; each picture shows the amounts of base and acid formed during the 5-minute interval preceding the picture. The pH indicator dye phenol red was used for detection of base and acid (see text for solution composition and lighting). Reproduced by permission of the *Journal of General Physiology*.

stationary chloroplast layer; the streaming endoplasm is just beneath this and is still within the focal depth. The smooth movement of particles and loose chloroplasts in the endoplasm can easily be seen. The streaming endoplasm is in the form of a continuous band which proceeds along the length of the cell on one side and doubles back on the other.

To demonstrate that either metabolism or a viscosity change, or both, are involved in the slowing down of the streaming rate upon cooling, a small piece of ice may be added to the dish. The temperature dependence is quite pronounced. It is possible to measure the velocity of streaming if a calibrated eyepiece micrometer and a stopwatch are available.

A sudden cessation of streaming usually means that the cell has received a mechanical shock strong enough to trigger an action potential. At room temperature, about 10 minutes may be required before the normal rate of streaming resumes following an action potential.

Use of *Nitella* in Membrane Physiology Exercises

Only brief mention will be made of two types of exercises for which internodal cells are especially suited, because of their large size. These are (a) measurement of resting membrane potentials and action potentials, with intracellular microelectrodes; (b) measurement of ion fluxes by means of radioactive tracers. Such exercises are quite complex both theoretically and in their practical details and require specialized equipment. It is essential for the instructor to have acquired a good working knowledge of the methodology and to have tested out all the practical aspects of the measurements. A summer spent with an expert in this discipline is highly recommended. These exercises should be limited to students with adequate backgrounds in physics and chemistry.

References

A. General

Hope, A.B.; Walker, N.A. The physiology of giant algal cells. London: Cambridge University Press; 1975.

This book concentrates on the biophysical aspects of giant cells, especially *Chara* and *Nitella*. Most of the coverage is devoted to membrane phenomena including acid and base formation. There is a chapter on protoplasmic streaming.

B. Acid-Base Formation

Lucas, W.J. The influence of light intensity on the activation and operation of the hydroxyl efflux system of *Chara corallina*. J. Exp. Botany 26:347–360; 1975.

The light-saturation of the OH^- efflux occurs at about 5 Wm^{-2}, equivalent to about 150 foot-candles of cool-white fluorescent illumination.

Lucas, W.J. Plasmalemma transport of HCO_3^- and OH^- in *Chara corallina*: non-antiporter systems. J. Exp. Botany 27: 19–31; 1976.

This work indicates that although there is a 1:1 stoichiometry of OH^- efflux and HCO_3^- influx, the two transport processes can occur in different regions of the cell surface. Thus, a common exchange mechanism is not responsible for the observed ratio. This suggests that other anions may also be absorbed via their own mechanisms.

Ryan, T.E.; Barr, C.E.; Zorn, J.P. Potassium transference in *Nitella*. J. Gen. Physiol. 72:203–218; 1978.

The possible relationship of K^+ uptake to H^+ extrusion is discussed. This appears to be more complex than the simple 1:1 stoichiometry observed between HCO_3^- uptake and OH^- efflux. Taken together with Lucas's work, the versatility of characean cells in taking up essential cations and anions as made possible by the equivalent release of H^+ and OH^-, respectively, is indicated. This is a concept that is currently the subject of active investigation.

Spear, D.J.; Barr, J.K.; Barr, C.E. Localization of hydrogen ion and chloride ion fluxes in *Nitella*. J. Gen. Physiol. 54:397–414; 1969.

This is the initial work on acid-base formation by characean cells and contains black and white photographs demonstrating the process in light.

C. Protoplasmic Streaming

Kamiya, N. Physics and chemistry of protoplasmic streaming. Ann. Rev. Plant Physiol. 11:323–340; 1960.

This is a comprehensive review containing information on the absolute value of the motive force of streaming, and discussion of the possible mechanisms involved in its generation.

Nagai, R.; Hayama, T. Ultrastructure of the endoplasmic factor responsible for cytoplasmic streaming in *Chara* internodal cells. J. Cell Sci. 36:121–136; 1979.

This report brings up to date the current ideas on the mechanism of protoplasmic streaming, especially in terms of an actin-myosin type of interaction. The roles of ATP and Ca^{++} are discussed.

D. Culturing of *Nitella* and *Chara*

Forsberg, C. Nutritional studies of *Chara* in axenic cultures. Physiol. Plant. 18:275–290; 1965.

Nitella clavata will not grow in the Forsberg medium but will grow in the modified version of it described in this article.

APPENDIX A
1000-Strength III 59

To make one liter of the above solution:

1. To 600 ml distilled water in a liter beaker add 1.89 g $FeCl_3 \cdot 6H_2O$ and 19.1 g nitrilotriacetic acid. While stirring magnetically, add enough 1.0 M NaOH to bring the pH to 7.0 after all materials have been dissolved. About 300 ml of NaOH solution are required.
2. Add to the above 50 ml of a solution made by dissolving 8.80 g $ZnSO_4 \cdot 7H_2O$, 0.142 g $MnCl_2 \cdot 4H_2O$, 0.162 g $CoCl_2 \cdot 6H_2O$ and 0.198 g $CuSO_4$ (anhydrous) in one liter of distilled water. Adjust the pH of this solution to 4.5 with HCl.

3. Adjust the final pH to 7.0 with the NaOH solution.
4. Bring the volume to 1.0 liter. Keep refrigerated.

APPENDIX B
1000-Strength IV 76.5

To make one liter of the above solution:

1. Add to 400 ml distilled water in a liter beaker 4.78 g nitrilotriacetic acid and 5.0 g humic acid (Aldrich Ghemical Co., Cat. No. 1675–2).
2. While the above is stirring magnetically, add enough 1.0 M NaOH to bring the pH to 9.3. About 75 ml of the NaOH solution are required.
3. Boil for one hour with magnetic stirring. A watch glass is used to cover the beaker to prevent excessive evaporation.
4. Allow to cool overnight.
5. Centrifuge to remove the particulate matter. Because of the large volume, 50-ml plastic centrifuge tubes are recommended with exactly 40 ml placed in each tube.
6. Collect all of the supernatant and bring the volume to 1.0 liter. The final pH is about 8.9. Refrigerate.

APPENDIX C
Kb Solution

Concentration	*Material*
1.0 mM	KCl
0.1 mM	NaCl
0.1 mM	$CaCl_2$
0.1 mM	$MgCl_2$
0.1 mM	$KHCO_3$

This solution is most conveniently made from two stock solutions, each 100-strength:

(a) a solution containing 100 mM KCl plus 10 mM each of NaCl, $CaCl_2$, and $MgCl_2$.
(b) a 10 mM $KHCO_3$ solution.

Add 10 ml of (a) and 10 ml of (b) to 980 ml distilled H_2O. Adjust to pH 6.0 with 1 mM HCl.

APPENDIX D
Stock Solution of Phenol Red

A 100-strength stock solution of the potassium salt of phenol red, pH 6.9, is made by neutralizing 1 millimole of phenol red (0.354 g) in 80 ml H_2O with KOH. If 0.1 M KOH is used, the volume required to bring the pH to 6.9 is less than 10 ml. The final volume is adjusted to 100 ml.

For the acid-base exercise, 1.0 ml of this stock solution is added to 100 ml of Kb solution.

APPENDIX E

1000-Strength Stock Solutions Used in Preparing Culture Solution
(Each solution made separately and kept in its own container)

Concentration of Stock Solution	*Material*	*Grams per Liter*
0.4 M	KNO_3	40.4
1.0 M	$MgSO_4$(anhydr.)	120.4
1.0 M	$NaHCO_3$(anhydr.)	84.0
1.0 M	NaCl	58.4
1.5 M	$CaCl_2 \cdot 2H_2O$	220.5
0.01 M	$NaH_2PO_4 \cdot H_2O$	1.38
0.01 M	$Na_2MoO_4 \cdot 2H_2O$	2.42
0.0037 M	H_3BO_3	0.23
6500 ppm	streptomycin sulfate (Sigma Chemical Co.)	6.5

Note: Add 1 ml of each of the above solutions to 990 ml of distilled water to make one liter of culture solution. Besides these 1 ml each of the 1000-strength III 59 and IV 76.5 stock solutions are to be added (See Appendices A and B for the preparation of these).

Chapter 3

Structural Organization of Living Cells

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Introduction

Students tend to visualize cells as static entities based on their experience with textbook photographs and microscope slides. By focusing on living eukaryotic cells, this laboratory demonstrates that cells are dynamic units and that movement and change are intrinsic properties of life.

Most of the unique features of the eukaryotic cell are visible in the light microscope. A number of cell organelles are readily observed and can be identified with a fair degree of confidence. In addition to examining and identifying cell organelles and cell structures, students are introduced to the techniques and concepts of microscopy and cytochemistry.

Living cells other than heavily pigmented plant cells are best visualized with a phase microscope. Phase microscopy gives extraordinary detail to cells by taking advantage of the small differences in refractive indices and thicknesses of various cell parts. Pleomorphic changes of mitochondria, cyclosis, and ciliar movement are spectacularly demonstrated.

The exercise was designed for second-year biology majors. The material can be modified for freshman-level courses by excluding some stains and reducing the number of specimens examined. The instructor might also eliminate the student use of the phase and polarizing microscope and retain these portions as demonstrations only.

Times involved:

Instructor:	4 hours reagent preparation
	2 hours purchase of fresh materials
Student:	6–8 hours (two laboratory sessions)

Student Materials

Background Information

The cells of all higher plants and animals are eukaryotic cells characterized by membrane-bounded nuclei and various membrane-constructed organelles which are lacking in prokaryotic cells. Most of the unique features of the eukaryotic cells are visible in the light microscope.

Certain cell organelles can be identified with certainty in unstained preparations. Chloroplasts are easily visualized with the light microscope without stains or other special preparation. Other organelles are not so easily identified, and for observing these a variety of techniques are used. Chemicals that stain living cells are known as vital stains. Organelles such as vacuoles can be made visible with a vital stain because of their functional tendency to take in water-soluble materials for storage. An aqueous solution of neutral red becomes concentrated in the vacuoles, making them visible. Use of the vital stain Janus

green B takes advantage of the functional activity of another cell organelle. The mitochondrion is the site of major reactions of biological oxidation and the electron transport system. When aqueous solutions of the blue-oxidized Janus green B diffuse into the cell, it is reduced in the cytoplasm to colorless compound and is locally re-oxidized in the mitochondria to the blue form.

Procedures

I. Observations of unstained cells

A. *Cell Surface*

Cell Membrane

Examine one or both of the following preferably with a phase microscope: amoeba or buccal cells. To examine the amoeba, place a drop of culture, taken from the bottom of the dish, on a clean slide. Place a piece of broken cover slip beside the drop and add a cover slip. Scan the slide with the microscope while reducing the light level slightly. When an amoeba is found, watch it for a time to see the membrane motion and changes that occur in the membrane and cytoplasm as the amoeba moves. Buccal cells are prepared by gently scraping the inside of the mouth with the flat end of a clean toothpick. Place the cells in a drop of 0.9% saline solution on a glass slide. Add a cover slip. How does the cell membrane differ from that of the amoeba? The nucleus and mitochondria should be visible.

Primary Cell Wall

With fine forceps remove a piece of epidermis from the purple underside of a leaf of *Rhoeo discolor* and place the tissue in a drop of water. Add a cover slip and observe with the microscope. Place a few drops of 10% sucrose at the edge of the cover slip and observe as the solution diffuses through the preparation. As the cell plasmolyzes, the cell wall will become distinguishable from the cell-membrane-bounded protoplast. If *Rhoeo* is not available, onion inner bulb scale epidermal peel may be substituted, but the onion tissue does not have the advantage of the cytoplasmic pigment found in *Rhoeo.*

Secondary Cell Wall

Make a thin section of *Coleus* stem and mount on a microscope slide. Under the microscope look at the vascular tissue to find cells with secondary thickenings of the cell wall. Note that *Coleus* also has some cells with cytoplasmic pigment.

Cilia

Ciliated protozoans generally move too fast for easy observation of cilia, so slow the organism by placing a drop of 1.5% methyl cellulose together with a drop of protozoan culture on a microscope slide. Add a cover slip before viewing with the microscope.

B. *The Cell Matrix and its Movement*

With fine forceps remove a stamen from a flower of either *Tradescantia* or *Rhoeo discolor*, place it in a drop of water on a slide, and add a cover slip. Streaming cytoplasmic strands should be visible in the cells of the staminal hairs. Observe with a phase microscope if available.

C. *Cell Organelles*

Nucleus

The nucleus is readily observed in onion bulb scale epidermis or in buccal cells. Use the slide of buccal cells prepared earlier in this exercise or prepare a slide of onion in the following manner. Cut a small triangle in the side of an onion bulb, pressing deeply enough to penetrate two or three layers of bulb scales. Remove the triangle and peel the epidermis from the inner bulb scale (see Figures 3.1 and 3.2). Place the epidermis in water on a glass slide. Add a cover slip. View the preparation with the phase microscope.

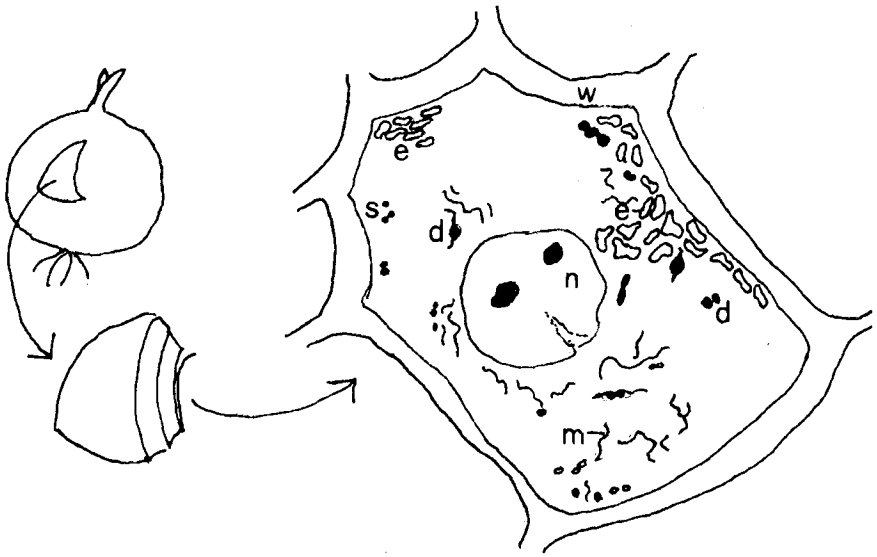

Figure 3.1. Diagram showing a cell from onion inner bulb scale epidermis as seen with phase optics. Active cytoplasmic streaming will be noted in specimens. Prominent features include the nucleus (n), nucleoli, cell wall (w), numerous droplets (d), spherosomes (s), mitochondria and vesicles (e) tentatively identified as smooth endoplasmic reticulum.

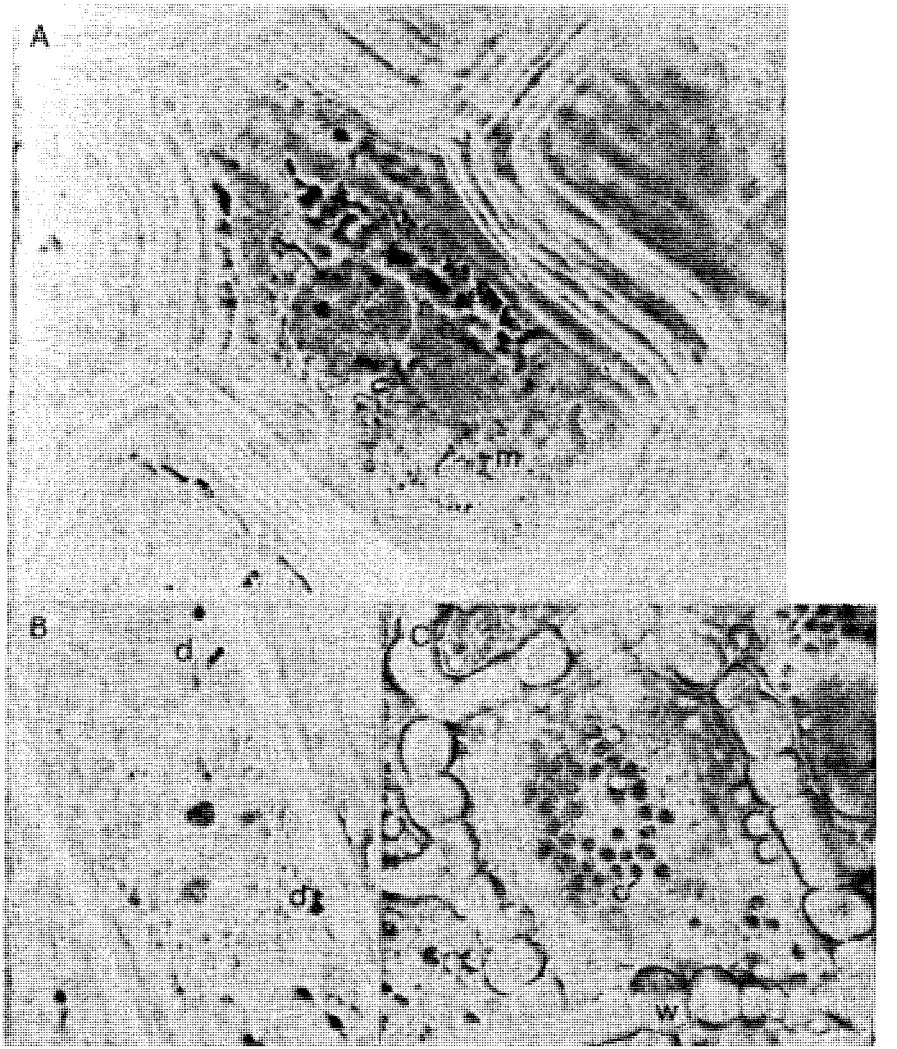

Figure 3.2. Cell organelles as seen with the phase microscope. At top: onion epidermal cell with numerous mitochondria (m). A large vacuole in the cell restricts the cytoplasm to the space between the cell wall and the vacuole. Area to the right above the nucleus contains vesicular elements suggestive of smooth endoplasmic reticulum (3). Bottom left: onion epidermal cell with large droplet structure (d) associated with mitochondria and vesicular structures. Bottom right: chromoplasts (c) of red pepper epidermis. Note distinctive cell wall (w).

Mitochondria

The onion bulb scale epidermal is one of the easiest cells in which to observe mitochondria. An epidermal peel of celery may also be used. Do the mitochondria move or change shape?

Chloroplasts

Examine one or more of the following: *Elodea,* spinach petiole epidermal peel, *Euglena* or *Spirogyra.* Prepare the spinach petiole epidermal peel by stripping a piece of epidermis from the stem or petiole of a piece of spinach. Mount in water.

Chromoplasts

Examine one or more of the following mounted in water: carrot, tomato, red pepper. Prepare the carrot by making as thin a section as possible and mounting the section on a slide in water. With tomato and red pepper, either pulp or epidermis can be examined (see Figure 3.2).

Endoplasmic reticulum

Examine the onion bulb epidermal peel with a phase microscope. You should see a number of different structures. Can you distinguish between the nucleus, mitochondria, vacuole, and smooth endoplasmic reticulum (Fig. 3.1)?

D. *Cell Communication*

Make thin cross-sections of one or both of the following, mount in water, and look for evidence of plasmodesmata: boiled date pits, cotyledons of growing seedlings.

E. *Cell inclusions*

Make a thin section of one of the following or mount a whole leaf of *Lemna* frond (duck weed): rhubarb stem, wax begonia petiole, *Aloe* leaf. Look for crystalline inclusions. Use polarizing lenses if they are available.

II. Cytochemical Identification of Cell Organelles and Molecules

A. *Identification of Vacuoles in Plant Cells with Neutral Red*

Place a few drops of 1% neutral red on a depression plate. Remove a root from an onion bulb and place the tip of the root in the neutral red. Crush the root gently with the rounded tip of a glass rod. Allow the root tip to remain in the solution 10–15 minutes. Carefully remove the root tip and mount it on a glass slide with a cover slip and a drop of the neutral red solution. Examine the slide and note the variation in shape of the vacuoles in the various regions of the root tip (root cap, meristem, region of elongation, region of maturation).

Sketch typical cells from the various regions of the root tip, noting in particular the relative size and shape of the cells and the difference in number, size, and shape of the vacuoles.

B. *Mitochondrial Identification with Janus green B*
Place a few drops of the working solution of Janus green B on a depression plate and float epidermal peels of celery stalk and the inner bulb scale of onion, peel-side down, on the solution for 5 minutes. Remove the peel and mount it on a slide with a cover slip and drop of stain. Examine the slide and sketch typical cells from the stained tissue, noting as much cellular detail as possible.

Can you see structures in either of the plant tissues that look like mitochondria?

Are any of the structures blue, indicating the localized oxidation of the stain by the mitochondria?

C. *Identification of Spherosomes with the Lipid Stain Sudan III*
Spherosomes are associated with lipid metabolism, and they tend to stain with Sudan III. Place drops of Sudan III in a depression of the depression plate and float epidermal peels of onion inner bulb scale epidermis, *Campanula* leaf, etc., on the stain for 5–10 minutes. Make a wet mount and observe with the microscope.

Have any structures absorbed the stain?
What color are they?
Do you think you have identified spherosomes?

D. *Cytochemical identification of cell molecules* (see Table 3.1).

1. Starch identification with KI_3
Make water mounts of any of the following materials: potatoes, ground rice seeds, corn starch, plant stems.

Observe thin cross-sections of the plant materials mounted in water with the microscope. If available, observe with a microscope fitted with polarizing lenses.

Note the characteristic pattern of the starch grains as seen with polarized light. The source of the starch can sometimes be determined based on its grain pattern in polarized light.

After observing the slide wih polarized light, view with ordinary transmitted light and add a drop of KI_3. The starch grains gradually darken and will stain red to blue to black, with the stain tending to darken with time.

2. Glycogen identification with KI_3
KI_3 can be used to identify other polysaccharides. Glycogen, the storage form of glucose in most animals and some protozoa, stains brown to violet in KI_3. Make a water mount of paramecia. After observing the unstained preparation, add a drop of KI_3 to the slide.

Table 3.1. *Chemical Composition of Cell Molecules Tested for in This Exercise*

starch	a carbohydrate—a polymer of glucose indicated by the formula $(C_6H_{10}O_5)_n$
glycogen	glucose polymer, storage form of glucose in animals
pectin	pectins are polymers of galactose, arabinose and galactouronic acid
lipids	fatty acids and esters of fatty acids, with glycerol
cutin and suberin	wax-like materials, highly complex fatty substances present in plants as an impregnation of epidermal walls. Makes the walls more or less impervious to water.
tannins	general term for a heterogous group of phenol derivatives
lignin	an organic substance or mixture of substances of high carbon content but distinct from carbohydrates; associated with cellulose in the walls of many cells
cellulose	a polysaccharide which is the chief component of cell wall of plants. Consists of long chain-like molecules the basic units of which are anhydrous glucose residues of the formula $C_6H_{10}O_5$
proteins	organic substances of high molecular weight formed by a number of amino acids united by a peptide bond

What visible change, if any, occurs with the addition of KI_3?

Does the glycogen appear to concentrate in vacuoles or is it dispersed in the cytoplasm?

3. Cellulose and lignin identification with the KI_3-sulfuric acid method and by zinc-chlor-iodide (Zn-Cl-I) reagent.

   Make thin cross-sections of the materials provided and place them in a depression plate containing either KI_3 or Zn-Cl-I reagents for about 15 minutes. Take a section that has been soaked in KI_3, blot off the excess, and place on a slide. Carefully add a drop of 65% H_2SO_4 and a cover slip. Cellulose will stain dark blue while other components of the cell wall such as lignin, etc., will appear orange or yellow, and pectin in the middle lamella, green. After examining each of the specimens soaked in KI_3, make mounts of the materials treated with Zn-Cl-I. Cellulose will appear blue while other components of the cell wall will appear orange or yellow. Components other than cellulose will sometimes block or interfere with the cellulose reaction of Zn-Cl-I. With other cross-sections make water mounts and compare these unstained preparations with the KI_3 and Zn-Cl-I-stained materials.

Do you get the same result with both stains?
Which method do you think is the more specific?

4. Lignin identification with phloroglucinol.

   Add phloroglucinol to a depression slide and float some thin sections of pear (fleshy part of fruit) on top for at least 10 minutes. Mount the material on a slide and examine. Lignin will appear reddish to violet.

   Can you identify lignin based on this reaction?
   Where does the lignin appear to be concentrated?

5. Pectin identification with ruthenium red.

   Place thin sections of *Vicia* roots, *Coleus* stems or apple (fleshy part of fruit) in ruthenium red in a depression plate for 10 minutes. Mount the material on a slide and examine. Pectin will appear pink to deep red.

   Where is the pectin located?

6. Cutin and suberin identification with Sudan IV.

   Examine water-mounted, thin cross-section of *Rhododendron* or other waxy-surfaced leaves.

   Remove the water; then add Sudan IV. Cutin and suberin will appear red.

7. Tannin identification with copper acetate.

   Examine water-mounted, thin sections of apple, potato, and eggplant. Remove water and add copper acetate. Tannins will appear dark brown.

   Can you identify the tannins in the cells?

8. Protein identification with Millon reagent.

   Examine a water mount of *Tetrahymena* or other protozoans; then add Millon's reagent. Protein containing tyrosine will form a red precipitate.

   Be careful in handling Millon's reagent. It is a toxic material containing mercury and nitric acid.

   Can you identify protein in the cells of *Tetrahymena?*

Instructor's Materials

Introduction

The success of the laboratory is dependent upon the proper use of the microscope and upon careful preparation of slides. It is important that students be given adequate instruction in the use of the compound microscope. When the instrument does not function well in their hands, students can quickly

become frustrated and lose interest. A number of references give good information about basic techniques and principles (Gray 1964; Novikoff and Holtzman 1976). One of the most frequent errors is improper positioning of the condenser. The majority of microscopes function best with the condenser racked almost all the way to the top so that the condenser lens is about 3 mm below the slide. Check to see that diaphragm and light source settings are correct. The student should be seated comfortably, and if binocular microscopes are used students should be taught to use both eyes in viewing. The majority of microscopes are parfocal so that little adjustment is needed once good focus has been obtained under low power. Changing to the oil immersion objective with water-mounted specimens will often cause the specimens to "swim" out of view. The problem can be avoided by removing excess water from the slide before switching to oil and sealing the cover slip edges with petroleum jelly or clear nail polish to prevent rapid dehydration. Once the student has used an oil objective it is often difficult to return to a lower magnification. The oil lens should be employed only when observations are completed at lower magnifications and no further information can be gathered.

Phase Microscopy

The phase microscope is the instrument of choice for many of the observations described in this lab. Ideally, several phase microscopes should be available for student use, but at least one phase scope is essential if mitochondria and smooth endoplasmic reticulum are to be studied. Adjustment of phase optics is simple and the scope manufacturer's instruction manual will give directions. Transmitted light microscopes without phase optics can be adjusted to give better visualization of living cells by setting the light source and diaphragm slightly below the levels used for prepared slides.

Polarizing Lenses

While it is possible to purchase polarizing lenses for most microscopes, inexpensive substitutes are available. American Optical Corporation supplies a complementary polarizing accessory that fits the AO microscopes and consists of a 31-mm disc and of a 20-mm disc of polarizing crystal bonded between two layers of acetate. The 31-mm disc is placed in the filter slot at the bottom lens of the Huygenian eyepiece or, with the Microstar scopes and in previous models, between the binocular body and the eyepiece. Fisher Scientific sells the same polarizing film in 15-cm and 30-cm squares (Cat. #13–789 A & B), and the film can be cut to fit the microscope. Since it is difficult to cut small circles of the film because layers tend to separate, larger pieces of film, 4–5 cm square, can be used. One piece is placed between the light source and the specimen (either on top of an instage light source or in the condenser filter holder), and the second piece placed between the specimen and the objective

lens. Although the set-up is crude and you probably will not be able to use the oil immersion lens because of the thickness of the film, it will work adequately at low magnifications for the types of qualitative observations described in this lab.

As one views the specimen one of the pieces of film is rotated. Substances such as starch or crystalline inclusions will exhibit a characteristic birefringent pattern when the lenses are crossed.

Microscopy takes a lot of patience. Attention to detail in the teaching of microscopy is rewarded by students taking an active interest in the lab. Seeing mitochondria squiggle around the cell or watching the effects of polarizing light on starch grains can be interesting and fun.

Teaching Aids—Video and Photomicrographs

Often students have a great deal of difficulty evaluating and analyzing microscope images and recognizing artifacts. To teach the above skills video cameras, videotape, and photomicrographs are invaluable aids. Most schools have video cameras, and it is simple to attach a video camera to a microscope. Special lenses are not needed, and the only requirement is that the microscope image must be received on the vidicon tube of the camera at a level that can be brought into focus. Any trinocular microscope with some sort of phototube will probably accept a video camera. The camera lens is replaced by a 5-cm x 2-cm tube adaptor with male connection threaded to match the camera lens mount threads. The video camera with adaptor is slipped inside the phototube. If the microscope has a photolens in the phototube assembly, the lens is removed and the video camera substituted for it. The camera can even be mounted in place of the eyepiece as long as there is adequate support for the camera.

It is much easier to point out features of a specimen on a video monitor than to have 15 or 20 students take turns looking at a demonstration microscope. The monitor is especially useful with materials such as onion epidermal peel when illustrating mitochondrial movement. Once students have seen the video demonstration it is much easier for them to return to their own preparations and benefit from their study. Videotapes can be made of the demonstration, eliminating the need for multiple preparations when dealing with numerous lab sections. Many schools can provide technical assistance with the making of tapes since taping from a microscope with video camera tends to be at least a four-handed job—one person at the microscope controls and a second at the video console. Short, ten-minute blank tape cassettes are available and are of convenient length.

Photomicrographs are excellent teaching aids. The photography is not difficult, and Kodak sells a good reference (Photography Through the Microscope) describing photomicrographic equipment and techniques. Color enlargements of a sampling of materials to be viewed in the lab will tend to

encourage students to match in their own preparations the quality of the photomicrographs.

A number of photographic films are available. If you wish to do your own developing, black and white film will be simpler. Color photographs will be more useful to students, however. With the color films the major decision will be whether to choose a negative film or a color reversal film. If the primary use of the photomicrograph will be as a projection slide, color reversal film will be the best choice. We have had excellent results with Kodak photomicrography color film 2483, a color reversal film with high contrast and very high resolving power. If the primary use of the photomicrograph will be as an enlarged print, commonly available color negative film may be used.

Slide Preparation

Good slide preparation is essential, yet simple. The majority of plant materials can be mounted on a slide in water. The specimen is covered with a cover slip by first touching the slide with one edge of the cover slip and then lowering the other edge with a needle to prevent the formation of air bubbles. Excess water should be blotted away. Glycerin may be used as a temporary mounting fluid as it offers the advantage of not evaporating as does water.

Epidermal peels tend to curl as they are removed from the plant so it is usually necessary to use a mounted needle or fine forceps to flatten the tissue. Plant tissues other than epidermal peels must be cut as thin as possible. Rest the plant material on a clean, solid surface such as a glass slide and using a single-edged razor blade, cut the section as thin as possible. Cut a number of sections, float them off the razor blade into a watchglass or petri dish of water and select the thinnest for study. It helps to work with a small piece of material and to make some angled cuts so that even if the whole section is not thin there may be a sufficient number of cells at the thinner edge of the angled cut.

The hand microtome is an old but useful instrument to produce thin, uniform sections. Elder pith and carrots are generally used to support objects for hand sectioning. European elder (*Sambucus nigra*) is recommended over the American elder (*S. canadensis*). It can be stored indefinitely and cuts with a clean, crisp action. The support material is cut to form a cylinder of the right diameter to fit the microtome, and split down the middle. To section a leaf the leaf is inserted in the split support cylinder and the support material placed in the microtome. For bulky specimens the support material must be hollowed out to an appropriate shape. The section is cut by drawing the razor across the microtome plate with gentle pressure and the section is then floated onto water in a petri dish.

Notes on Student Exercises

I. *Observations of unstained cells*

Cell surface and matrix

Most of the observations described in the lab are straightforward and present no difficulty. *Rhoeo discolor* is the best specimen to use for a demonstration of the cell wall and plasma membrane. Cytoplasmic pigment in *Rhoeo* allows the student to identify the plasma membrane as distinct from the cell wall when the cell is slightly plasmolyzed. A number of materials give nice demonstrations of cyclosis: *Nitella,* onion bulb scale, epidermal peel, *Amoeba,* and staminal hairs. Each of the materials will show active cytoplasmic streaming as well as interesting features peculiar to the particular specimen. The authors favor the staminal hairs because the cells are very attractive, and the material is not used in any other parts of the exercise. Students sometimes confuse staminal tissue for the staminal hair cells. The hair cells appear as a delicate chain of cells with interesting thickenings in the walls. Numerous pollen grains are usually present.

Cell organelles

The nucleus and mitochondria are clearly visible in both the buccal cell preparation and the onion bulb scale epidermal peel. The plant material provides the clearer demonstration because the cell and organelles are larger, and the mitochondria are visually distinguishable from other organelles, droplets, and particles in the cytoplasm. A phase microscope is necessary to clearly see the mitochondria.

The materials selected to demonstrate chloroplast structure show a variation in chloroplast shape and size. The chloroplasts of *Elodea* and spinach have small spots visible in the light microscope that correspond to the grana of thylakoids.

The main difficulty with the chromoplast materials is finding cells that have clearly visible chromoplasts, although the tissues are obviously colored, due to chromoplast pigments. The carrot tissue must be thinly sliced so that some single layers of cells are available for observation. The tomato tissue, either pulp or skin, will have quantities of granular material from ruptured cells.

Most textbooks state that the endoplasmic reticulum is visible only with the electron microscope. Junqueira (1971) has, however, redrawn a diagram from Bloom and Fawcett illustrating the appearance of cell organelles in the light microscope including smooth endoplasmic reticulum and Golgi bodies. Structures identical in appearance to those identified as smooth endoplasmic reticulum by Junqueira are visible with the phase microscope in onion bulb scale epidermal peel (Fig. 3.1).

The authors have sought tissue to use for the demonstration of Golgi bodies in living cells but thus far without great success. Gray (1964) recommends earthworm ovaries as a source, but the identification of ovarian tissue in small specimens is difficult. With a larger specimen, positive identification of the ovaries, located in the 13th metamere, would be easier. If the organ were isolated and squashed gently on a slide in physiological saline, it should be possible to see the Golgi bodies with the phase microscope.

Plasmodesmata are visible in a variety of plant tissues. Date pits are a good tissue to use although they are sometimes difficult to find in the market. The pits must be slightly softened either by boiling for several hours or autoclaving for a shorter period of time. If other materials are being autoclaved put the pits in for two or three runs. The relative hardness of the tissue is an advantage since it is possible to cut thin sections easily with a single-edged razor.

Many plant tissues contain crystalline inclusions, and it is interesting to try a variety of different species. *Lemna* frond (duckweed) is an excellent material. It is widely available, does not need to be sectioned, and contains abundant inclusions. The small leaves are mounted in either water or glycerine, a cover slip added, and the preparation flattened with a little pressure. Examine the material first in unpolarized light, and then, with the polarizing lenses in place, rotate one of the lenses until the lenses are crossed.

II. *Cytochemical identification of cell organelles and molecules*

Certain cell organelles can be readily identified and better studied with the use of cytochemical stains. Plant vacuoles concentrate neutral red, making the vacuole visible. If a material such as a root tip is used, students can see the increase in size of the vacuole, from cells in the meristem with multiple small vacuoles to a single large vacuole in mature regions of the root.

The root must be gently crushed in the neutral red solution but not broken in pieces and must remain in the solution long enough to absorb sufficient quantities of stain. Because of the large number of cells in the root tip, students will have to examine the preparation carefully to find material free of debris. First-year students often find "DNA" in this preparation (i.e., helical thickened xylem).

We have included a classic stain for mitochondria, Janus green B, although we feel that this stain produces far from striking results. Small organelles in the cytoplasm produce a diffraction halo that matches the color of Janus green B. On occasion we have gotten good results, unfortunately not highly reproducible. As noted previously, mitochondria can be positively identified in onion bulb scale epidermal peels without benefit of stain.

Spherosomes are small, spherical structures in plant cells and are associated with lipid metabolism. Both spherosomes and lipid droplets will stain

with Sudan III, and the distinction between the two is possible only with the EM. Spherosomes are osmophilic because of their enzyme content. Some commercially available solutions of Sudan III are supersaturated and contain stain crystals. The stain should be filtered before use.

Starch identification with KI_3 is virtually a never-fail test. Students should first examine a water mount of the tissue and then add a drop of KI_3 to the edge of the cover slip and watch the color change as the KI_3 diffuses beneath the cover slip. Potato tissue is recommended because students can see how the potato cells are completely packed with the starch grains. The thin pieces of potato should be placed directly on the glass slide so that free starch grains can be retained on the slide. Examine a piece of potato and samples of other starches mounted in water without added KI_3 with the polarizing microscope for an extremely effective demonstration of polarized light effects.

KI_3 used to identify glycogen in paramecia also causes the paramecia to release their trichocysts. Glycogen in KI_3 is golden brown, not unlike the color of the stain solution in the bottle. The entire organism stains, indicating a uniform distribution of glycogen. Other protozoans could be used, preferably using one species or a mutant of a species that stores glycogen and one that does not.

Cellulose and lignin can be identified by KI_3-sulfuric acid and Zn-Cl-I reagent and lignin by phloroglucinol. The reagents differ in their specificity, and it is good to compare the same tissue stained with the different reagents. The use of sulfuric acid must be monitored carefully since it is possible to damage microscope lenses and stages. You may choose to omit the reagent because of the risk involved. To date we have had no problems, but we keep bicarbonate of soda easily accessible to students at their microscopes to neutralize spills and caution them to clean their microscope stages of any spilled liquids. *Coleus* stems give a clear bright orange reaction for lignin in the vascular tissue.

The phloroglucinol test for lignin in pears is probably the most vivid reaction in the exercise. The student should examine a thin slice of pear mounted in water and try to locate the stone cells. A second slice of pear is placed in phloroglucinol for at least ten minutes. The stone cells, rich in lignin, will turn a brilliant pink-magenta visible to even the unaided eye.

Pectin is found in the middle lamella and a number of fruits have a high pectin content. If an apple is sectioned or if cells are scraped onto a slide and tested with ruthenium red, the cells will turn bright red.

Sudan IV will stain cutin and suberin a bright red. The leaves should be cut in a thin cross-section. *Rhododendron* gives a reliable reaction but other waxy-surfaced leaves may be tried.

Any vegetable that darkens when cut almost certainly contains tannins. When certain types of apples are used, the tannin concentration is high enough that dark brown crystals form in reaction with the copper acetate.

Caution should be used in the handling of the Millon's reagent because of its mercury and nitric acid content.

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APPENDIX I

Materials for a Class of 20 Students

Sufficient microscopes for a class of 20 students, including at least one scope with phase optics.
Razor blades—2 packs single-edge, laboratory-style
Fine forceps
Slides
Coverslips
Lens paper
Distilled water in dropping bottles
Petri plates or watch glasses—20
Depression plates—20

I. Observations of unstained cells

A. Cell Surface

Cell membrane: one or more of the following:

a. amoeba—one culture *Amoeba proteus* (Carolina Biological Supply Cat. #L1)

b. buccal cells

1 box broad-ended toothpicks
0.9% NaCl—100 ml

Cell Wall: one or both of the following:

Rhoeo discolor—one plant (Carolina Biological Supply Cat. #15–7500)

Onions—3–5 onions will be sufficient for this and succeeding parts of the exercise

Coleus—one vigorous plant is sufficient for all parts of the exercise (Carolina Biological Supply Cat. #15–7310)

Cilia

Any ciliated protozoan; one good choice is *Paramecium multinucleatum* (largest paramecium)—1 culture (Carolina Biological Supply Cat. #L2)

B. Cell matrix and its movement: one or more of the following:

Staminal hairs: a number of plants, e.g., *Tradescantia* and *Rhoeo discolor*, possess fine hairs on the flower stamens. Three to four flowers are sufficient for a class of 20.

Tradescantia: (Carolina Biological Supply Cat. #15–7560)
Rhoeo discolor: (Carolina Biological Supply Cat. #15–7500)
Elodea tips: (Carolina Biological Supply Cat. #15–7340)
Nitella tips: (Carolina Biological Supply Cat. #15–1285)

C. Cell organelles

1. Nucleus

A number of materials will give good visualization of the nucleus including the onion bulb epidermis, staminal hairs, and *Elodea*.

2. Mitochondria: one or more of the following:

celery: several stalks
onion

3. Chloroplasts

Spinach: several leaves with petioles

Spirogyra—1 culture, (Carolina Biological Supply Cat. #15–2225)

Elodea

Euglena—1 culture (Carolina Biological Supply Cat. #15–2800)

4. Chromoplasts—one or more of the following:

Carrot—several
Tomato—two or three
Red pepper—two to three

D. Cell communication

Date pit softened in pressure cooker or autoclave
Cotyledons of germinating seedlings

E. Cell inclusions—one or more of the following:
Lemna frond (duck weed)—20–25 plants
rhubarb— several stalks
Aloe— several leaves
wax begonia— one plant

II. Cytochemical identification of cell organelles and molecules
Reagents should be in dropping bottles (see Appendix II).

A. Identification of vacuoles in plant cells with neutral red.
1% neutral red
glass rod

B. Mitochondrial identification with Janus green B
celery or onions
working solution Janus green B (see appendix)

C. Identification of spherosomes with Sudan III
Sudan III
onion
celery

III. Identification of cell molecules

A. Starch
KI_3-Iodine Potassium Iodide
—potato (2 or 3)
—small quantities of one or more of the following: peas, ground rice seeds, corn starch, plant stems
—polarizing lenses for microscope (advanced level course)

B. Glycogen identification with KI_3
KI_3
Paramecium

C. Cellulose and lignin identification with the KI_3-sulfuric acid method and by zinc-chlor-iodide reagent
—KI_3 reagent
—ZnCl-I reagent
—65% H_2SO_4 in dropping bottle
—*Vicia* roots
—*Coleus* stems
—other plant stems or leaves

D. Lignin identification with phloroglucinol
—pears (give best reaction)
—*Coleus* stem
—*Vicia* roots
—phloroglucinol

E. Pectin identification with ruthenium red
—*Coleus*
—*Vicia* roots
—apple
—ruthenium red (Gallard-Schlesinger is possibly the ony U.S. source—Cat. #3406625 ruthenium oxychloride ammoniated)

F. Cutin and suberin identification with Sudan IV
   —Sudan IV
   —*Rhododendron*
   —or other waxy-surfaced leaves
G. Tannin identification with copper acetate
   —copper acetate
   —apple
   —potato
   —eggplant
H. Protein identification with Millon's reagent
   —Millon's reagent
   —*Tetrahymena*

APPENDIX II
Reagent Preparation

1. Neutral red: 1% aqueous solution.
2. Janus green B: to make the working solution add 1 or 2 drops of a 1% aqueous solution of Janus green B to 50 ml 10% sucrose. Make up the working solution at the time of the lab. Cover the container and shake vigorously to completely oxidize the stain.
3. Sudan III: Make a stock solution of 2 g powdered dye in 100 ml absolute alcohol. When ready to use, dilute the stock solution with an equal amount of 45% alcohol.
4. KI_3: Dissolve 0.3 g iodine (I) and 1.5 g potassium iodide (KI) in 100 ml distilled H_2O.
5. Ruthenium red: pectin
   0.2 g ruthenium red dissolved in 50 ml distilled H_2O.
6. Sudan IV: cutin and suberin
   50 ml 95% ethyl alcohol saturated with Sudan IV; add 50 ml glycerin
7. Sudan III: lipids
   2 g Sudan III in 100 ml absolute alcohol; add 100 ml 45% alcohol
8. Phloroglucinol: lignin
   Dissolve 1 g phloroglucinol in 82 ml 95% ethyl alcohol, add 18 ml concentrated hydrochloric acid.
9. Copper acetate: tannins
   7 g copper acetate dissolved in sufficient distilled H_2O to bring final volume to 100 ml.
10. Zinc-chlor-iodide: Testing for cellulose
    50 g $ZnCl_2$ (zinc chloride) and 16 g KI (potassium iodide) dissolved in 17 ml distilled water (This formula is correct! The resulting solution is very concentrated.)

11. KI_3-sulfuric acid: Testing for cellulose
    KI_3 as prepared in item 4 is used, plus a drop of 65% H_2SO_4 is added at edge of coverslip when the slide is made.
12. Millon's reagent: Testing for proteins. Use CAUTION in the preparation of this reagent.

    Dissolve 10 ml liquid mercury in 188 ml concentrated HNO_3 with CAUTION! Use a hood while dissolving the mercury in the HNO_3. When the evolution of brown fumes stops, add twice the volume of distilled water. Decant the supernatant liquid and store in a glass-stoppered bottle.

Chapter 4

The Use of Plant and Animal Tissue Culture in Biology Laboratory Instruction*

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*The author wishes to acknowledge the help of Bob Setteneri in preparation of the section "Methods in animal tissue culture."

I. Introduction

Tissue culture is a very exciting and integral part of the study of cell biology today. Its historical development and current technology of growing cells of living organisms outside the normal body (*in vitro* culture) has made possible a great range of biological experimentation and knowledge about cells' development and behavior under given situations. Whether one is studying how a nerve cell axon makes synapse with a muscle cell or the effect that viruses or chemicals have on cancerous growing cells, the value of studying these living processes under controlled *in vitro* conditions is intuitively obvious.

Tissue culture can be a strategic educational tool in introducing the student to the concepts of cell and tissue growth and nutritional growth requirements. The concepts of environmental oxygen and carbon dioxide gas tensions, pH or acid-base dynamics and buffering are additional valuable concepts for the student to grasp. The nutrient binding and buffering capacity of proteins (e.g., a fetal calf serum in tissue culture media) are not normally given a place in general instruction but can be emphasized so that the student can understand the normal physiological processes involved in cell growth and development.

Perhaps the most interesting feature for the student is learning to grow cells in a tissue culture situation. This allows an opportunity to observe firsthand the actual appearance and structure (cytology) of living cells (see figure 4.1).

There is a no more effective teaching method than allowing the student the opportunity to inductively observe actual processes such as cell movement, pinocytosis, brownian motion, cell division, rotation of the cell nucleus, bacterial contamination, etc. An actual dynamic process such as a cell attaching to a surface or rounding up and detaching because of a lack of calcium (e.g., EDTA chelating action of the subculture procedure--section II-B) cannot be effectively taught using any other method. The concept of epithelial cells growing sheetlike in a flask monolayer or being broken up by a simple trypsin or EDTA treatment such as is done in a subculture routine is an ideal graphical representation of cell behavior and growth within the body. As is also the case in most laboratory work, the student will probably experience some difficulty in initially culturing the cells and will be able to visualize dead or dying cells (shiny appearance under phase optics) in a culture situation. This is good also because it is a more realistic portrayal of the complete life history of cells (cell cycle) than examining a static prepared tissue slide, for example, or seeing only living cells in an instructor prepared demonstration. Additionally, the idea of the student being able to observe the natural appearance of a nucleus, nucleolus, or mitochondria with phase optics and then to observe the appearance after staining or electron microscopic-type magnification can be an important learning bridge in the area of cytology.

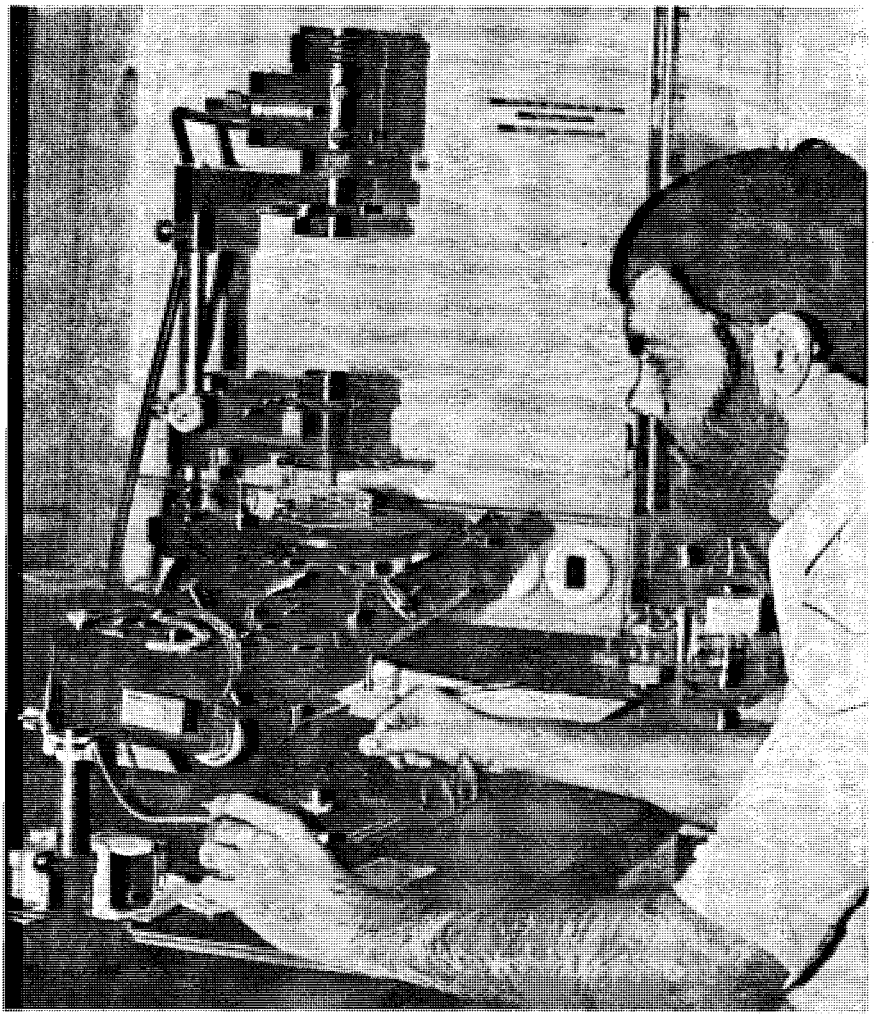


Figure 4.1. Examination of cultured cells with a Nikon model MS inverted microscope, phase optics with 35 mm camera attachment.

Much of the procedural format is contained in the ensuing sections, so at this point it only needs to be emphasized that the purpose of this type of experimentation is as follows:

a. to study cell cytology and tissue structure;
b. to study the behavior and life history of the cell;
c. to learn the techniques and theory of tissue culture;
d. to develop skill in using the equipment routinely used in maintaining and subculturing cell cultures;

e. to learn basic characterization procedures such as cell staining, slide making, vital staining, counting, size measurement, drawing, photography and darkroom techniques;
f. to become experienced in aseptic transfer and handling techniques;
g. to gain experience in keeping a research notebook and scientific writing.

As a person practices tissue culture and develops expertise and confidence in the method there is an unlimited range of specific experimental applications for the biology laboratory. A few of the most commonly used applications are using tissue culture cells in respiration experiments or the effect of chemicals or viruses on cell growth and division and monolayer attachment. An example would be the effects of colchicine, various ions, cyclic AMP, etc., on cell division, growth, and behavior. The flat monolayer lends itself especially well to work with radioisotopes used in metabolic studies in autoradiography. Special histochemical staining reagents may be employed to give a visualization of different cytological components in the cell such as lipids, glycogen, DNA, or RNA.

Another emphasis deemed productive is the use of photography and darkroom techniques as an adjunct to cell culture. Cells viewed and photographed with phase optics are particularly striking (figure 4.2).

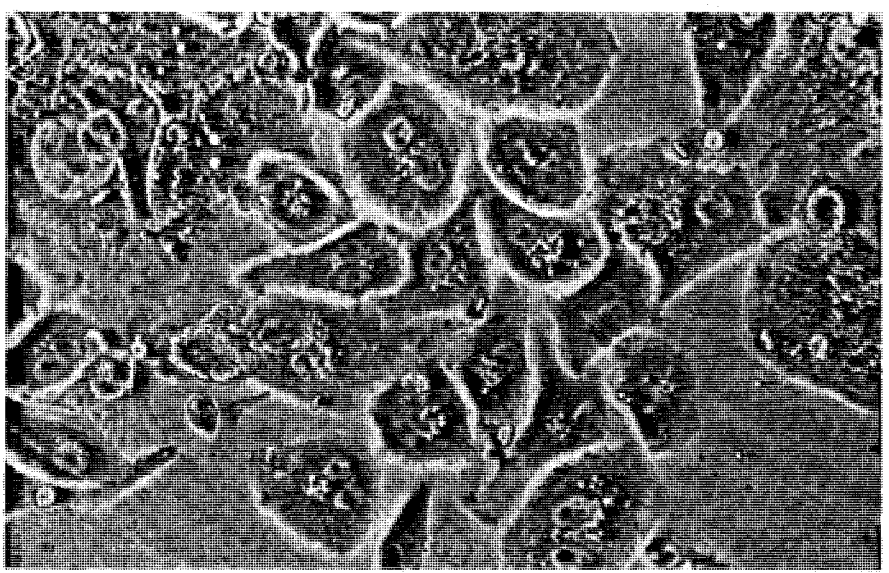


Figure 4.2. HeLa cells in tissue culture, 200x, phase optics, Plus X film.

It is relatively simple to adapt a camera to a microscope, take a black-and-white photograph, and develop the negative. The negative can then be contact-printed (sufficient for most student work) and used by the student as a part of the project results. This added dimension and visualization usually helps maintain high student motivation towards the project. In addition, important photomicroscopy techniques are added to the repertoire of the biology student. I have also found it useful to explore microscope optics at this point as well as using a discussion of photographic theory as a bridge to a preliminary introduction to the technique of autoradiography.

One prevalent concern on the part of the student or potential laboratory instructor is "How do I get my feet wet?"

Exposure to the basic equipment and methods of tissue culture can best be obtained by visiting a university research laboratory or pharmaceutical house where tissue culture is routinely employed. This also may represent an inexpensive source of cell lines and glassware (on a limited level). This would also provide the opportunity for a student tour where many methods, equipment, glassware, and research applications can be assimilated in one initial experience.

Most laboratories have the essential equipment: an incubator, sterile hood, hemocytometers, staining dishes, etc. It is important that one has microscopes equipped with phase optics (inverted microscopes are preferred) and photographic capability. Tissue culture glassware is commercially available and if sparingly used or interfaced with petri dishes, etc., does not represent a real financial burden. Specialized culture equipment is not essential and can be added as money and interest permit.

Section two concerning general student information summarizes the basic methods of tissue culture and skills needed. Notes and suggestions directed to the student in this section represent important information and experimental ideas for the instructor as well. The following instructor's section details the methods normally employed in a teaching approach to tissue culture.

I firmly believe that giving the student the opportunity to work with the living material and dynamic of tissue culture does much to maintain a strong state of student motivation. This motivation is essential to learning the process of science—observing, wondering and asking questions, theorizing, gathering data, and answering questions.

II. Student Materials

Plant and Animal Tissue Culture Techniques

The term tissue culture indicates the cultivation of tissue cells in a medium and container external to its normal position (*in vitro,* as contrasted with *in vivo*). This type of living tissue preparation facilitates ease of observation and study of cell structure (cytology), study of cell differentiation and interaction

with its environment (histology) and allows the experimenter ease in adjusting environmental parameters (pH, temperature, nutrients, etc.) in the case of metabolic or physiological studies. Much of our experimentation will be primarily concerned with the basic techniques of cell culture and manipulation. Analysis and presentation of results will involve drawing, photography, time-lapse photography, and some simple cytological staining methods.

Tissue culture may be initiated through subculture of an existing pure cell line (examples are HeLa cancer cell, monkey kidney cell) or explant of a cell mixture from a donor animal such as a chick embryo. In either case the *resultant cell suspension* can be subjected to several subsequent preparations. For instance a monolayer culture employs a plastic falcon flask for growing the cells. Test tubes with a flat surface on one side used with a glass cover slip for cell attachment may be used in maintenance of a culture; these are called Leighton tubes. The cells can be grown in a perfusion chamber such as a Sykes-Moore or Rose chamber, where media is being constantly replenished. The Sykes-Moore chamber allows the cells to grow without media changes or manipulation, and facilitates long-term microscopic observation such as is needed for time-lapse photography.

Plant cells in culture are similar to animal cells in that they can be grown *in vitro* if the proper microenvironmental conditions are met. The parameters important to a cell in tissue culture would be temperature pH, moisture, mineral salts, amino acids, vitamins, and a sugar energy source. An example of these requirements is a chemically defined culture medium such as White's basal medium for plant cell culture. These media also contain salts for buffering the pH and an excess acidity visual indicator such as chlorophenol red. Coconut milk contains a factor(s) that produces rapid cell division and so may be added as a supplement in some cases.

The plant cell culture method is interesting in that each cell of the plant contains the genetic potential not only for cell division, enlargement, and differentiation, but also for producing a complete organism. When grown in an appropriate liquid medium the explanted cells (a sample taken from an original plant source) will divide, form colonies, and produce a baffling array of forms. Sometimes, recognizable forms may be produced from an organized cell clump such as embryos, root nodules, shoots, etc. If an embryo plantlet is formed, it is possible to transfer it (or any other cell clump) to an agar plate where it will receive sufficient support to grow into a mature plant. A good source of explant tissue for this latter technique would be an embryo taken from the seed of a mature plant.

Another type of culture that is professionally used in maintenance of animal cells is the suspension method, where the media is modified (calcium omitted) so that the cells won't stick to a surface. The culture tubes are usually rolled with a constant motion so there is maximal exchange of nutrients and wastes between cells and the medium. The large scale production method

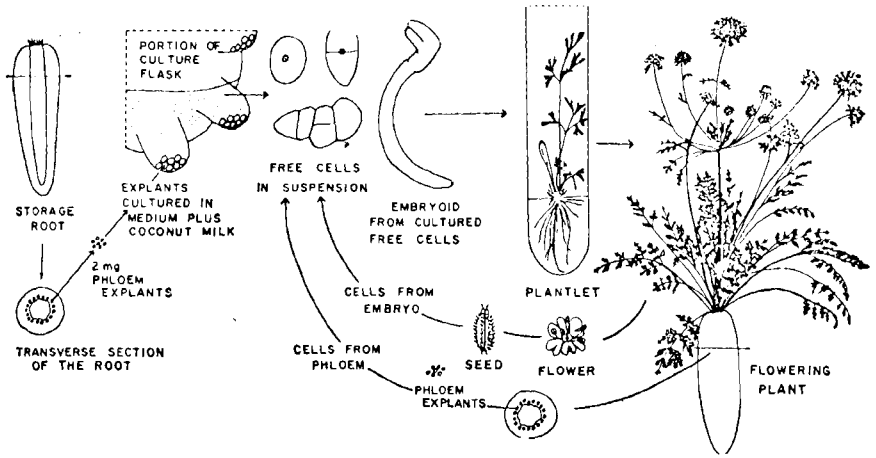


Figure 4.3. Diagrammatic representation of the cycle of growth of the carrot plant; successive cycles of growth are linked through free, cultured cells derived from phloem, or from the embryo. From "Growth and Development of Cultured Plant Cells," Steward, F.C. et al, *Science*, Vol. 143, January 1964. Copyright 1964 by the American Association for the Advancement of Science.

is used extensively in research and pharmaceutical research houses where the cells are being tested with viruses or chemicals routinely. Long-term storage of tissue is usually accomplished by placing representative cultures and flasks in glycerin and freezing.

Another aspect of culture technique is cell counting, including total and viable cell counts. This is important because optimal cell growth is achieved in a specific range of cell number per media volume (section III-D for method). This concept is referred to as plating efficiency. Other techniques such as staining and specific culture procedures are found in section III—instructors materials, the appendices and the Ward's Cell and Organ Culture Booklet.

The importance of maintaining aseptic conditions at all times cannot be overemphasized. Always use tools, glassware, and media that have been sterilized. If possible, perform explants or transfers in a fume hood (with air suction on) or glove cage. When containers have to be opened, etc., observe standard flaming techniques.

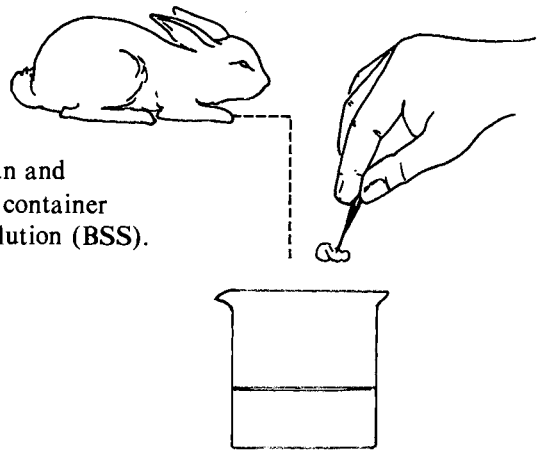
The following contains diagrammatic representation of:

A. Preparation of animal primary tissue for monolayer cultures.
B. Subcultivation of established animal cell cultures.

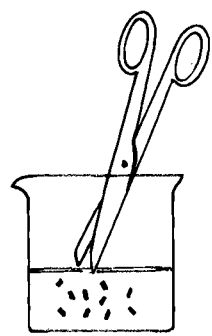
This should help you visualize the techniques that you will be using in this tissue culture laboratory. You may wish to refer to Appendix I—Tissue Culture Vocabulary to review some of the main terms associated with tissue culture procedures.

A. Primary Culture of Animal Cells

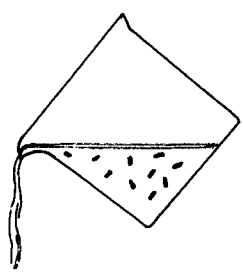
1. Obtain tissue or organ and immediately place in container with balanced salt solution (BSS).

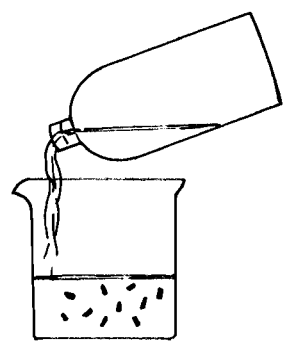


2. Mince tissue into fragments of approximately 1-mm dimensions.



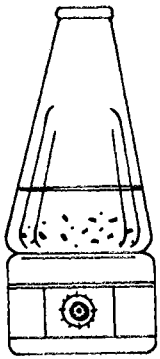
3. Wash fragments several times in fresh delbecko's or Hanks BSS.



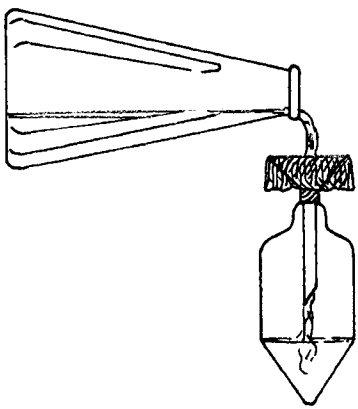


4. Transfer fragments in BSS to trypsinizing flask containing a Teflon-coated stirring bar. Add trypsin and place on magnetic mixer.

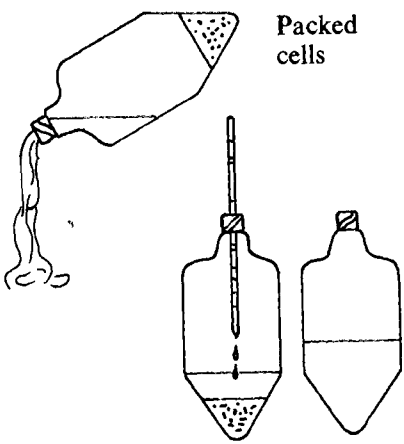
   Note: The amount of trypsinization necessary depends to a large extent on the density of the tissue, and may be controlled by varying temperature, time, pH, degree of agitation, and concentration of trypsin.



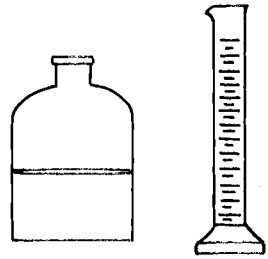
5. When trypsinization is complete, strain suspension through gauze into a centrifuge tube and centrifuge at 500–900 rpm for 5–10 minutes.



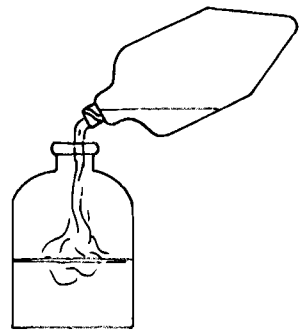
6. Pour off supernatant from packed cells and add a measured amount of nutrient medium. Resuspend cells by vigorous pipetting.



7. Perform a viable cell count using trypan blue stain and a hemocytometer.

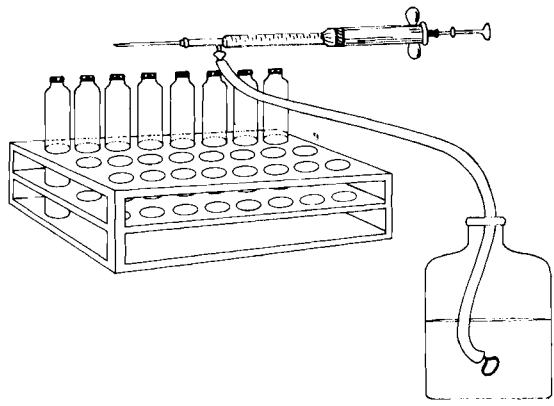


8. Transfer suspension from centrifuge tube to suitable container.

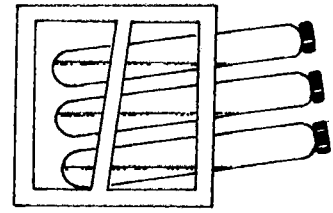


9. The volume of the suspension may now be adjusted with nutrient medium to obtain the desired cell concentration. Concentration of cells for optimal monolayer growth vary as much as from 150,000 to 500,000 cells/ml depending upon the nature of the tissue.

10. The cells should now be planted by inoculating tubes, flasks, or other appropriate containers with the cell suspension. Round tissue culture tubes (16X125mm) are usually planted with 1 ml of suspension.

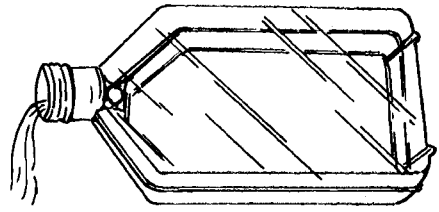


11. Incubate cultures at 37°C.



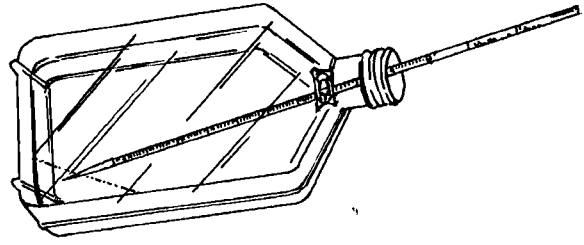
B. Subculture of Established Cultures

1. Discard medium from monolayer culture.



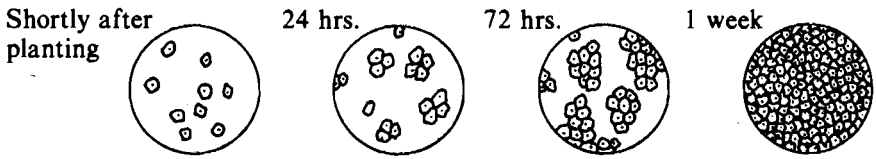
2. Loosen cells from surface of culture vessel with trypsin/EDTA treatment; add 0.25% trypsin/EDTA solution to the culture vessel covering the cell sheet. Incubate at 37°C until cell monolayer lifts off as a sheet.

3. After cell sheet is broken up, add a measured amount of fresh medium. Pipette solution vigorously to break up cell clumps.



4. After performing a viable cell count, the suspension may be adjusted to desired concentration and cells planted by inoculating suspension in appropriate containers and incubating at 37°C.

Examine microscopically at intervals for characteristic growth under optimum conditions.



III. Instructor's Materials

The discussion presented below provides the actual procedural steps for initiating tissue culture in the laboratory as well as performing the supporting methods of cytological analysis, staining and preparation of permanent slides.

A. Methods for animal tissue culture
B. Notes and theory correlated with successful animal tissue culture
C. Plant tissue culture
D. Cell counting procedures
E. Tissue fixation and staining procedures

A. Methods of Animal Tissue Culture

Cell monolayer cultures in 120-cm^2 flasks are purchased from a commercial source or local research laboratory. For routine student work the green African monkey kidney cell line (Veros) is recommended. It is a hardy fibroblastic type of cell line that grows well with most tissue culture media formulations and student 'mismanipulations' (see figure 4.4).

The planting media consists of the following components: Eagles Minimum Essential Medium (MEM) with Earle's salts and HEPES buffer supplemented by adding the following substances as specified to 100 ml of MEM:

10 ml of fetal calf serum (FCS) (final concentration = 10%)
1 ml of L-glutamine (200 Molar)
1 ml of 10,000 units penicillin, 10,000 streptomycin and 10,000 neomycin mixture.

Appendix 5 contains a list of the above components as well as a sample order for materials that would support laboratory experimentation by twenty students.

The monolayers are subcultured in the following manner:

1. The media are poured off, and the monolayer washed two times with approximately 50 ml per wash of calcium and magnesium free phosphate buffered saline (PBS), at a pH of 7.2.
2. One ml of trypsin-EDTA solution containing 0.5 gm trypsin (1:250) and 2.0 gm EDTA/liter of Hanks balanced salt solution (HBSS) without Ca^{++} and Mg^{++} is added at 37°C to each flask and dispersed over the monolayer with gentle shaking.

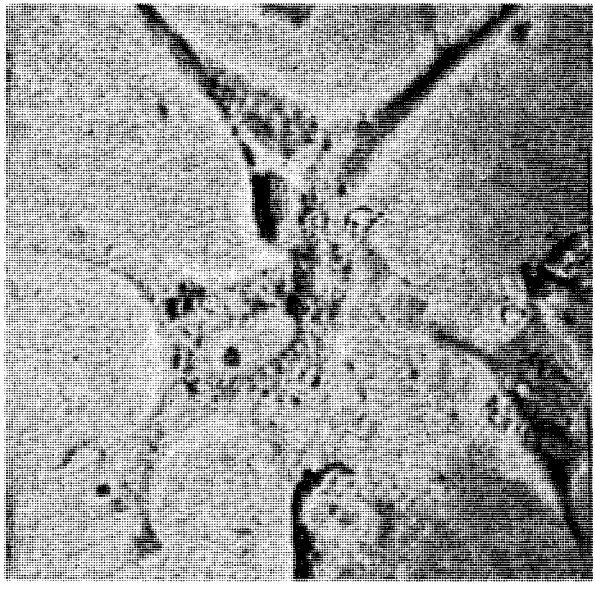


Figure 4.4. Veros Cell Monolayer (green African monkey kidney), 100x, phase optics, Plus X film.

3. The flasks are then placed in an incubator at 37°C for approximately 3–5 minutes, depending on the time required to dislodge the cells. Shaking by hand is required.
4. Ten ml of planting medium is added to each flask and the cells are dispersed by aspirating and expelling the suspension from the pipette. This is done ten times.
5. The contents of a series of flasks are pooled and the cells in the suspension are diluted with planting medium to 5 x 10^5 cells/ml.
6. The cells are subcultured into falcon flasks, Sykes-Moore chambers, Rose chambers or Lab Tek flaskettes.
7. The monolayers are used for experimentation when they reach almost confluent growth (approximately 1–2 days).
8. Separate flasks are used for maintaining the cell lines. Maintenance media consists of MEM plus supplements with FCS reduced to 5% final concentration.

B. Notes and Theory Correlated with Successful Animal Tissue Culture

1. Trypsinization
   For primary explant work you may wish to trypsinize actively using a trypsinizing flask with a magnetic stir bar or shaker. Trituration of the cell clumps with a 10-ml serological pipette will help break up

the tissue also. If you wish to dilute off the trypsin/EDTA solution, centrifuge @500 rpm for 5 minutes and decant the solution. Resuspend the cells in tissue culture media. For trypsinizing during subcultures, leave the 1 ml of solution on the monolayer until it lifts off the surface as rafts of cells. It will look like a scum lifting off the surface of the flask.

2. Planting cells

   After you develop a feel for the routine of tissue culture you can probably dispense with the viable cell counts when subculturing. Simply dilute the trypsinized cells with approximately 10 ml of fresh media and pipette out two 3-ml aliquots for planting two new flasks plus the one parent flask.

   If you wish to plant coverslip cultures simply add 1 ml of cell suspension to a 16x120mm test tube in which the 12x24mm coverslip has been placed. If incubated in a horizontal position the cells will settle on to the coverslip within a few hours and begin monolayer growth. A simple way to prepare coverslip cultures for general student use in laboratory is to scatter ten to thirty coverslips over the bottom of a sterile petri dish and cover them with a fresh cell suspension from one trypsinized flask monolayer.

   The advantage of preparing coverslip cultures in advance of the laboratory is threefold—they can be placed on a slide as wet mount for brightfield examination; they can be stained and then placed on the slide in a permanent mount; they can be used in making a perfusion chamber setup by simply putting together a Sykes-Moore chamber sandwich around the cover slip and injecting medium. Be sure the coverslip is cell-side up and is facing the chamber's interior and medium.

It is vitally important that organically clean glassware be used for planting the cells. Commercially available plastic flasks are the easiest to work with. If glass vessels or cover slips are to be used they should be dichromate/sulfuric acid or nitric acid-cleaned followed by rinses of ethyl alcohol and distilled water.

Autoclave the glassware in a larger container (e.g., cover slips in a petri dish partially open with a cotton plug); the container can subsequently be used to dispense the glassware during laboratory as well as maintaining sterility.

3. Sterile technique

   A sterile hood is not really needed if you are observing aseptic flaming procedures. You can construct a simple sterile hood by cutting one

half of a long side out of a large cardboard box and hanging a germicidal fluorescent light in the box. You can determine the sterile work area in the hood by exposing bacterial inoculated plates in different sectors, incubating and observing the germicidal activity.

The student work area can be sponge disinfected daily with a lysol solution.

4. pH and growth dynamics
   To prevent loss of CO_2, be certain that all vessels are tightly stoppered (be sure screw-caps have rubber liners). The Earle's salts maintain pH very well in a tightly closed flask or flasks that are loosely stoppered and gassed with 5% CO_2 in a gas flow incubator. Hank's salts maintain pH best in an open container or one that is loosely stoppered and exchanges gases with air. This is one reason why Hank's BSS solution is used extensively as a routine tissue bathing solution where washing and manipulation of cells is required. Remember—for Earle's salts in an open container, the pH indicator will turn purple, indicating a rise above pH 7.6; Hank's salts in a closed container will turn yellowish indicating acidity (pH below 7.4).

It is a good idea to remind the student of the following acid-base buffering dynamic:

$$CO_2 + H_2O \rightleftharpoons \underset{\text{Carbonic Acid}}{H_2CO_3} \rightleftharpoons \underset{\text{Bicarbonate}}{HCO_3^-\ (Na^+)} + H^+$$

You may wish to research this area more fully and introduce additional information on pH optima of the specific cell line used, osmolarity, and comparison of buffering capacity of the buffering systems used in tissue culture.

The use of Hepes buffered media is suggested for student work. As a hydrogen ion buffer, the pK_a is 7.31 at 37°C and it tends to stabilize and resist rapid pH changes in the medium (Hepes, N-2-Hydroxyethylpiperozine-N'-Ethansulfonic Acid).

Examine the cultures daily and adjust the pH or change medium as indicated.

HeLa cells will probably require a medium change every 2–3 days. Adjustment of pH may or may not be necessary between medium changes, but pH should not be allowed to drop below 7.0. A dense monolayer will be formed within 7–9 days.

The advantage of the rainbow trout gonad cells mentioned in Appendix 5 is that they grow slowly at room temperature and need to have a media change every four to five days. They only need routine subculturing or culture transfer once every two weeks and can be held for several months in the refrigerator without destroying the culture.

5. Interpretation of Cellular structure
   One of the major problems an educator has in this area is to secure a labeled diagram of a tissue culture cell as visualized by phase optics or brightfield observation. It is important that the students compare the cellular images they are visualizing with known structures at a light and electron microscopic level. You should consult cytology and histology atlases or textbooks to piece together the information you need to demonstrate these relationships clearly to the students. One such attempt is pictured in figure 4.5.

C. Plant Tissue Culture

The carrot plant (*Daucus carota* L.) is a convenient and popular plant source for tissue culture studies.

1. Remove 2–3-mg explants from the secondary phloem of the root or embryo and disperse in a small amount of medium. Dispersion may be accomplished by gently crushing, pipetting up and down or using a filtering method with bolting silk or cheesecloth.
2. Add the cells (equal to one explant) to a falcon flask (30-ml) or tube to which a cover slip has been added and culture in White's medium plus deproteinized coconut milk.
3. Observe and check daily for tissue growth, pH balance, etc. Replace medium as required.

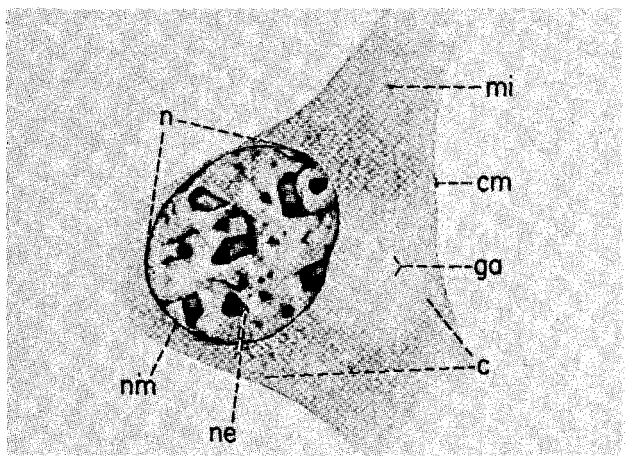


Figure 4.5. Tissue culture cell with identified morphology: n-nucleus, nm-nuclear membrane, ne-nucleolus, mi-mitochondria, cm-cytoplasmic membrane, ga-golgi apparatus, c-cytoplasm. From "The Living Animal Cell" Wards Curriculum Aid #750–8021, Courtesy Wards Natural Science Establishment, Inc., Rochester, NY.

4. In about a week or whenever cell growth is evident, make subcultures from the original suspensions. It is at this point that your students should design their own experiment.

A subculture of cells in White's basal medium only will tend to minimize cell division and maximize cell enlargement and differentiation.

White's medium with added deproteinized coconut milk will promote cell reproduction. Plant cells can be cultured on a solid surface (agar preparation) with as much ease as with a liquid suspension.

A typical formulation of White's medium for plant tissues is as follows:

Ingredients per Liter (in mg)			
Calcium Nitrate $4H_2O$	200	Manganese Sulfate H_2O	4.5
Magnesium Sulfate $7H_2O$	360	Zinc Sulfate	1.5
Sodium Sulfate	200	Boric Acid	1.5
Potassium Nitrate	80	Glycine	3.0
Potassium Chloride	65	Thiamine	0.1
Monosodium Phosphate H_2O	16.5	Niacin	0.5
Potassium Iodide	0.75	Pyridoxine	0.1
Ferric Sulfate $6H_2O$	2.5	Sucrose	20

This medium may be used either unsupplemented, or with the addition of coconut milk.

White's Medium is supplied at pH 4.8 ± 0.2.

To cultivate cells on solid media:

Make up 3% agar solution with distilled water and sterilize. Cool to 50°C and mix with equal portions of the culture media (50°C). Distribute or pour the agar preparation into the appropriate flasks and allow to solidify. Use an inoculating loop to transfer cultures.

The Ward's plant tissue culture kits (carrot cloning) are perhaps the easiest materials to work with.

Plant tissue culture can be realistically a semester-long project and is perhaps best suited for individualized student projects.

Detailed information and procedures are available with the Ward's kits (Appendix V).

D. Cell Counting Procedures

1. Total cell count: Hemocytometer

   The hemocytometer count is a feasible means of routine cell count in most laboratories. A single count, done carefully, may require 10–15 minutes and even under the best conditions, may present up to 10% variation.

a. Clean a hemocytometer and coverglass by first rinsing in water followed by absolute ethanol with a final rinse of acetone. Dry and polish with bilbous paper. Seat the coverglass *firmly* on the hemocytometer so that it covers both counting chambers.
b. Take a 1-ml sample of suspension for counting. Dilute 1 ml of the suspension with 1 ml of trypan (0.4%) blue to get a 1:2 dilution. This dilutent step is helpful in staining the nucleus of cells and preventing clumping thus facilitating a viable cell count. Aspirate several times to mix thoroughly and break up the cell clumps.
c. Charge counting chambers of the hemocytometer (without flooding) and count the unstained cells in 10 squares (5 squares in each chamber). Each square is 0.1 ml cubed therefore 10 times 0.1 mm^3 equals 1.0 mm^3. There are 1000 cubic millimeters in a one milliliter volume.
d. Calculate the concentration of cells per milliliter of original sample. Example:

Total cells counted (10 squares)	= 100
Multiply by dilution factor. . . .	x 2
	200 cells per mm^3

Add 3 zeros = 200,000 cells/ml

Note: The total number of cells counted in the ten squares x 1000 x the dilution previously made equals the number of cells per ml of the *original* suspension.

2. Viable cell count: Viable stains

A number of "vital" staining procedures have been developed in an effort to differentiate between viable and nonviable cells. With some dyes, e.g., methylene blue or various tetrazolium salts, the dye is used to indicate metabolic activity although the relationship of metabolic activity to viability is not at all clear. With others, e.g., eosin Y (2), trypan blue, and erythrosin B (3), the ability of viable cells to exclude dye is the criterion used.

a. Place 0.5 ml of cell suspension (diluted to contain $1x10^5$ to $2x10^5$ cell/ml) in a 12x75 mm tube. Add 0.1 ml of 0.4% trypan blue and mix thoroughly. Allow to stand for 5 minutes but not more than 15 minutes.
b. With a capillary pipette, fill a hemocytometer as for cell counting.
c. Make a total cell count and a count of unstained cells. Assuming the unstained cells to be viable, express the results as % viable cells.

3. Ocular micrometer
   A more rapid and convenient method, though less precise, is the use of a calibrated ocular micrometer. The diameter of a colony or explant may be measured in two or more measurements; one can calculate roughly surface area from the formula for the area of a circle.

E. Tissue Fixation and Staining Procedures

1. Formalin
   Make up a 10% solution in an isotonic solution (Ringer's solution). This works very well as a general purpose fixative for cells, tissues (animal and plant) and whole organisms.

   Ringer's solution:
   sodium chloride 0.9 g
   potassium chloride 0.042 g
   calcium chloride 0.025 g
   distilled water 100.0 ml

2. Formalin-Acetic Acid-Alcohol (FAA)
   FAA is recommended for monolayer cultures or for explants to be paraffin-embedded and sectioned. It is a good fixative which preserves most proteins. Fix for at least 30–40 minutes.

80% ethanol	90 ml
Glacial acetic acid	5 ml
Neutralized formalin (40% formaldehyde)	5 ml

   (Neutralize formalin with an excess of M_8CO_3. This fixative is best made up fresh.)

3. Bouin fluid
   Bouin fluid is recommended for general histology and may be used on monolayers. For explants to be paraffin-embedded and sectioned, fix for 2–16 hours, wash in 50% ethanol and then in several changes of 70% ethanol to remove traces of picric acid. Such explants may be stored in 70% ethanol for future dehydration and paraffin infiltration. Monolayers should be fixed for ½– 2 hours, washed in 50% and 70% ethanol as above. Further treatment will depend upon the stain to be used.

Picric acid (1.4 gm/100 ml dist. H_2O)	75 ml
Formalin (40% formaldehyde)	25 ml
Glacial acetic acid	5 ml

Staining

1. Toluidine blue
   For a 0.25% solution, dissolve 0.25 gm in 100 ml of distilled water.
2. May-Grunwald-Giemsa Stain
   This stain is recommended for monolayer cultures to demonstrate differentially ribo- and deoxyribo-nucleoproteins (RNA-protein and DNA-protein). DNA-protein stains red-purple while RNA-protein stains blue. Ribonuclease treatment for one hour may be used as a control.
   Materials:

   Stock May-Grunwald Stain 2.5 gm
   Dissolve in absolute methanol to 1000 ml. Age 1 mo. Filter.

   Stock Giemsa 1.0 gm
   Dissolve in 66 ml of glycerol at 55–60°C for 1.5–2.0 hours; add 66 ml of absolute methanol.

Procedure

1. Wash the cells in three rinses of warm BSS.
2. Fix for 5 minutes in *absolute* methanol. Agitate during fixation.
3. Stain for 10 minutes in filtered stock May-Grunwald solution.
4. Stain for 20 minutes in dilute Giemsa solution (dilute 1:15 in distilled water just before use).
5. Rinse rapidly in distilled water (10–20 sec).
6. Quickly rinse in 2 changes of acetone to dehydrate tissue. *Do not let coverslip or slide dry.*
7. Clear by rinsing three times in acetone-xylol (2:1), three times in acetone-xylol (1:2), and 10 minutes in fresh xylol.
8. Mount in balsam.
9. Examine the stained cells under the microscope (see figure 4.6 for normal appearance).

The Ward's animal tissue culture kit contains FAA fixative and May-Grunwald/Giemsa stains as well as equipment and glassware needed for animal tissue culture (see Appendix V). The materials and supplies routinely needed for a class size of twenty students are also listed in Appendix V.

F. Suggested Laboratory Schedule of Experimentation and Assignments

1. Laboratory scheduling
   Some theory, cytological observation and the techniques of subculture and making a primary explant can be initiated during the *first period.* The *second period* can be used to reinforce microscope optics (phase)

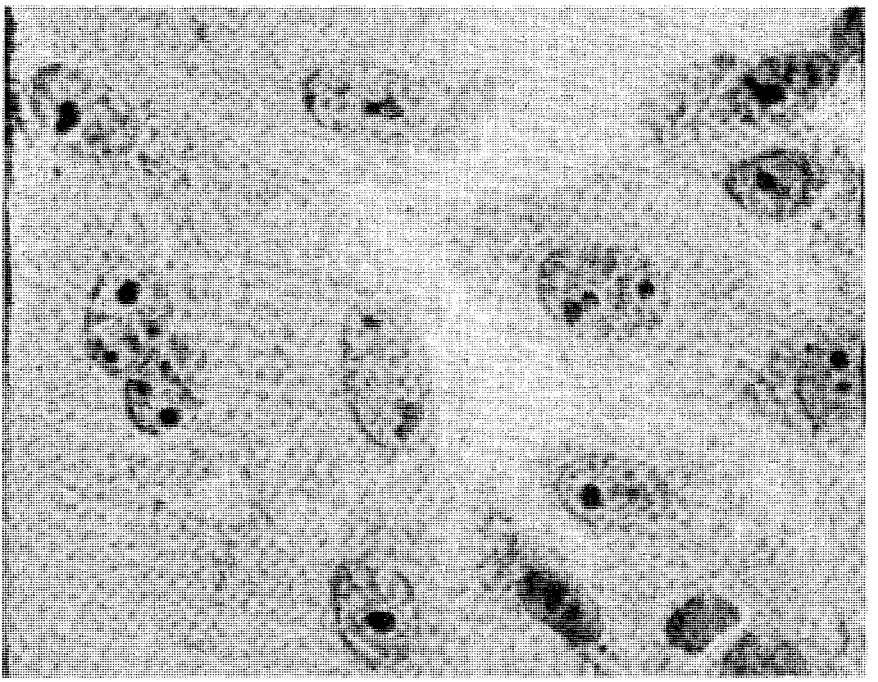


Figure 4.6. Veros cells stained with May Grunwald/Giemsa stain, 800x, Ektachrome (ET with 82A Wrattan filter), printed on black-and-white Panalure paper (Kodak).

and microscopy procedures (cell counting, etc.) as well as continuing the feeding of subcultured cells, observing cytology, and perhaps staining and making permanent slides. Staining would be practical only if laboratory sessions were once a week and the subcultured cells of week one were ready for subculture. One flask could be subcultured and the other used for staining. A modified approach would be to subculture some of the cells (week one) into specialized chambers (Sykes-Moore) or culture tubes containing cover slips so that a monolayer on glass would be ready for staining the second week. The third and fourth periods may be devoted to mini-research projects using monolayer subcultures as well as continued maintenance, cytological observation, staining, and photography. The student should have submitted a research proposal by the second period for third and fourth period research efforts. Part of a total laboratory period can be reserved in two weeks for reporting and class discussion of the research (mini-symposium style).

The two-week time period is realistic considering the time involved for photographic processing (assuming Ektachrome tungsten film is used) and/or drying time for permanent mount microscope slides.

2. Laboratory assignments
   The initial student assignment will be to culture an assigned cell line as well as prepare a primary explant from a chick embryo (i.e., Wards format in Appendix V) or another tissue type. This can be a relatively independent study, so the student should be encouraged to engage in preliminary reference reading and organize a time-table of experimentation. Following this, a proposal should be submitted to the instructor for approval. The following techniques could be tried; many of the animal tissue culture techniques can be performed concurrent with plant tissue culture studies should they be attempted.

The results of the plant tissue culture study should reflect the experimental design and may be expressed in several ways. Some of the more obvious studies would be a study of plant cytology including drawings, staining, photography, time-lapse photography, effect of nutritional additives, or cell specialization culminating in organ and/or plantlet development.

The chick embryo (or other tissue) primary explant should be attempted. You could use the Wards brochure—"The living animal cell: cell and organ culture" that accompanies their tissue culture kits as listed in Appendix V. In this section of the laboratory the student should make observations (possibly photography) of monolayer cultures, perform subcultures when appropriate and study the monolayer cells after they have been fixed and stained.

A third approach would be to have the students culture and subculture a cell line obtained commercially (see Appendix V). They should become proficient at subculturing into flasks, cover slip cultures or perfusion chambers. Once the student is comfortable with this technique it would be relatively easy to use the technique for setting up experiments in autoradiography, time-lapse cinematography, and routine characterization of the cell type through staining and/or photography.

The student research notebook and project write-up should contain as a minimum—observations, labeled diagrams and photographs of cell cytology. Both phase optics observations and brightfield microscopy of stained monolayers should be a part of this.

In addition a substantial portion of the student laboratory report should be concerned with the methods and techniques of all culture, subculture and starting a primary explant.

References

Eddington, L.C. Teaching cell biology: experimental format. The American Biology Teacher 34:6:329–331; 1972.

GIBCO. Price and reference manual. Grand Island Biological Company; Grand Island, NY; 1979.

A good source of information on media formulations and standard procedures in tissue culture.

Krause, P.F., Jr; Patterson, M.D., Jr., editors. Tissue culture: methods and applications. Academic Press; New York, NY; 1973.

An excellent compilation of specific methods for culturing various types of animal tissues. Recommended highly.

Merchant, D.J.; Kahn, R.H.; Murphy, W.H. Handbook of cell and organ culture. Burgess Publishing Company; Minneapolis, MN; 1964.

A basic guide to media and procedures for tissue culture.

Murashige, T. Plant propagation through tissue culture. Annual Review of Plant Physiology 25:135–166; 1974.

A detailed review of experimental rationale for current plant culture formulations.

Paul, J. Cell and tissue culture. Churchill-Livingstone; Edinburgh; 1975.

An extremely well written textbook on the science of tissue culture.

Steward, F.C.; Mapes, M.O.; Kent, A.E.; Holstein, R.D. Growth and development of cultured plant cells. Science 143:20; 1964.

An overview of the growth requirements and metabolism of plant cells grown in culture.

Steward, F.C.; Mapes, M.O.; Smith, J. Growth and organized development of cultured cells, I.-Growth and division of freely suspended cells. American Journal of Botany 45:9:693-703; 1958.

Steward, F.C.; Mapes, M.O.; Mears, K. II.-Organization in cultures grown from freely suspended cells. American Journal of Botany 34:10:709–713; 1958.

The above three references provide a good detailed insight into the techniques of plant tissue culture. They are available together as a Bobbs-Merrill reprint number B-305.

Steward, F.C. Analysis of growth: the responses of cells and tissues in culture in plant physiology: a treatise. Volume VI. Academic Press; New York, NY; 1969.

A comprehensive review of plant tissue culture.

Tissue Culture Association. Decennial review conference on cell tissue and organ culture. National Cancer Institute; Bethesda, MD: Monograph 26; 1967.

An interesting overview of tissue culture with some experimental applications.

Ward's. The living animal cell: cell and organ culture. Ward's Natural Science Establishment, Inc.; Rochester, NY; 1965.

Recommended brochure for simple laboratory experiments using the chick embryo. Written by D.J. Merchant.

Willmer, E., editor. Cells and tissues in culture. Volumes I and II. 1965.

A classic authoritative source of information on various methods and approaches to tissue culture, a strong emphasis on invertebrate culture technology.

APPENDIX I
Tissue Culture Vocabulary

Tissue Culture—Tissue culture is concerned with the study of cells, tissues, and organs explanted from organisms and maintained or grown *in vitro* for more than 24 hours. Dependent upon whether cells, tissues, or organs are to be maintained or grown, two methodological approaches have been developed in the field of tissue culture:

Cell culture—The growing of cells *in vitro* including the culture of single cells. In cell cultures the cells are no longer organized into tissues but exist as individual cellular units.

Tissue or organ culture—The maintenance or growth of tissues, organ primordia, or the whole or parts of an organ *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function.

Explant—An excised fragment of a tissue or an organ used to initiate a primary culture.

Monolayer—A single layer of cells growing on a surface of a plastic or glass flask or specialized chamber.

Suspension culture—A type of culture in which cells multiply while suspended in medium. They are prevented from attaching to the surface due to a deficiency of calcium in the medium.

Primary culture—A culture started from cells, tissues, or organs taken directly from organisms (see explant). A primary culture may be regarded as a cell line once it is subcultured.

Planting—The process of adding cells and new medium to a culture vessel for subculture purposes. The term may also denote the attachment of cells in a chamber.

Clone—A population of cells derived from a single cell through mitotic cell division.

Subculture—The transfer of cells from one culture vessel to another (see planting).

Trypsinization—The process of adding trypsin to cells to break down and dislodge the cell—cell attachments. The term may also be used generally to refer to the addition of EDTA solution (Versene) for the purpose of chelating calcium and thus facilitating cell—cell disattachment.

Balanced Salt Solution (BSS)—A mixture of buffer salts and ions having a pH, ionic strength, and osmolarity (isotonicity) compatible with mammalian tissues.

Cell Generation Time—The interval between consecutive divisions of a cell.

Population Doubling Time—When referring to an entire population of cells and indicates the interval in which, for example, 1×10^6 cells increase to 2×10^6 cells; not synonymous with "Cell generation time."

Viable Cell Count—Addition of a 'vital' stain such as trypsin blue to cells. Those that exclude the blue dye are living or vital. A viable cell count is necessary when performing a primary explant.

Plating Efficiency—The percentage of individual cells which give rise to colonies when inoculated into culture vessels. Generally a density of 500,000 cells per milliliter produce a successful subculture.

Confluence—A condition of tissue culture flask saturation with cells. Confluence would indicate that subculture is needed.

Fibroblasts—Fibroblasts are spindle or irregular-shaped mesodermal cells that give rise to muscle, cartilage, bone, or connective tissue cells. In cell cultures many other cell types are morphologically indistinguishable from fibroblasts.

Epithelial-like Cells—In cell cultures epithelial cells may assume various shapes but tend to form sheets of closely adherent polygonal cells.

APPENDIX II
Perfusion Chamber Techniques

Time-lapse cinematography is an excellent method for studying and recording the normal activity of single cells *in vitro* when used in conjunction with a perfusion chamber. In addition, the effect of agents such as drugs, hormones, detergents, or carcinogens may be demonstrated in the living cell with this technique. A number of ingenious and practical designs for continuous perfusion have been proposed. Essentially all such systems consist of a chamber in which (a) cells may be maintained for short periods of time, (b) the fluid medium may be perfused over the cells in an interrupted or continuous flow from an external reservoir, and (c) the cells may be visualized easily with phase contrast optics. The Sykes-Moore perfusion chamber and Rose chamber are especially convenient systems. Depending upon the technique to be used to add cells, the chamber can be autoclaved completely disassembled or only partially assembled. In the method described here, the cells will be grown as a monolayer culture on a coverslip. When the monolayer is established, the coverglass is aseptically transferred in an inverted position to the partially assembled sterile chamber of the Sykes-Moore chamber and the top screwed on to complete the assembly. The chamber is then filled with media. There are two sizes of silicone rubber "O" ring gaskets that can be used with the chamber. The thicker of the two gives a 2.5 mm working distance and is preferred for routine work. The thinner (1.5 mm working distance) gasket is particularly recommended for use in time-lapse cinematography where Kohler illumination is required.

A. Materials:

One Sykes-Moore perfusion chamber
Three sterile round coverglasses, 25-mm, #1
One "O" ring gasket, silicone rubber, 1½ mm thick
One holder for perfusion chamber
One wrench assembly
One sterile curved forceps
One petri dish, 60-mm
One sterile 1 ml hypodermic syringe
Two sterile 4 oz. widemouth French square bottle with sterile #7 rubber stopper, or tube with cover slip
Monolayer culture of cells in mid-log growth phase
25 ml sterile media

B. Procedure:

1. Assemble the bottom half of the perfusion chamber by placing a round coverglass inside of the deeper half of the chamber (part with inside threads). On top of the coverglass, place the "O" ring gasket. The assembled bottom assembly is placed inside a 60-mm petri dish. The top (outside threads) is placed alongside the bottom assembly *with the wrench holes up.* Sterilize by autoclaving.
2. Two round coverglasses are placed along one wall of a widemouth 4 oz. French square bottle or tube, and autoclaved with an aluminum foil cover.
3. Harvest cells as directed in section III-A.
4. Add 10 ml of the suspension of cells (1.5–2 x 10^5 cells/ml) to the sterile French square bottle or tube so the coverglasses are completely covered by the medium. Replace the aluminum foil with a sterile rubber stopper. Incubate at 35–37°C until the cells are attached to the coverglass (a minimum of two

hours). When the cells are attached, aseptically remove a coverglass with the sterile forceps, invert and place on top of the sterile "O" ring gasket in the bottom assembly. The top part of the chamber is aseptically set in place and carefully tightened with a wrench. *BE CAREFUL! DO NOT OVERTIGHTEN!!*

5. A sterile hypodermic needle is introduced into the chamber through one of the four access holes in the assembly and through the rubber gasket. This needle will provide an air escape when the chamber is filled.
6. Aseptically inject 1 ml of medium with the second needle introduced into the chamber as directed in step 5, using the hole opposite the air escape needle. The medium should be added SLOWLY in order to maintain cell attachment and to avoid creating bubbles within the chamber.
7. Remove both needles and place the chamber in the holder. Cells may be observed and photographed immediately or incubated at 35–37°C.

   Note: Two types of perfusion techniques may be practiced:

   a. Constant—where the media slowly enters and leaves the chamber. A gravity-flow technique can be designed.
   b. Intermittent—for most purposes, this may be the easiest. The old media may be withdrawn and new media injected at appropriate intervals.

The Sykes-Móore perfusion chamber is recommended by reason of its availability (see Appendix V). The Rose chamber has been classically used in research cinematography studies but is usually hard to secure commercially.

The common plastic flask (Falcon or Corning line) or flaskette is a very convenient flask for primary culture or subculture (figure 4.7a). Its use is dependent on the availability of inverted microscopes in the laboratory. The flask plastic ware is the most convenient vehicle for maintaining routine subcultures of a cell line in the laboratory.

APPENDIX III
Laboratory Photographic Procedures

The following observations are presented to help you master a few of the common techniques used in laboratory photography and subsequent darkroom processing procedures.

A. PHOTOGRAPHY UNDER DAYLIGHT CONDITIONS—For outdoor macrophotography use a 35mm single lens reflex camera and follow the rule of thumb where at an F stop of 16 it is customary to shoot a fraction of second that matches ASA of the film. For example Tri-X black and white film at an ASA of 400 using an F stop of 16 may be shot at 1/400 second exposure. If time permits it is instructive for the students to take a roll of outdoor photographs followed by a roll of indoor laboratory shots. The exposure would have to be determined by use of a light meter.

B. MICROSCOPE PHOTOGRAPHY— Select an appropriate camera/body tube adaptor or commercial microscope camera equipment (refer to figure 4.1). If your microscope does not have a trinocular head or separate photographic tube, a regular camera adaptor may have to be ordered through a local camera store. If tungsten illumination is available use a number 82a wrattan filter (blue) for Ektachrome (ET) color film. Use through-the-lens light metering or an external light meter to get an f-stop reading. You can predict that a 15-watt illumination

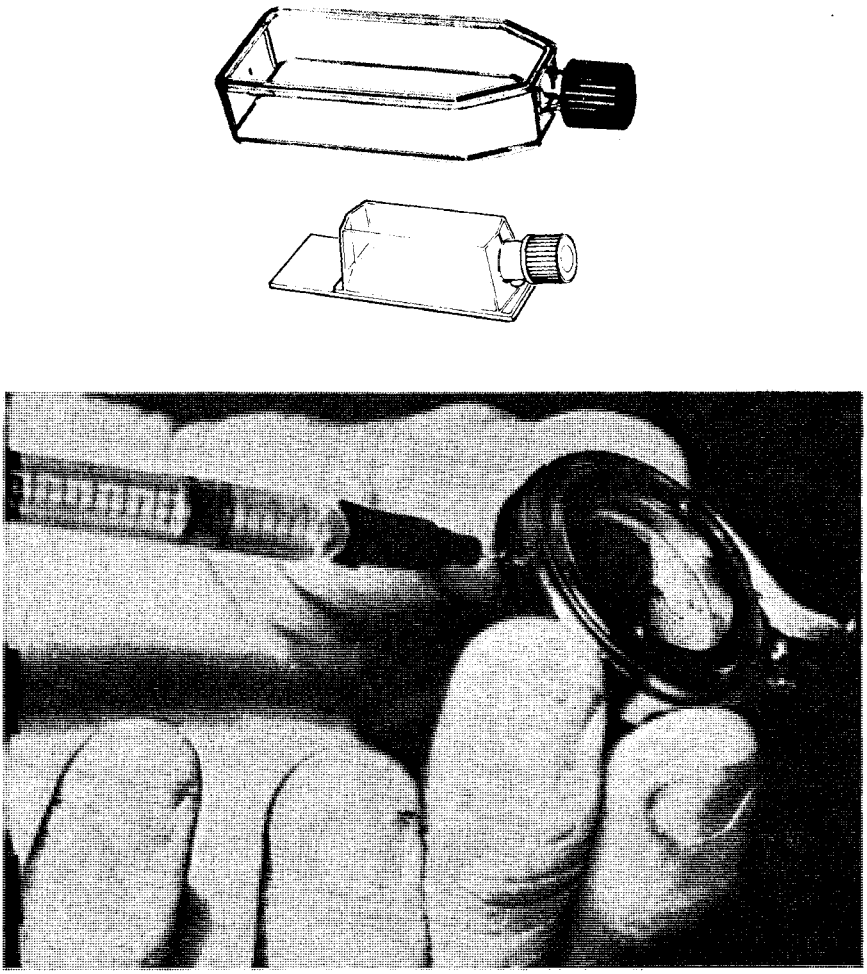


Figure 4.7. A. Typical tissue culture flask and flaskette (lab tech). B. Filling a Sykes-Moore Chamber.

source at 8 volts will give a plus x film (ASA 125) exposure of 1 second with a 10x phase objective, and a tri-x film (ASA 400) exposure of ¼ sec. using a 10x phase objective. Black-and-white polaroid film is faster and exposure can be as fast as 1/15–1/60 sec. with a 10x brightfield objective.

You may be successful in adapting the following table for use with your microscope and illuminator. This was originally determined by the author by simply shooting a series of test strips at different exposures, developing the negative and judging the best exposure range to use with cells or tissues, bright field or phase optics. Most laboratories use 8-volt tungsten illumination with 12-watt bulbs; therefore the condition may be quite similar.

Table 4.1. Summary of photographic conditions used in tissue culture photomicroscopy (illuminator voltage at 8 volts).

Film Type	*(ASA)*	*Optics*	*Objective*	*Exposure*	*Wrattan Filter*
Plus X	(125)	Brightfield	4x	1/15–1/30	58
			10x	1/4–1/8	58
			20x	1/4–1/2	58
			40x	1/2–1	58
			100x	>2	58
		Phase	10x	1/4–1	58
			20x	1–2	58
Tri-X	(400)—cut exposure by ¼ from plus x setting				
ET (Ektachrome Kodak)	160	Brightfield	4x	1/250	82a
			10x	1/250–1/125	82a
			20x	1/125–1/60	82a
			40x	1/60–1/15	82a
			100x	1/15–1/4	82a

Note: A quartz-Halogen illumination source (100 watts) must be used with the automatic exposure control or using above exposures at 6 v.

You may wish to design a form which would have categories like the above so that the student can match exposure and negative number with the specimen photographed. Having the student keep such information helps reinforce the understanding of film types, ASA speed, and the relationship of objective magnification, numerical aperture, light relations, color correction filters, and optimal illuminator filament temperature (voltage). Emphasize the importance of keeping photographic records and encourage the students to attach the negatives (stored in envelopes) to the record form and file in a folder.

C. PHOTOGRAPHIC THEORY—Photons of light may be captured by $Ag^+ - Br^-$ salt crystals suspended in an emulsion; this causes a reduction of silver (Ag) and forms a 'latent image'. Films having larger $Ag^+ - Br^-$ crystals generally have a higher ASA rating, which indicates they are more efficient at capturing available light during an exposure. This also means that they tend to be a coarse-grained film, therefore permitting less fine latent image detail or resolution. Plus x film (ASA 125) appears to be the best all around film for laboratory use. Treatment with a developer solution makes the latent image visible. A rapid darkening of the emulsion occurs in approximately thirty seconds when the reduced silver particles in the grain are oxidized to elemental silver (Ag_2). This occurs by the combined action of hydroquione and sodium hyposulfate ($Na_2S_2O_2$) chemicals in an alkaline solution (Na_2CO_3). During development the multilayered emulsion may swell and result in fogging or distortion. Decreasing the concentration of the developer (1:1, 1:2, etc.) may help slow the developing time and thus produce smaller clumps of Ag grains and a finer-grained less contrasty appearance.

The acid-stop bath step lowers the pH of the emulsion and inhibits further developer reaction. Generally a 3% acetic acid solution is used.

The fixation step solubilizes the unused developer chemicals and the unreacted $Ag^+ - Br^-$ grains. It contains sodium hyposulfate ($Na_2S_2O_3$) and an alum salt which helps stabilize the elemental Ag grains and hardens the emulsion. The final wash step completes the removal of previous chemicals and continues the hardening of the emulsion. Cold running tap water should be used. Prints may be air-dried if a double-weight paper is used; otherwise, blot dry with toweling and place in a paper dryer. Place the emulsion side next to the ferrotype plate to produce a glossy finish; emulsion-side-up to give a matte finish. Press the prints in a book until they stay flat.

D. ENLARGER AND DARKROOM TECHNIQUE—The enlarger is used for making positive prints from a negative and functions essentially like a slide projector. Place the negative end in the appropriate-sized holder, emulsion-side-down, and project on to the easel. Adjust the magnification setting, easel framing, and focus. It is generally wise not to enlarge over 10x. Printing a 4x5 black-and-white negative at an f stop setting of 11 would need a 2–5 sec. exposure—depending upon the contrast of the negative. Always focus on eyes (portrait) or nucleolus (cell). If you are not sure of correct exposure times it is best to cut some small test strips and do an exposure series so as not to waste printing paper. Overexposure always results in a fast darkening of the paper. When printing several pictures at one time it is advisable to mark the print number on the back with a pencil before it is exposed. Select the printing paper on the basis of the desired objective. For example, normal photographs are printed on F-2 paper so as to give black-gray-white soft tones; cells generally give a low-contrast and brilliance of the picture. Refer to table 4.2 for a general schedule of photographic development procedure.

Acufine developer used straight doubles the ASA index and means that you could compensate for an underexposed negative. Use a Kodak OC safelight for black and white printing. The filter transmits light at a wavelength that lies outside the normal color sensitivity range of the emulsion and therefore prevents fogging. Use an 8-watt bulb at a minimum of four feet from the working area and hang up the developed negative roll with a weight so that it will dry straight.

Table 4.2. Schedule for Photographic Negative Development and Printing:

Type of film or Printing paper	*Develop* @ 70° F*	*Acid Stopbath*	*Fixer*	*Water*	*Photoflo and Rinse*
Plus X pan ASA 125 D-76 (1:1)	7 min	45–90 sec	5–6 min	20–60 min	yes
Tri X pan ASA 400 D-76 (1:1)	10 min	45–90 sec	10–15 min	20–60 min	yes
Microdol X	10 min	45–90 sec	10–15 min	20–60 min	yes
Kodabromide printing paper					
Dektol or D-72 (1:2)	30–90 sec	45–90 sec	10–15 min	20–40 min	—

*Agitate every 30 seconds when running a small tank.

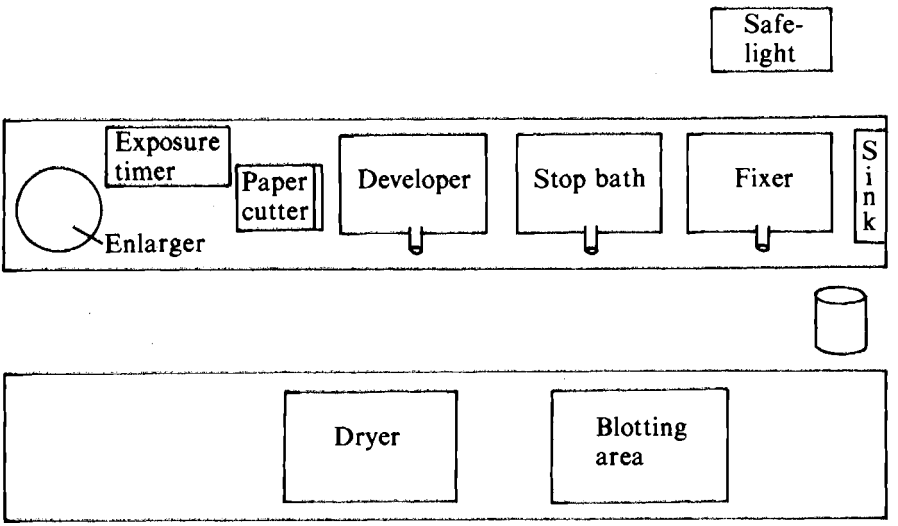


Figure 4.8. Diagram of a typical darkroom design.

Used developer can be saved for about one month. The fixer can be reused until it turns yellow. Do *not* mix fixer and developer at any time. Store solutions in well-marked amber glass jugs.

E. DARKROOM RULES

a. Arrange darkroom work areas, equipment, glassware, and solutions before starting (see figure 4.8).
b. Keep toweling handy and *wipe up* all spills immediately.
c. *Clean* all equipment, glassware, and working area thoroughly before leaving.
d. Do not leave your negatives or prints in the work area.
e. When making solutions always date and initial the container.

APPENDIX IV
Keeping a Student Research Notebook

An integral part of the tissue culture section of laboratory is the format of data collection and experimental write-up (see Eddington 1972).

It is suggested that the student enter laboratory work in a notebook using the format below:

(Left Page)	(Right Page)
Rough Data Page and Calculations	Lab # <u>Name</u> Date Purpose and Introduction: Methods: Results: Discussion and Conclusions:

This resembles closely the accepted reporting style of most scientific journals. It is helpful to communicate this fact to the student during the pre-laboratory session.

LABORATORY OR RESEARCH PROJECT WRITE-UP

Experiment Number and Title

Name of student
Date of experiment

Purpose and Introduction: A statement of the central principle(s) the experiment is to demonstrate. The experimental hypothesis and/or method theory and pre-laboratory lecture notes may be entered here as well as pertinent background reading and citation of other scientific work. The student may wish to leave one page for PURPOSE alone so that the introductory information gathered from reference sources can be summarized. If the tissue culture-related experiments are to be written up separately from the notebook, the purpose can be changed to an "abstract" and the introduction can be extended as a literative review and summary of theory.

Methods: This section may be compressed or lengthy depending upon the amount of experimental format given in the experiment. If the complete procedure is given the student can enter "given in experiment." If supplemental procedures are given by the laboratory instructor then they should be recorded in this section. In all cases it is useful to have the student submit a flow chart or outline of the experiment beforehand and this then can be added into this section when the experiment is written up.

Results: The data obtained from the experiment, whether it be drawings, graphs, charts, short statements, or demonstrations, etc., should be completely labeled and identified. All observations made with the microscope must include drawings that give the source of the material, stain used, and the magnification.

Discussion and Conclusions: In this section the experimental results are analyzed, discussed and related to the original purpose, if possible. Individual as well as class results should be dealt with in the section. There also may be further literature to cite as it correlates with the general experimental design and results in the experiment or by the instructor. Any questions given should be answered or included in this section as well as a final summary of the major accomplishments of the experiment.

Bibliography: Reference sources cited according to the following example—Pelczar, M.J. and Reid, R.D., 1965, *Bacteriological Techniques*, Science 14:111; 213–215 (Title of article, Source, Volume, Number, Pages)

One important precaution should be noted at this point—students will have a tendency to become "caught up" with the techniques of tissue culture to the exclusion of learning the theory of cell culture, staining, microscope optics, etc. The laboratory instructor's suggestions and requirements are important in helping the student focus on real goals and objectives. Learning tissue culture techniques may be only one of the objectives.

APPENDIX V
Tissue Culture Supplies, Equipment, and Publications

A. SUPPLIES AND EQUIPMENT

The following list represents an approximation of materials, equipment, and supplies needed to support twenty students' experimentation for two or four laboratory periods. It is assumed that one instructor will be working with an assistant

and that tissue culture experimentation will be self-contained in one laboratory facility.

Equipment:

(1) Refrigerator with freezer compartment
(1) Incubator set at 37.5°C
(2) Clinical centrifuge (lab top)
(1) Autoclave or pressure cooker
(10) Microscopes—three or four objectives
(2–4) Inverted microscopes (preferably with phase optics)
(20–40) Sykes-Moore chambers (optional)
(100) Sterile tuberculin syringes with 26-gauge ⅝-inch needles. (optional)
(100) One ml disposable pipettes—volumetric
(50) Five ml disposable pipettes—volumetric
(100) disposable pasteur pipettes
(20) pipette bulbs
(50) 16x125 mm culture tubes
(100) tissue culture flasks-plastic, 75 cm^2
(S/P catalogue number T4160–75)
(2 boxes each) coverslips—#1
9x24 mm
25 mm round
(2) Repipettes—for dispensing plant medium
(1–2) hood with germicidal light

It is assumed that common laboratory items are available: glass rods, curved-tip scalpels, microscope slides, coplin dishes, test tube racks, bunsen burners, beakers, measuring cylinders, volumetric flasks, petri dishes, centrifuge tubes, 100-ml blank bottles, and a glass still or source of deionized water.

All glassware used for holding media or tissues and dissecting instruments should be sterile.

I have found it very convenient to dispense media into sterile 100-ml blanks and formulate complete media in 100-ml aliquots. This insures against contamination and wastage of the bulk supply. Repipettes are extraordinarily useful in dispensing media aliquots to subculture flasks and tubes. The repipette with media may be held in the refrigerator on non-lab days.

SUPPLIES

The following represents the main tissue culture media components and are listed in the format of a purchase order to Grand Island Biological Company (catalogue 1979).

			Price
2x500 ml	310–4190	Pbs. Dulbeccos (1x) w/o Ca^{++}, Mg^{++}, $NaHCO_3$	6.75 ea.
20 ml	320–5030	1-glutamine (200mM) (100x)	3.75
2x100 ml	610–5300	Trypsin-EDTA Solution (1x)	2.85 ea.
20 ml	600–5240	Antibiotic-Antimycotic Solution (100x)	4.20
100 ml	630–5250	Trypan Blue Stain 0.4%	3.05

2x500 ml	380–2360	MEM Eagle w/Earles Salts, W/25mM Hepes buffer, w/o 1-Glutamine	14.85 ea.
100 ml	200–6140	Fetal bovine serum	8.80
2x100 ml	560–1740	Whites Medium 5–3 for plant culture w/ Chlorophenol red	3.90 ea.

Please ship to arrive day, date, year.

Mailing address: Grand Island Biological Co.
519 Aldo Ave.
Santa Clara, CA 95050

If teaching kits are used (see Ward's catalogue) then most supplies and media are provided. Tissue culture media packs can be ordered separately as well as viable chicken eggs if one is going to perform a primary explant.

A recent communication with Ward's Natural Science Establishment, Inc., indicated that by 1980 the following tissue cultures kits would be available:

Plant Tissue Culture Kit — Ward's #88W030
Animal Tissue Culture Kit — Ward's Present #88W8000
Sub Kit #1—Dispensed whole embryo project
Sub Kit #2—Propagation of cells in monolayer
Sub Kit #3—Histogenesis of bone project

The idea behind the reorganization of the existing teaching kits is to offer difficulty graded kits as well as sub kits allowing educators the opportunity to initiate tissue culture with a lower initial cost.

Companies that carry plant and animal culture media and supplies are:

Grand Island Biological Company
519 Aldo Avenue
Santa Clara, CA 95050
or
4175 Staley Road
Grand Island, NY 14072

Ward's Natural Science Establishment, Inc.
P.O. Box 1712
Rochester, NY 14603

Flow Laboratories
936 West Ayde Park Blvd.
Inglewood, CA 90302
(213) 674–2900

Science Dimensions*
P.O. Box 582
2157 Miner Street
Costa Mesa, CA 92627
(714) 545–4498

*This is a small company but offers good quality educational type kits for the beginner using (HeLa and Veros cell lines). They are also in the process of marketing a super eight-time lapse drive and microscope adapter available at a very reasonable cost.

CELL LINES 120 cm^2 FLASK MONOLAYER

Green African monkey kidney (Veros)	GIBCO #720–1002
HeLa	Flow laboratories #03–117
Rainbow trout gonad	Flow Laboratories #02–734

Most monolayer flask cultures cost approximately $25–35.00 per unit. Leighton tube cultures with coverslip monolayers are available (they are convenient for a single lab) but a minimum order is sometimes prohibitive.

B. TISSUE CULTURE EQUIPMENT AND GLASSWARE SUPPLIERS:

Bellco Biological Glassware

Bellco Glass, Inc.
340 Edrudg Rd.
Vineland, NJ 08350, telephone (609) 691–1075

Corning Products for tissue culture and cell biology

Customer Service Department
Science Products Division
Corning Glass Works
Corning, NY 14830, telephone (607) 974–4126

Lab-Tek Tissue Culture Chamber/Slide

Miles Laboratories, Inc.
Lab-Tek Division
30 W. 475 N. Aurora Rd.
Naperville, IL 60540

Plastic Tissue Culture Products

Flow Laboratories
1710 Chapman Ave.
Rockville, MD 20852, telephone (301) 881–2900

Tissue Culture Labware:

Gibco Laboratories
3175 Staley Rd.
Grand Island, NY

Tissue Culture Association, Inc.

1 Bank St., Suite 210
Garthersburg, MD 20760, Telephone (301) 869–2900

General publications pertinent to Tissue Culture Association

TCA Report
TCA Manual
In Vitro

The latter is a journal of the Tissue Culture Association.

The Tissue Culture Association also supports a center which hosts seminars and educational workshops in the areas of Tissue Culture Techniques, education and research methods. You may be placed on this mailing list by writing to:

W. Alton Jones Cell Science Center
Lake Placid, NY

Chapter 5

Use of a Plant Pathogen to Examine Koch's Postulates*

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*Funds for the development of these materials were provided in part by the National Science Foundation in their program for undergraduate instructional improvement.

I. Introduction

This exercise allows students to examine Koch's postulates, the foundation of pathogenic microbiology. It does not require the expense or facilities involved with the use of test animals, nor does it involve any of the dangers associated with using animal pathogens. Students are able to: (1) reproduce Koch's postulates using a vegetable pathogen, *Erwinia carotovora,* and a carrot as a model host tissue; (2) distinguish between the ability to grow on a host and the ability to damage the host using a second organism, *Escherichia coli.* These two points highlight the distinction between normal flora and pathogens.

This exercise has been used for several years in a microbiology service course for agriculture, home economics and humanities majors. The students ranged from freshmen to seniors and from those with no previous biology or chemistry courses to those who had completed organic chemistry. This exercise is the first in an investigative project dealing with pathogens and, as such, extensive detail is provided for the students. This was done intentionally to acquaint students with the components of experimental design; succeeding experiments in the project require the students to be responsible for increasingly greater portions of the experimental design. The basic element of the exercise is quite straightforward, making it suitable for a variety of introductory-level laboratories in which Koch's postulates are to be examined. The exercise can be used in botany, general biology, microbiology, or plant pathology laboratories. The key modifications would fall into three categories: (1) modifying the student materials to remove the present detail; (2) changing the initial source of the pathogen by having students isolate their own pathogens from a mixed population on a rotted vegetable (obviously, a pure culture of *Erwinia* is not a "natural" situation); (3) expanding the section that deals with the characterization of the pathogen. The last modification could be a good place to introduce those biochemical tests used in clinical identification exercises in many courses. Preparation time and performance time depend, in part, on the students' training and available facilities. The only techniques students must know are basic aseptic technique and the Gram stain. Preparation for this exercise requires that the following be done:

1. Maintenance of the organism: The *Erwinia* culture must be transferred to a fresh nutrient agar slant or carrot slice every 4–6 weeks.
2. Preparation of infected carrot slices for students: This involves about one hour for a class of 20 students, one to two days before the exercise is begun.
3. Preparation of materials for the exercise: This will vary depending on the extent to which one provides materials for the students or has them prepare the materials. If the instructor does this, it will take approximately three hours, for media preparation and pouring plates.

The time involved for student performance can be apportioned in several ways. If no storage points are used, the exercise will require approximately one hour/day for four days running, assuming that the students have had prior experience in aseptic transfer and the preparation of Gram stains. If they have not, a simple laboratory exercise in aseptic technique and Gram stain should be done first. These techniques are described in Appendix II and Appendix III. The exercise need not be done on consecutive days. Generally, the time intervals (see student materials) may be increased to two days; if a longer interval is necessary the materials can be stored in a refrigerator or cold room between steps.

I will describe several follow-experiments that have been used with this exercise to highlight various aspects of pathogenic microbiology and how to use this material in an investigative format in Appendix I.

II. Student Materials

Upon first realizing that we exist in a world filled with bacteria, most people's immediate response is "Ugh!" There are microorganisms on every object with which we deal and in and on many locations on our bodies. You must realize that this is the natural circumstance and learn to differentiate between those organisms whose existence is either harmless or beneficial to us and those which produce disease, the pathogens.

A pathogen is something that generates suffering. The word comes from the Greek words "pathos," meaning suffering, and "genesis," meaning origin. Thus, a pathogenic or disease-causing organism is not simply one which will grow on a host, but one that damages the host in some way by its presence.

The first step in the study of a pathogen and the disease it causes is to isolate and identify the pathogenic organism. This can be quite difficult since a variety of organisms is often present during the diseased state and the pathogen must be distinguished from the other organisms. In bacterial rots, for example, as the primary pathogen rots the vegetable, the rotted tissue provides a food source for many microorganisms and many different types may be present.

One of the major achievements in the early history of microbiology was the development by Robert Koch of a set of criteria by which one can identify the organism responsible for a particular disease.

Koch's Postulates:

1. The presumptive pathogen must be present in all cases of the disease.
2. It must be isolated in pure culture from a diseased organism.
3. Inoculation of the isolated pathogen into a healthy organism should cause the same disease.

4. One must be able to re-isolate the pathogen from the newly infected organism.

This exercise deals with the study of pathogens and disease. Due to the difficulty one has in obtaining human subjects, we will confine our studies to the investigation of "sick" vegetables. We will investigate plant pathogens and use the destruction of vegetable tissue as a model disease system. In this exercise, you will (1) use Koch's postulates to show that a particular organism is a pathogen and (2) demonstrate that another organism which also grows on the vegetable is not necessarily a pathogen within the context of our definition.

Koch's Postulates Exercise

Part 1—Day 1

1. Obtain or pour a nutrient agar (NA) plate.
2. Prepare carrot slices as follows. Cut 3 slices of carrot approximately ¼" thick. Using forceps, dip one slice into alcohol. Pass the slice through a flame. The carrot will catch on fire. Let the flame burn out and IMMEDIATELY place the carrot slice into a sterile plastic petri plate. Repeat for the other two slices, placing each in a separate plate. Add 5 ml sterile water to each plate.
3. From the rotting carrot provided, aseptically transfer one loopful of rotting material to the surface of a carrot slice in a plate. Label the plate #1. Examine the rot organism under the microscope using the Gram stain.
4. Using a sterile loop, mock-inoculate a carrot stick in a second plate. Why is this done? Label the plate #2.
5. Immediately after the mock inoculation, streak the loop onto an NA plate. Why? Label the plate NA #2.
6. Transfer a loopful of *E. coli* to the carrot slice in a third plate. Label the plate #3.
7. Incubate the three carrot plates and the NA plate at 30°C for 24 hours.

Day 2

8. Check the plates for (a) growth of microorganisms on each carrot slice (check by observing under the microscope using the Gram stain) and (b) apparent rot of slices. The centers of the slices often show the first signs of rot; they appear macerated and torn/shredded, lacking in firmness when compared to the tissue of a healthy carrot. The slices may even become liquefied, or nearly liquefied, due to the rot organism which attacks the material that makes the carrot tissue firm. You can test for firmness by poking the slice with a sterile loop.

Part 2: Can be done on Day 2

1. Obtain or pour 2 NA plates.
2. Obtain isolated colonies from plates #1 and #3 by streaking a small amount of material from the inoculated slices in each plate onto separate plates of NA. Use the dilution streak technique. Label the plate made from material in plate #1, NA #1, and the plate from the material in plate #3, NA #3.
3. Incubate the NA plates at 30°C for 24 hours.
4. The carrot plates #1 and #3 may be kept until you are sure there is growth on the NA plates. They may then be discarded, along with plate #2, by sealing with tape and placing in a special garbage pail.

Part 3: Day 3

1. 48 hours from the beginning of the experiment, prepare carrot plate #4 using the procedure in Part 1. Inoculate with a loopful of material from an isolated colony from plate NA #1. Be sure to use a rod-shaped organism.
2. Incubate carrot plate #4 at 30°C for 24–48 hours.
3. Examine the isolated colonies on NA #1 and NA #3 from Part 2 using Gram stains.

Part 4: Day 4

1. Obtain or pour an NA plate.
2. Check for rot in carrot plate #4. (Use criteria described in Part 1: #8)
3. Do a Gram stain on the material from liquid or carrot in plate #4.
4. Obtain isolated colonies from carrot plate #4 by streaking a small amount of material from the inoculated slice onto an NA plate. Label the plate #4 NA.
5. Incubate plate for 24 hr. at 30°C. Check an isolated colony with a Gram stain.

Discussion

1. Explain the way in which the results you obtained relate to Koch's postulates.
2. Does the Gram stain alone provide enough information to prove that the disease-causing organism you finally isolate is the same one with which you started? What else should be done?
3. What effects would contaminants have on your conclusion as to the cause of the rot?
4. Would you conclude that *E. coli* is or is not a pathogen in the context of this experiment?

III. Instructor's Materials

This exercise is technically simple, but does require care at several steps. For the exercise to be successful, you must use a pure culture of *Erwinia carotovora,* which must be maintained in an uncontaminated state, particularly on the carrot slices given to students. While students can reproduce Koch's postulates starting with a mixed culture (possibly a good idea for an advanced class), students in introductory courses have great difficulty when faced with contaminants.

A. Obtaining culture: The organisms used in this exercise are *Escherichia coli* (any strain) and *Erwinia carotovora* ATCC #25270. The *Erwinia* can be obtained from the American Type Culture Collection. The ATCC catalog lists other strains that cause soft rot, but this is the strain which we used. If you use this strain, you must order the culture several months ahead of time since it is a plant pathogen and you must receive clearance from your state agricultural commission to obtain the organism. Forms for this purpose will be sent to you by ATCC on their receipt of your order. The forms must be forwarded to the proper authority, who will notify ATCC. Clearance generally does not pose a problem in obtaining a culture. Because this is a plant pathogen, *all infected materials must be autoclaved before discarding.*

B. Setting up stock culture:
   1. ATCC provides a lyophilized culture. Instructions for starting the culture are sent by ATCC. You will need nutrient broth (NB-Difco). A culture grown up in NB can be stored in the refrigerator (4°C) for several months.
   2. After starting the culture in liquid, streak a loopful of culture onto nutrient agar (NA-Difco) to obtain isolated colonies (see Appendix II) and incubate 24–48 hrs./30°C.
   3. Check an isolated colony with a Gram stain (see Appendix II) to make sure it contains only small, Gram-negative rods. Use this same colony to inoculate a carrot slice to check for rot. See below.
   4. You should identify several colonies in this way and prepare a stock culture from each. This can be done in the following manner:
      a. Streak onto the surface of an NA slant, incubate 24–48 hrs./30°C and store in a refrigerator (4°C) for up to six months.
      b. Stab a tube of NA with the culture. Incubate 48 hrs./30°C. Tape the tube shut and store for 12–14 months in a refrigerator.

         Use one of these two methods for storage of permanent stocks.

      c. Inoculate a sterile carrot slice (see below, Section D) by touching a drop of culture to the surface of the carrot. Maintain at room

temperature. Transfer some of the rot material every 2–3 weeks to a fresh carrot slice. This is not the method of choice for long-term stock maintenance.

5. A simpler procedure for culture maintenance may be used, but there is a much higher risk of contamination. After starting a liquid culture of the *Erwinia,* inoculate 100 ml of NB with a drop or two of culture and incubate for 24–48 hrs./30°C. This culture may be used to inoculate the carrot slices and they can be stored for several months in the refrigerator. A loopful of this may be transferred to fresh NB for subsequent cultures.

C. Once a year, it is a good idea to key out the culture using Bergey's *Manual* and the strain data provided by ATCC to make sure you are carrying the original strain.

D. Preparation of a carrot slice and inoculation.

1. Start with a healthy carrot. Wash carrot with alcohol using a cotton swab. Peel. Cut three slices of carrot approximately ¼″ thick. Using forceps, dip one slice into alcohol. Pass the slice through a flame. The carrot will catch on fire. Let the flame burn out and IMMEDIATELY place the carrot slice into a sterile plastic petri plate. Repeat for the other two slices, placing each in a separate plate. Add 5 ml sterile water to each plate.
2. Transfer a loopful of *Erwinia* culture onto the surface of a carrot slice. Incubate inoculated carrot slices for 24–48 hrs./30°C. Check for rot simply by observing the slice and by touching it with a sterile loop. It is quite clear from the feel whether the carrot is rotting.

E. A deficiency in the experimental protocol is the use of the Gram stain as the only means of determining that the *Erwinia* remains the rot-producing organism and not a contaminant. The organism should actually be keyed out thoroughly in a more rigorous experiment. You may streak a line of cells from any sample to be tested, particularly NA1, NA3 and NA4, onto a plate of Simmon's citrate agar. This will differentiate between *Erwinia* and *E. coli* and is a simple way to help confirm the identity of the *Erwinia.*

F. Time course of experiment for students

1. First lab: obtain infected carrot
   transfer infection to fresh carrot
   interval: 24–48 hours/30°C or plates can be stored at room temperature for up to a week
2. Second lab: streak to isolate
   interval: 24–48 hours/30°C. Plates must then be stored in the refrigerator if not used immediately. Can be stored one week.

3. Third lab: carrot slice
   interval: 24–48 hours/30°C or can be stored at room temperature for one week
4. Fourth lab: streak to isolate
   interval: 24–48 hours/30°C. Plates must then be stored in the refrigerator (up to one week) if not used immediately.
5. Fifth lab: check second streak

G. Materials and suppliers for Koch's postulate exercise:

1. Suppliers

Difco Laboratories Detroit, Michigan 48232	Nutrient broth Nutrient agar Gram stain kit
Scientific Products 1750 Stoneridge Dr. Stone Mountain, GA 30083 or Fisher Scientific Products P.O. Box 829 Norcross, Georgia 30091	Plastic petri plates (100 × 15 mm) (Disposable plates are strongly recommended, for the carrot slices are difficult to clean out of glass plates. Disposable plates can be autoclaved with rotted material in them.)
American Type Culture Collection 12301 Parklawn Drive Rockville, Maryland 20852	

2. Materials for 20 students

80 sterile plastic petri plates (for carrot slices)	20 scalpels or spatulas (to slice carrots)
60 glass petri plates (sterile)	20 forceps
60 NA pours (25 ml/pour) (plates may be pre-poured for students)	20 bottles of 30 ml sterile water each
20 beakers of 10 ml 95% or absolute ethanol	60 ml stock culture of *E. coli* grown for 24–48 hrs. in NB
20 slices of *E. carotovora*-infected carrot in 20 separate petri plates	30°C incubator
	20 bunsen burners
	20 inoculating loops
	glass slides

20 Gram stain kits: dropper bottles of
(a) crystal violet;
(b) Lugol's iodine
(c) 95% alcohol
(d) safranin
50°C water bath for cooling melted agar

References

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APPENDIX I

Use of Related Materials in an Investigative Format

The exercise on Koch's postulates was developed as part of a course in which students participated in a multi-level investigative project. The investigative format is used to develop an understanding of what constitutes scientific investigation that transcends the piecemeal approach which stresses the learning of loosely related facts and the pursuit of cookbook experimentation. The factual and conceptual material is better understood and provides a basis for the integration of new information as it arises. The students are engaged in the same process as scientists, working toward a goal, as in actual scientific investigation.

In the course, students first learn basic laboratory technique and are then introduced to experimental design through a series of experiments. The first experiment in the series is presented with all details provided, from background information to ways in which results are interpreted. Subsequent experiments require that the student provide more and more of the design components. Finally, the students are given a catalog of techniques and assays and asked to develop a project in a particular area.

The Koch's postulates exercise was developed as the first experiment in a project that is intended to introduce the student to the area of pathogenic microbiology.

Once the identity of the pathogen has been established, a number of aspects of the disease may be studied. The means by which the organism damages the plant tissue can be examined. This is mediated by a set of enzymes which degrade the pectin found in the tissue. The activity of these enzymes may be measured as a function of colony growth or by measuring their presence in the growth medium. The vegetables

may be examined for the presence of other organisms that cause rot, or soil and other locations may be checked for this particular pathogen. Students can study the course of the disease itself, the spread of the organism or how it enters the vegetable. Students may also examine the effect of different storage conditions on the course of the disease or those factors involved in resistance to the disease or loss of virulence of the pathogen. The following are capsule forms of exercises we have used successfully as followups to the Koch's postulates experiment.

I. Sick potato– live tissue
   A. Protocol exactly as given in student materials except for step 1 in part 1-day 1, where potato blocks are used instead of carrots. Prepare potato blocks as follows: scrub and rinse a potato. Soak potato for 10 min in a 7:3 ethanol: clorox mixture. Rinse with sterile water. Slice ends off potato with a sterile knife, then make 4 cuts to obtain rectangular block.
   B. This is an alternative to using carrot, but is not used routinely in large groups due to the difficulty in sterilizing the tissue. Can be made to work in small groups.

II. Sick potato—dead tissue
   A. Again protocol is the same as given except for tissue preparation. In this case, potatoes are peeled, cut into small blocks, placed in test tubes to which 5 ml water is added and autoclaved for 10 min.
   B. This is another alternative to using carrot but is not strictly Koch's postulates since the tissue is not live. It is easy to set up a large lab and the students are generally not aware of the difference between live and dead tissue.

III. Assay

Pectic substances occur in nature solely in the cell walls of plants, usually as the insoluble structural material, protopectin. A group of more soluble derivatives of this native substance can be extracted from plant tissues; these are the pectic acids and pectin. Pectic or polygalacturonic acid is usually considered to be a homopolysaccharide made up of unbalanced chains of α—(1,4)—linked D-galacturonic acid residues; pectin itself is pectic acid with varying extents of methyl esterification (Starr and Chatterjee). Two basic types of pectin enzymes exist: pectin esterase which simply removes methoxyl residues from pectin, and a range of depolymerizing enzymes . . . which can be distinguished under three headings: whether pectin or pectic acid is the preferred substrate; whether they act by transelimination or hydrolysis; and whether cleavage is random (endo-) or endwise (exo-) (Progress in Industrial Microbiology).

Pectin is available from several sources; Na polygalacturonate came from Sigma.

   A. Growth on solid media
      1. PEC-SSA medium (Starr, Chatterjee, Starr, Buchanan). This is a gel of polypectate medium; if pectin digestion activity is present, colonies will sink into the agar. This medium is used to show pectin degrading activity wih moderately vigorous digesters; for weak digesters, omit the agar from the recipe.

         Place 100 ml distilled water and a magnetic stirring bar in a 1-liter Erlenmeyer flask on a heater with a magnetic stirrer. Add the following ingredients in order, while stirring and heating: 0.6 ml 10% aqueous $CaCl_2$ $2H_2O$; 1.0 ml of 0.1% aqueous bromothymol blue in 6.4×10^{-4} N NaOH: 0.5 g Difco yeast extract; 3.0 g sodium polygalacturonate; and 0.3% Difco agar. Heat almost to boiling, watching carefully, as material has a tendency to foam. While solution is hot, adjust pH to 7.3 with 1 N NaOH. Autoclave and pour plates (PEC medium cannot be remelted).

2. PEC-YA medium (Starr, Chatterjee, Starr, Buchanan). Pectate digestion activity is shown by flooding a 24-hour plate with 2 N HCl. The areas around colonies that have digested pectate will show as clear against a hazier area of precipitated pectate. This is a good medium for spreading and isolating organisms. Replica plating can be done when isolating colonies. Theoretically, you can use pectin in this medium, but it has worked better for us with polypectate. Individual batches of polypectate should be tested before classroom use.

   Place 100 ml distilled water and a magnet stirring bar in a 1-liter Erlenmeyer flask on a heater with a magnetic stirrer. Add the following ingredients in order, while stirring and heating: 1.0 g polygalacturonic acid; 1.0 g yeast extract; 1.0 ml of 1.0% aqueous BTB in 6.4×10^{-4} N NaOH; and 1.5 g agar. Heat as described for PEC-SSA, adjust pH to 7.3 and autoclave. Pour plates. To use this medium, streak or spot cultures on surface and allow to grow. Flood plates with 2 N HCl. Score by appearance of clear halos around and beneath colonies in otherwise turbid medium. See special instructions in Starr *et al.* (1974).

B. Enzyme activity in culture medium: viscosity

Viscosity measurements have found widespread use for determination of pectinolytic activity. This assay is based on the loss of viscosity of aqueous solutions of pectin or sodium pectate following decrease in chain length (Progress in Industrial Microbiology).

While there are quantitative assays using the Oswald viscometer and similar methods, we have found that a similar method using a narrow bore, long-tipped pipet gives sufficient accuracy in a classroom situation.

Protocol:

1. Prepare a 5% solution of pectin in water.
2. Place 1 ml pectin solution/tube. Prepare one tube for each solution to be tested and one control.
3. Add 1 ml solution to be tested to pectin. Add 1 ml water to control. Mix well.
4. Mix 0 time reading as follows: fill 1-ml pipet with pectin mixture. Time solution as it runs out of pipet to the 0.8-ml mark.
5. Take readings of run-out time at 5, 20, 15 min or as required.

IV. Isolation of rot-producing organisms: Crystal violet pectate medium (Cuppels and Kelman)

A. This method allows one to isolate Gram-negative rotters from field soil and distinguishes *Erwinia* from *Pseudomonas*. Using this medium you can quantify the amount of soft-rot organisms present in soil samples. We have had some problems with this medium; the pH determination is critical.

PROCEDURE FOR PREPARATION OF CRYSTAL VIOLET PECTATE MEDIUM FOR ISOLATION OF PECTOLYTIC BACTERIA (Cuppels and Kelman, 1974)

1. Preheat 5-cup Waring blender by rinsing with hot water.
2. Place 500 ml boiling water (distilled) in the blender.
3. Using a rheostat to control the speed of the blender, start the blender at low speed and add:
   a. 1.0 ml 0.75% (w/r) aqueous crystal violet solution (final concentration = 1.5 ppm crystal violet)
   b. 4.5 ml 1 N NaOH (8 g NaOH/200 ml)

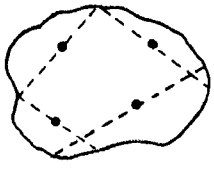
c. 3 ml 10% $CaCl_2 \cdot 2H_20$ (Use fresh solution. Do not store stock solution for more than 2 weeks)
d. 2.5 g Difco agar
e. 1 g $NaNO_3$

4. Blend at high speed for 15 sec.
5. Slowly add 15 g sodium polypectate while blending so that the sodium polypectate will not clump. Blend at high speed for 15 sec.
6. Place medium in a 2-liter flask. Add 0.5 ml 10% sodium lauryl sulfate (SDS-laboratory grade).
7. Cap with aluminum foil rather than a cotton plug. Autoclave for 25 min at 120°C and 15 lb pressure. Allow pressure to drop *slowly* to avoid bubbles.
8. Pour plates as soon as possible. Plates should be permitted to dry for 48 hr at room temperature before being used. *No surface water should be present at time plates are used.* Note: This is a very important aspect in the successful use of this medium.
9. (Optional) Add 0.9 ml 1% thallium nitrate-filter sterilized (final concentration = 0.0018%) to the hot medium (500 ml).
   Addition of solution of thallium nitrate to give a final concentration of 0.0175 mg/ml in CVP medium will greatly enhance reduction of background populations, but also slightly reduce recovery of *Erwinia*. Medium containing thallium nitrate should be used immediately. There is an increase in toxicity to bacteria including *Erwinia* if this medium is stored.

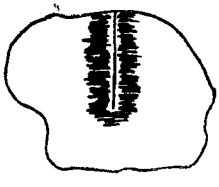
V. Environmental factors affecting rot in vegetables

The key to examining these factors is a tissue system in which the amount of rot can be quantified. Once this is developed, one can examine the effect of environmental factors on the course of the disease. The tissue we use is potato. Its use yields relatively reproducible results.

A. Use a firm, healthy potato with no surface lesions. Swab the potato surface with alcohol. Using a sterile needle dipped into a liquid culture of the pathogen, inoculate four sites on the potato, making the inoculations as far from each other as possible. Use one potato for each environmental variable you wish to study. Incubate each potato in a beaker covered with foil. Environmental variables may include temperature, time, atmosphere (nitrogen, carbon dioxide, etc.), relative humidity, etc. At the time selected, remove the potato from the beaker and slice through the point of inoculation perpendicular to the surface. The rot will have spread perpendicularly to the initial stab, so you may measure the distance it has spread from the initial inoculation line. It is very easy to distinguish healthy tissue from rotted tissue. The average of four sites is used to increase the reproducibility of the data. One can then plot the spread of rot against the variable examined.



• Site of inoculation
Cutting line



Spread of rot from site of inoculation (cross-section)

B. A flamed dissecting needle will work for making the inoculations, although the precision of the inoculation can be increased by using a Hamilton syringe. In this case, you may inject a maximum of 5 microliters/site.

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APPENDIX II

I. Procedure for basic smear preparation and a simple stain

A simple stain involves the use of a single dye and enables us to see the general shape and arrangement of cells.

Materials

crystal violet	various cultures will be available in the laboratory
safranin	
methylene blue	slides

Protocol

1. Pass a clean glass slide through a flame to clean off lint, etc. Place slide on table, flamed side up.
2. Hold an inoculating loop using the thumb and index finger.
3. Insert the loop in the upper area of the flame at a 60° angle until the entire wire portion is heated red-hot to kill any organisms on the loop.
4. Hold the culture tube in the palm of the left hand with the sides of the tube resting against the insides of the fingers. (If you are left-handed, reverse the directions.)

Hold the tube in place with the thumb. Remove the tube cap with the little finger of the hand holding the loop.

5. Pass the lip of the tube through the flame once to kill any organisms that might be present.
6. Insert the loop into the tube, jiggle it and withdraw a loopful of broth.
7. Flame the lip of the tube, bring the cap to the tube to close, and replace the tube in the rack.
8. Place the material on the loop onto the slide and spread the drop to cover an area approximately that of a dime. Reflame the loop before putting it down on the lab bench.

   The smear is made by spreading a *small* amount of cellular material from a liquid culture on the surface of a clean glass slide. (The directions for preparing a smear of a culture on an agar plate are given following step 14.) The resulting film is air-dried and "fixed" to the slide by passing it through the flame of a bunsen burner. Fixing not only kills the cells but also coagulates the cellular proteins, thus fastening or "fixing" the organisms to the slide. The material is then ready for staining. Fixed smears are exposed to a stain for a specific amount of time, then washed, blotted dry and examined under the microscope.

9. Allow the smear to air dry. Do not apply heat before the smear is dry since this may cause boiling, which will result in broken cells. You should be able to see the smear, but it should be thin and fairly transparent. Circle the smear area with a wax pencil.
10. When the smear is dry, fix it as described above. Do not overheat. The slide should be uncomfortably hot but not intolerable when touched to your wrist.
11. Apply about 5–6 drops of dye to the slide. Stain for 45–60 seconds if using crystal violet or safranin. Methylene blue requires 2–3 minutes for good staining.
12. Pour off the stain and rinse gently with slowly running water. Do not allow the water to drop directly onto the smear.
13. Dry the slide by blotting *(do not rub)* between pages of bibulous paper.
14. Examine the slide under the oil immersion lens of your microscope.

NOTE: The same procedure may be followed for staining bacterial material taken from agar with the following exception: Place a drop of tap water on the slide, then follow steps 1–5. To remove cells, merely touch the loop to a colony. This should remove sufficient cells. Emulsify the cell material in the drop of water and continue with step 7. Continue as described.

II. The Gram stain—a differential stain

A second type of stain widely used in microbiology is the differential stain. As the name implies, it takes advantage of a differential response to the stain procedure. This differential response may be between different cells or between different portions of the same cell. Differential stains may involve the use of more than one dye and, sometimes, a specific de-staining step between the dye applications.

The most widely used differential stain is the Gram stain. It is used to divide nearly all bacteria into two major groups: Gram-positive and Gram-negative. Gram-positive organisms (such as *Staphylococcus* and *Bacillus*) retain the initial violet stain; Gram-negative organisms (such as *Escherichia* and *Pseudomonas*) lose the primary stain when decolorized and are then restained by the red counterstain. The Gram

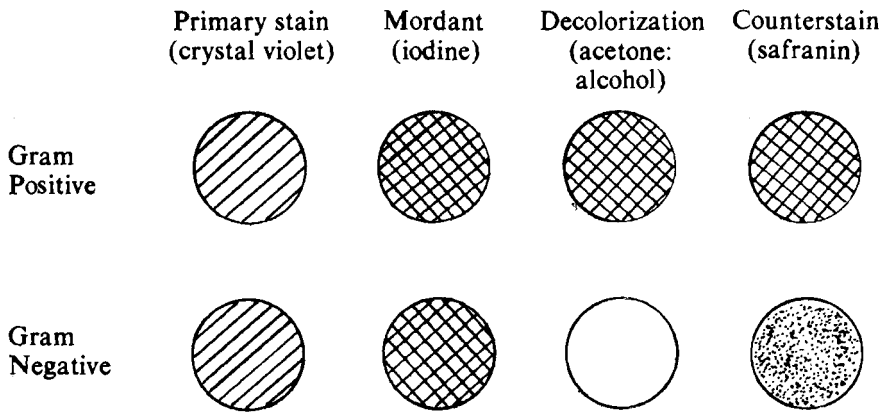
reaction, besides being a constant characteristic (although there are some exceptions) of a cell and of great value in identification, correlates well with a variety of cell properties. (See chart.)

Gram-Positive	*Gram-Negative*
More susceptible to penicillin, sulfonamides and basic dyes	Less susceptible to penicillin, sulfonamides (except G-diplococci)
Less susceptible to streptomycin	More susceptible to streptomycin
Lysozyme degrades cell wall	Lysozyme digests cell wall only with addition of EDTA or alkaline pH
Relatively thick cell walls, few acids, only 1–4% lipid	Cell walls slightly thinner, but more complex, consisting of several layers; most of the amino acids found in proteins, 22% lipid
Nutrient requirements often complex	Nutrient requirements usually relatively simple

The basis for the differential response is one of rate rather than an absolute characteristic of bacteria. Thus, the procedure must be performed with great care. Too long a decolorization will result in Gram-positive organisms appearing to be Gram-negative. Also, many Gram-positive bacteria become Gram-variable as the culture ages, so only young cultures should be used in determining the Gram reaction.

Although the mechanism of the Gram reaction is still unknown, it seems to be related to differences in the cell wall structure. The thicker, Gram-positive walls appear to be impermeable to the dye-iodine complex, while the dye-iodine complex washes out quickly in Gram-negative cells exposed to the lipid solvents acetone and alcohol. While we do not generally deal with them in lab, you should be aware that there are two other Gram reactions: the Gram-variables *(Neisseria)*, which may be either Gram-positive or Gram-negative, and the non-reactors which do not stain or stain very poorly *(Spirochaetes, Mycobacteria)*.

The sequence of steps in the Gram stain is shown below:



Materials

(Solution I) 1% aqueous crystal violet
(Solution II) 2% aqueous iodine
(Solution III) 95% ethanol:acetone (7:3)
(Solution IV) 2.5% safranin in 95% ethanol and water (1:9)
slides
various cultures will be available in the laboratory

Protocol

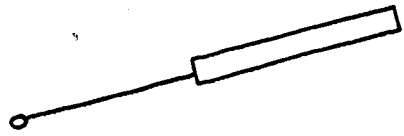
1. Prepare a smear of 12–18-hour culture. (See directions in Simple Stain.)
2. Fix the smear.
3. Stain I: Cover the smear with Solution I. Stain for 1–2 minutes. Pour off stain and rinse gently with a small amount of tap water.
4. Cover with Solution II for 2 minutes. Wash briefly with tap water.
5. Decolorize with Solution III by dripping it over the tilted slide until the wash is colorless. This is the most critical step and proper timing is important. It should not take more than 15–20 seconds.
6. Counterstain: Cover with Solution IV for one minute.
7. Rinse with tap water and blot carefully on bibulous paper.
8. View under oil immersion lens. Gram-positive bacteria are purple; Gram-negative bacteria are pink.

APPENDIX III
Aseptic Technique

Organisms must often be transferred from one container to another to begin growth in fresh medium or in a different medium, when changing growth conditions, for isolation, or for enumeration. This must be done without contaminating the test organism or having it contaminate anything. Since microorganisms are found everywhere, the test organism can be contaminated by another organism from the air, glassware, or people, thus spoiling the test results. Also, while we do not routinely work with disease-producing organisms, all microbes are potentially harmful under certain conditions, so we must control their spread. The methods of transfer all employ specific steps to minimize the possibility of contamination or spread. This general approach, termed aseptic technique, is not a particular method, but is a concept that covers almost every manipulation performed in the laboratory.

In this section we will describe the techniques for transfers used in growing microorganisms. In the section on stains you were told one method for sampling that involved aseptic technique. In the section on media you will learn how to perform transfers for isolation and enumeration.

There are two common tools to transfer organisms: the inoculating loop and the pipet. The inoculating loop we use is a thin metal wire formed into a loop at one end and held in a metal holder.



Pipets are basically calibrated glass tubes, tapered at one end, which are used like a straw for transferring a specific amount of liquid. If you have not previously used a pipet, the TAs will give a demonstration and a TV tape on pipetting is available for viewing.

Transfers are made of organisms growing in either solid or liquid media to either solid or liquid media. They may be from one tube to another tube (tube-to-tube), from a tube to a plate or from a plate to a plate.

The directions given below are for a right-handed person. If you are left-handed, merely reverse the directions.

Transfers Using a Loop

1. Tube-to-tube transfer
   a. Loosen the tube caps, but do not remove them.
   b. Hold both tubes in the palm of the left hand, with the sides of the tubes resting against the insides of the fingers and using the thumb to hold them in place. The tube containing material to be transferred should be nearest the little finger.
   c. Hold the loop between the thumb and index finger of the right hand. Insert the loop into the upper portion of the flame at about a 60° angle. Flame the loop until it is red-hot, remove from the flame and let it cool. Briefly count to ten.

      The loop is flamed to kill any organisms that are on it. It is then cooled so it does not fry organisms in the culture.

   d. Using the ring and little fingers of the right hand, remove the cap from the tube containing the culture and flame the lip of the tube, passing it through the flame only once.

      The tube is flamed to kill organisms around the lip, but it is flamed only briefly so the tube does not get too hot.

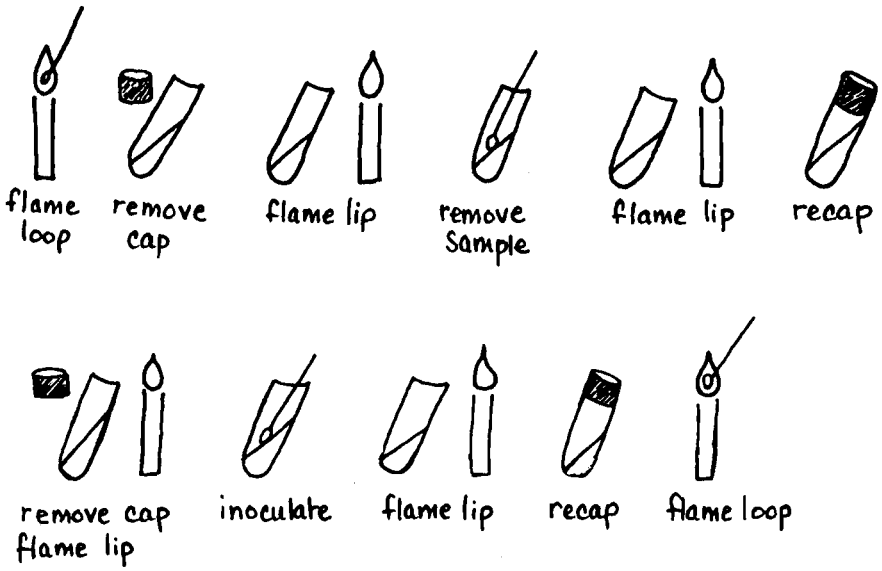
   e. Insert the cooled loop without touching the sides of the tube and just touch the loop to the culture material. (With liquid medium, just immerse the loop in the top portion of the liquid after vortexing the tube to ensure distribution of the organism.) This is usually sufficient to obtain the proper number of cells for transfer.
   f. Withdraw the loop, flame the lip of the tube and replace the cap by bringing the cap to the tube.

      The culture tube is capped before continuing with the manipulation to protect the original culture. The cap is brought *to* the tube, rather than vice versa, to minimize the possibility of contamination by organisms in the air.

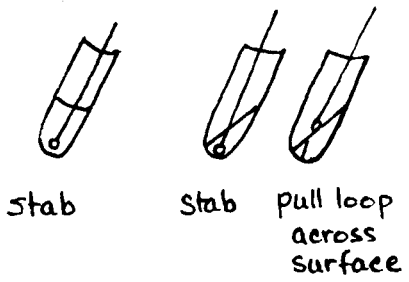
   g. Remove the cap of a fresh tube, flame the lip, insert the loop to the bottom of the slanted area of solid medium and draw it up across the surface to disperse the organisms on the fresh medium. (If transferring the liquid medium, insert the end of the loop into the liquid and gently jiggle the loop.) Withdraw the loop, flame the lip of the tube and replace the cap. Flame the loop.

      Flame the loop before putting it down on the lab bench to kill any organisms remaining on it.

   h. Tighten the caps on the two tubes and replace them in a rack.
   i. Summary:



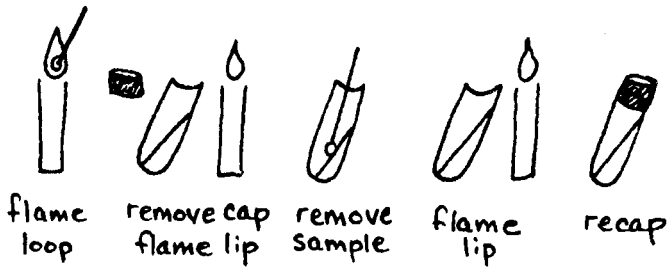
2. Stab/Stab and streak. These two methods are variations of the tube-to-tube transfer. In this procedure, the organism is removed from the first tube as described above. The fresh tube is inoculated by stabbing the inoculating loop through the medium to the bottom of the tube. Draw the loop straight out. For a stab-and-streak, stab to the bottom of the tube, then draw the loop back up across the slant surface. Complete the steps as described above.

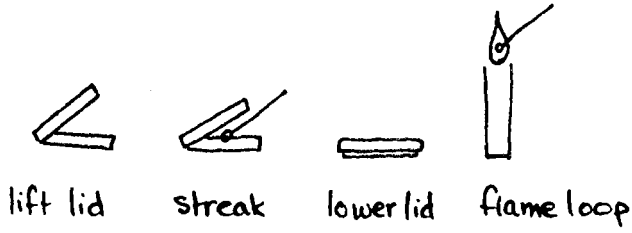


3. Tube-to-plate
   a. In this method of transfer, material is removed from the culture tube as described.
   b. Using the left hand, lift the lid of the plate to approximately a 45° angle.
   c. Insert the loop to touch the surface of the agar at the furthest point from the open side and zig-zag the loop across the surface.

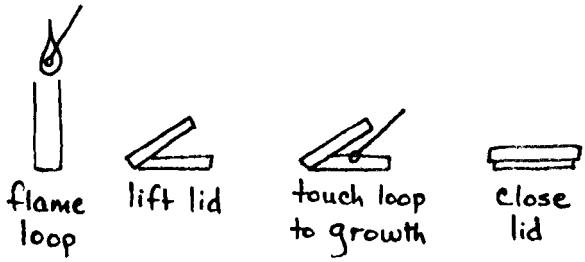
      In streaking the loop on an agar surface, you must be careful not to break into the agar with the loop.

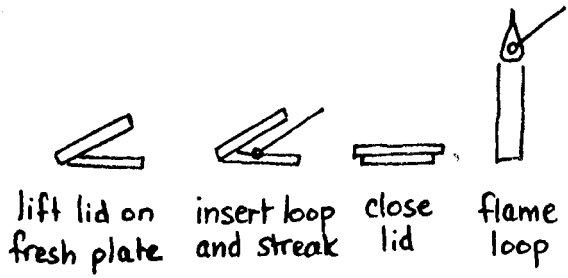
   d. Withdraw the loop, lower the lid and flame the loop.
   e. Summary:





4. Plate-to-plate:





Transfers Using a Pipet

When bacteria are in a liquid medium and must be transferred from one container to another, pipetting may be the method used, particularly when a specified quantity of material is to be transferred. This method is not used when dealing with a dangerous

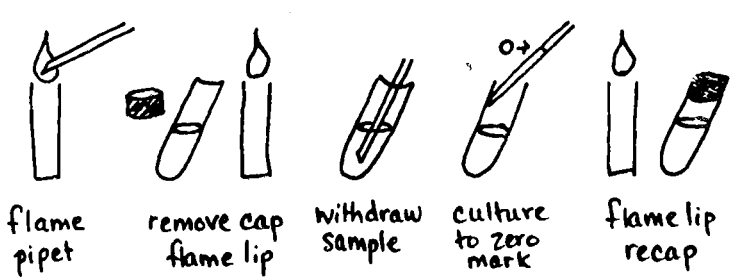
or potentially dangerous organism, as the possibility of ingesting some of the liquid while pipetting is too great.

Sterile pipets are kept in the lab in round or square metal cans labeled with the size pipet they contain. Bring the can to where it will be used. *Do not* carry sterile pipets across the room or allow them to touch anything or they will no longer be sterile. To remove sterile pipets from cans, lay the can on its side with the edge against the edge of the bench. Use the right hand to pull out a pipet from the top of the stack in the can, being careful not to touch the tip of the pipet to the ends of other pipets or to the lip of the can as these areas are usually not sterile. When using a sterile pipet, pass it through a flame only once to kill any surface bacteria before using it in sterile liquid. Do not overheat as this can be injurious to both bacteria and chemical solutions.

1. Tube-to-tube transfer
   a. Loosen the tube caps, but do not remove them.
   b. Hold both tubes in the left hand (see procedure for tube-to-tube transfers with a loop).
   c. Remove a sterile pipet from a pipet can using the method described above.

      If the pipet does touch something, discard it and take a fresh pipet. Do not put an unsterile pipet back in the can.
   d. Hold the pipet between the thumb and middle finger of the right hand, with the index finger resting loosely on top of the pipet. Flame the pipet by passing it once through the flame.

      An overheated pipet will kill many or all of the bacteria you are transferring. This can cause serious error when you are counting a specific volume of cells and is a factor to be considered if no growth appears after such a transfer.
   e. Using the little finger and palm of the right hand, remove the cap from the tube containing the culture. Flame the lip of the tube.
   f. Insert the pipet into the tube and withdraw culture to above the zero line on the pipet. Rest the tip of the pipet against the inside wall of the tube and let culture run out until the liquid is at the zero mark.
   g. Withdraw the pipet, flame the tube lip and bring the cap to the tube to close.
   h. Uncap the fresh tube, flame the lip, touch the tip of the pipet to the inside of the tube and let the required amount of inoculum run in.
   i. Withdraw the pipet, flame the tube lip and replace the cap.
   j. Immediately place the pipet, tip down, in a dirty pipet bucket. Never put a dirty pipet on the lab bench. Tighten the tube caps and replace the tubes in a rack.
   k. Summary:



2. Tube-to-plate transfers are similarly performed. As in transfers with a loop, the plate lid is lifted to a 45° angle. Inoculate the plate by touching the tip of the pipet to the center of the agar and letting the required amount of liquid run out. Remove the pipet, lower the plate lid and immediately place the pipet, tip down, in a dirty pipet bucket.

Chapter 6

Digestive Enzymes of the Cockroach

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Introduction

This experiment on digestion in cockroaches is an important part of my senior-level course in animal physiology, not only because it reinforces the lectures in digestion and enzymes, but also because it clarifies the fundamentals of statistics and introduces students to the critical thinking required in transforming data into a scientifically valid conclusion. The experiment also has the advantage of employing current research techniques. (See for example Bland and House 1971.) Yet, even freshmen in a general biology laboratory can complete the experiment in three hours, except for the protease test which requires brief observations overnight. Not the least advantage, from the instructor's point of view, is the study's inexpensiveness and ease of preparation. It uses common reagents and inexpensive cockroaches (see Appendix A), and requires less than three hours to prepare if the materials are at hand.

My upper-level undergraduates work in pairs, with each pair preparing enzyme extracts from homogenized salivary glands, crop, midgut and hindgut of a single cockroach, and also a control consisting of a heated mixture from the other extracts. Each pair of students then tests each extract and the control for either sucrase or lipase or amylase and protease. (Since the last two assays are so simple, the same students do both, using the same extracts.) Students rank the enzyme activities for each section on a scale from 0 to 3, with the control defined as 0. The instructor compiles the data and returns them to students to calculate the means and standard errors (optional) for each section. They then use the sign test to determine whether activity in each section is significantly greater than that in the control, or greater in one section than in another section with significant activity. In the sign test, P is determined simply from the proportion of cases in which the activity from one section was greater than in the control or in another section. (See Appendix B.) This nonparametric test is so transparent that students find that it clarifies basic principles of statistics, even (or especially!) if the students have already had a statistics course. Because the conclusions are based on compiled data, the results are immune to the effects of an occasional aberrant cockroach or student.

From the data, students can deduce which sections of the cockroach digestive system contain the enzymes. However, inferring from this which section actually secretes the enzyme is not always straightforward. Enzymes may travel from one section to another and not be rinsed off the tissue before the extract is prepared. Thus, even though all the enzymes are supposed to be secreted only by the midgut, except for amylase which is also secreted by the salivary glands, the crop sometimes shows significant activity. Students should be able to infer that this activity could be due to the much greater mass of tissue adsorbing enzymes from other sections. Students should also be able to suggest that significant enzyme activity in the hindgut might be due to its being "downstream" and hard to clean. Secretion of enzymes by

the hindgut would make little sense, because nutrients could not be absorbed from the hindgut into the hemolymph. Initially students are apt to view these deviations from what "the book says" as failures. Perhaps the most valuable lesson from the experiment is that whoever wrote the book was smart enough not to accept data at face value. Of course it is necessary to emphasize the difference between fudging data to get the "right" conclusion, and interpreting data in the discussion of a report. In addition to interpreting data, I also suggest that in report discussions students compare functions of the sections of the cockroach digestive system with analogous organs of mammals.

Student Material

The strategy of digestion in cockroaches is similar to that in humans and in other vertebrates: food passes from mouth to anus through a tube where it is mixed with digestive enzymes. A cockroach's meal, like yours, is first mixed with saliva. It then enters the crop (foregut), which can store up to a two month's supply of food. The contents of the crop eventually pass through the proventriculus (gizzard), which crushes solids and acts as a valve. The food then enters the midgut, which includes eight ceca at the anterior end. Almost all of the absorption of nutrients takes place in the midgut. The remaining material goes into the hindgut, which is joined to the midgut at the site of attachment of the Malpighian tubules, which are analogous to the vertebrate kidney. Finally, feces are formed in the rectum.

Although the overall process of digestion in cockroaches is similar to that in vertebrates, there are important differences in detail. For example, nutrients are not absorbed into capillaries in cockroaches, which lack circulatory vessels. Instead, the nutrients are absorbed directly into the body fluid (hemolymph) surrounding the digestive system. A second major difference is the absence of mucus to protect the inner lining of the cockroach gut. Instead, there is a continuously formed semi-permeable sac—the peritrophic membrane—which contains the food in the midgut, and a layer of impermeable chitin to protect the crop and hindgut. (Chitin is the material which also forms the exoskeleton.)

Cockroaches are notoriously omnivorous. We therefore expect them to employ a variety of digestive enzymes. From experience we know that they digest sucrose, proteins, starch and fats, so we would expect that somewhere in their digestive systems are secreted sucrase (invertase), protease, amylase and lipase. The question we wish to answer is "Where in the cockroach digestive system are these four kinds of enzymes secreted?". The first step in answering that question is to test the ability of extracts of the salivary glands, crop, midgut, and hindgut to digest sucrose, gelatin, starch and olive oil. High digestive activity in a section *suggests, but does not prove* that the enzyme is produced and secreted in that section.

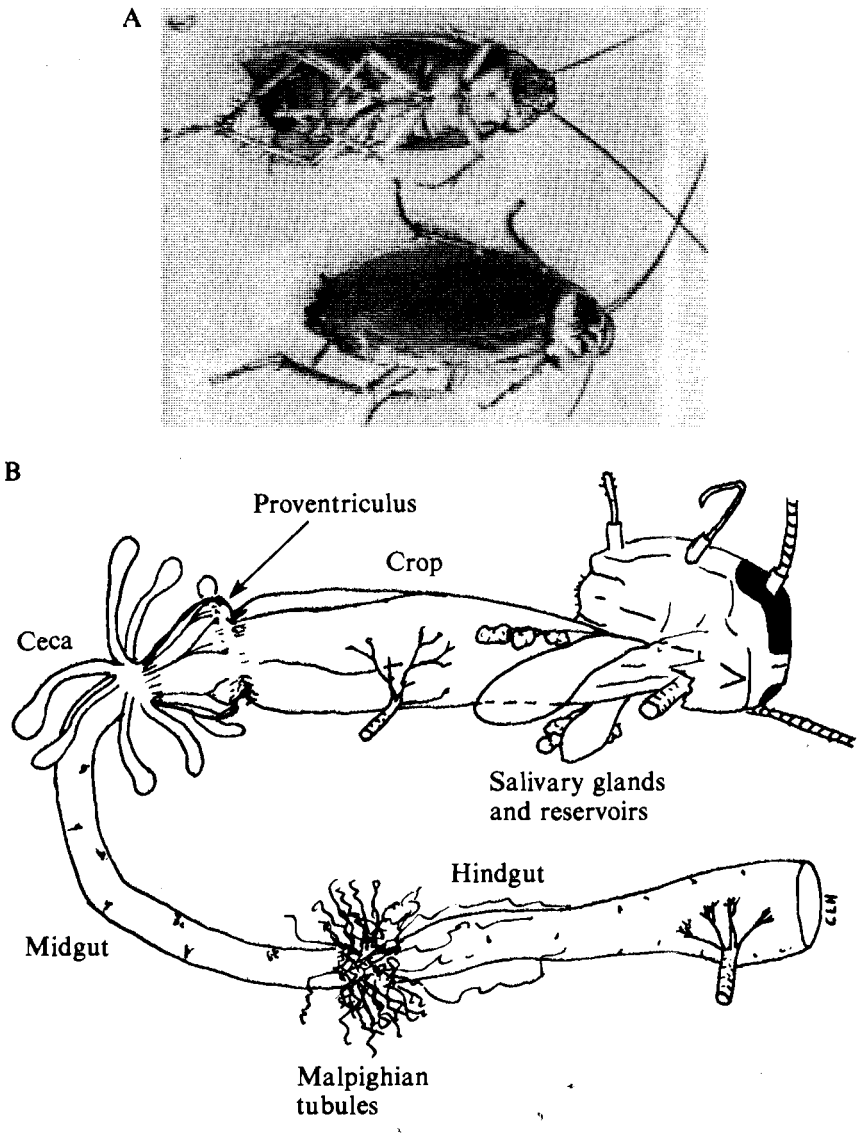

Figure 6.1. A. Adult American cockroaches, *Periplaneta americana*. Male, ventral view; female, dorsal view. Males may be distinguished from females by their more slender body and by the pair of styles on the anal segment. One style is visible between the hind legs of the male. The length of each cockroach, excluding antennae, is approximately 2 cm. B. Digestive system of the American cockroach. The gut is brown, the salivary glands are white, and the reservoir is transparent. The Malpighian tubules are yellow, and the tracheae are white.

Instructor's Materials

I. Preparation of enzyme extracts

A. Obtain a large cockroach which has been immobilized by CO_2, by cooling in a freezer for 15 min, or by holding it under warm water.

B. Cut the anus transversely to free the rectum, and remove the digestive tract by carefully pulling on the head. The entire digestive tract should come out with the attached head. Often the tract breaks at the proventriculus; if this happens cut open the abdomen and pull out the midgut and hindgut. Place the digestive tract into a watch-glass containing insect saline.

C. Using the dissecting microscope, identify and separate the salivary glands, crop, midgut and hindgut. The salivary glands are particularly difficult; they look like small white globules of fat just behind the head. Often the salivary glands are lost. If the glands are not present, leave a blank under S in the data. Discard the proventriculus and large tracheae. Also discard the part of the gut where the Malpighian tubules are attached. Small trachae may be included since they do not secrete digestive enzymes.

D. Cut open the crop and rinse. If possible, gently squeeze solids out of the midgut and hindgut, since these may have absorbed enzymes from other parts of the digestive tract.

E. Label 4 depressions of a spot plate S, C, M and H. Add 5 drops of water to each of the 4 depressions. Place the sections isolated in step C above into the appropriate depressions:

S salivary glands (with reservoirs)
C crop
M midgut and ceca (hereafter referred to as midgut)
H hindgut

F. Grind each section with the blunt glass rod to release enzymes from the cells. IMPORTANT: rinse the glass rod thoroughly when going from one section to another.

G. Use a separate Pasteur pipette to withdraw the liquid extract from each depression. Combine 1 drop of each extract into a fifth 10-ml test tube labelled K; the rest of the extract in each pipette goes into the appropriate 10-ml test tube labelled S, C, M and H.

IMPORTANT: keep the pipettes in correct order. They will be used in step I and for the protease and amylase tests.

H. Each extract should just fill the rounded bottom of the test tube (.2 ml). If necessary, add water to increase volume.

I. Place test tube K, containing the mixture from the four sections, into a boiling water bath for 15 min. This will be used as a control. (Why is this a control?)

J. Each group of students will do one of the following:
   1. Sucrase (procedure II.)
   2. Protease (III.) and amylase (IV.)
   3. Lipase (V.)

K. Data from all groups will be combined. Base your conclusions on the combined data.

II. Sucrase

A. Add to each of the 5 test tubes S, C, M, H and K, 1 ml of 5% sucrose solution. Incubate the tubes at 35°C for 1 hr.

B. During inoculation, label 5 additional 10-ml test tubes S, C, M, H and K, and to each test tube add the following:

   4 drops of distilled water
   1 drop of Fehling's solution A
   1 drop of Fehling's solution B

C. After incubation add 1 drop of incubated solution from each test tube in part A to the corresponding test tube in part B. Heat these test tubes for 10 min in a boiling water bath. (Save the remaining solutions from part A in case you have to repeat the test.)

D. A reddish copper precipitate will form in the test tubes containing reducing sugars (glucose and fructose)—that is, in the test tubes in which sucrose was hydrolyzed during incubation. Estimate the sucrase activity by the amount of precipitate formed. Enter the data on the Data Sheet.

III. Protease (This test must be done before any others using the same extract.)

A. Mark each of 3 strips of exposed and developed photographic film in 5 sections labelled S, C, M, H and K. (Write in pencil on the emulsion—non-glossy—side.)

B. Apply 1 drop from each extract on the appropriate section of the emulsion, using the Pasteur pipettes.

C. Place the strips of film into a petri dish on moist filter paper, being careful not to smear the drops.

D. Take the petri dishes home, and at approximately 6-hour intervals rinse off 1 strip with water.

E. A transparent spot on the film indicates digestion of the emulsion (a gelatin). Estimate the protease activity by the speed of digestion of the emulsion. Enter the data on the Data Sheet. *Return the petri dishes to the instructor.*

IV. Amylase

A. Add 10 drops of 1% starch solution to each of the five tubes S, C, M, H and K. Shake each tube to mix thoroughly.

B. Using the appropriate Pasteur pipette, immediately mix 1 drop from each tube with 1 drop of IKI solution in a depression of the porcelain

spot plate. A blue precipitate indicates that starch is still present in the solution. If the mixture remains amber, the starch has been digested in the tube. Use this indicator to judge how fast the starch is hydrolyzed by the extract of each section of the digestive system.

C. Repeat the procedure in step B every 10 min until you are satisfied that no further change will occur. Generally this will take less than 1 hr. Estimate the relative amylase activity by the speed of digestion, and enter the results on the Data Sheet.

V. Lipase

A. To each of 5 10-ml test tubes add the following, shaking frequently:
   5 drops of olive oil
   5 drops of ethanol
   5 drops of saturated phenol red solution
   a few drops of 10 mM NaOH, to make each solution definitely pink, but not claret

B. Add the contents of one of the above tubes to each of the tubes of extract from S, C, M, H and K. Shake. It is likely that the mixture in one or more tubes (especially M) will turn yellow. If it does, add drops of 10 mM NaOH until the color in all five tubes is the same.

C. Incubate the five tubes at 35°C for 1 hr. Check the tubes periodically and note which mixtures turn yellow fastest, due to acidification by the liberation of fatty acids from the olive oil. Enter the results in the Data Sheet.

DATA SHEET

Turn in one copy per experiment to the instructor.

Group # _____

Members of group _____

_____

Enzyme tested _____

	S	C	M	H	K
Relative activity					Zero

Zero indicates activity no greater than that of control K. 3 is maximum activity.

In case of ties use the same number more than once.

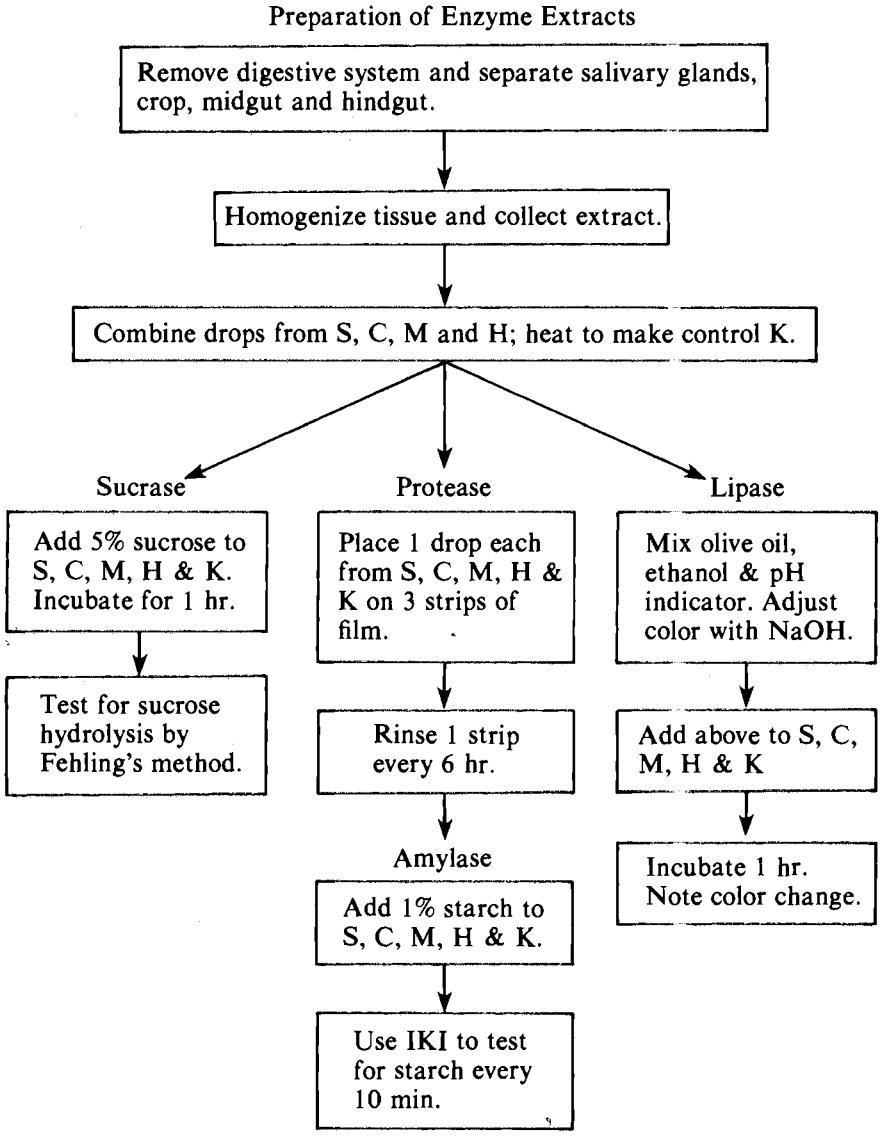

Figure 6.2. Flow diagram outlining the experimental procedures.

Insect Saline:

NaCl 16 g/l

Fehling's Solutions:

A: $CuSO_4$ 6.93 g/l

B: 25.0 g of KOH plus 34.6 g of sodium potassium tartrate

Add H_2O to make 1 liter.

Materials

For each group:

2 glass spot plates (3 depressions each)
1 blunt stirring rod (5-mm glass rod heated and pressed in depression of spot plate)
1 watch glass
5 Pasteur pipettes
2 250-ml beakers (serve as test tube racks)
10 10-ml test tubes
1 large cockroach, at least 2 cm long (See Appendix A. Cockroaches should normally be maintained on a mixed diet, such as lab chow or dog food, but should be starved for 1 week prior to these experiments. Starving makes cleaning of the gut easier, although it is said to reduce the enzyme activity. Guthrie and Tindall 1968, p. 270.)
1 dissecting microscope
scissors
forceps
probe

For each bench:

1 150-ml flask of saline with Pasteur pipette
1 hot plate or Bunsen burner
1 400-ml beaker for hot water bath

For each lab:

1 incubator set at 35°C
freezer or CO_2 to immobilize cockroaches

Additional material for each sucrase group:

1 1-ml pipette

Additional material at each sucrase bench:

1 small flask of 5% sucrose (5 g of sucrose in 100 ml H_2O)
1 dropper bottle of Fehling's solution A
1 dropper bottle of Fehling's solution B

Additional material for each protease group:

1 petri dish
1 filter paper
3 pieces of exposed and developed photographic film, approx. 5 × 20 mm

Additional material for each amylase group:

1 porcelain spot plate
1 dropper bottle of IKI (or iodine) solution

Additional material at each amylase bench:

1 small flask of freshly boiled and cooled 1% soluble starch with Pasteur pipette (1 g of starch in 100 ml of H_2O)

Additional material at each lipase bench:

1 dropper bottle of olive oil
1 dropper bottle of ethanol (at least 90%)
1 dropper bottle of saturated phenol red solution
1 small flask of 10 mM NaOH with Pasteur pipette (0.40 g of NaOH in 1 liter of H_2O)

References

Bland, K.P.; House, C.R. Function of the salivary glands of the cockroach, *Nauphoeta cinerea*. J. Insect Physiol. 17:2069–2084; 1971.

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APPENDIX A
Raising Cockroaches

For this experiment any species of large cockroach is suitable; the most easily obtained species are *Periplaneta americana, Leucophaea maderae* and *Blaberus craniifer*. One or more of these species is available from Ward's Natural Science Establishment, Inc. and Carolina Biological Supply Co., or they may be obtained from entomology departments. Since cockroaches are also useful for studies of neurophysiology, behavior, parasitology and insect morphology you may wish to start a permanent culture. Large numbers of cockroaches can be raised in a galvanized tub covered with a tight-fitting mesh. Inside the tub should be stacked sheets of stiff plastic separated by 1-cm spacers to provide more resting area. A 250-ml beaker inverted in a petri dish makes a convenient waterer, and any dry lab chow will serve for food. In very dry areas humidity can be retained by covering the tub with a plastic sheet with a 1-cm hole. The temperature should be approximately 27°C for rapid growth, but lower temperatures are tolerated. Covering the inner edge of the tub with petroleum jelly prevents escapes. It is also desirable to keep the tub in a room sealed with weather

stripping, screen, etc., not only to prevent infestations of the building, but to confine to one area the odor which some find objectionable. As an added precaution against infestation one can work only with males. These are easily collected by luring them into a fish bowl with tissue saturated with the sex-attractant of females. The tissue becomes saturated if kept in a container with females confined since the larval stage to insure their virginity.

APPENDIX B

The Use of the Sign Test on Typical Results

Table 6.1 presents typical results from two laboratory sections using *Periplaneta americana*. A glance at the mean relative activities suggests that different sections of the digestive system differ in activity for a particular enzyme, but we need a more precise way to judge the probability that those differences are real. Because the data are merely rankings, rather than quantitative measures, we cannot use the Student's t test or any other parametric statistical test. Instead we must use the sign test. The objective remains the same as with the t test: to find the probability (P) that the difference between two sets of data is due only to chance. By convention, a P equal to or less than 0.05 will justify our conclusion that the difference is statistically significant. (Whether the difference is biologically significant is another question.) The procedure for obtaining P in the sign test is even more simple than the procedure in the t test. One obtains two numbers, N and x, and then refers to the table of probabilities (Table D in Siegel 1956, p. 250). N is simply the number of times a datum from one set of

Table 6.1. Typical results using the American cockroach. The first part of the table shows data as relative enzyme activity, from 0 (minimum) to 3 (maximum). All the data from a single row under one enzyme are from the same cockroach. (/ means no datum). The second part of the table gives the mean relative enzyme activities ± SEM (n).

Sucrase					*Amylase*					*Protease*					*Lipase*				
S	C	M	H	K	S	C	M	H	K	S	C	M	H	K	S	C	M	H	K
0	0	3	0	0	0	0	3	0	0	0	3	3	2	0	1	1	3	3	0
0	1	3	1	0	2	1	3	0	0	0	0	3	0	0	0	3	3	2	0
/	0	3	0	0	3	0	2	0	0	0	0	3	1	0	0	1	3	2	0
0	1	3	2	0	3	0	2	2	0	0	0	3	2	0	0	0	3	0	0
3	1	2	1	0	3	1	2	1	0	/	2	3	1	0	2	1	3	2	0
1	1	3	2	0	3	2	1	0	0	0	0	3	3	0	0	0	3	0	0
3	1	3	2	0	3	2	2	1	0	0	2	3	2	0	0	0	3	2	0
0	3	3	2	0															

	S	*C*	*M*	*H*
Sucrase	1.0 ± .53(7)	1.0 ± .33(8)	2.9 ± .13(8)	1.3 ± .31(8)
Amylase	2.4 ± .43(7)	.9 ± .34(7)	2.1 ± .26(7)	.6 ± .30(7)
Protease	0.0 ± .00(6)	1.0 ± .49(7)	3.0 ± .00(7)	1.6 ± .37(7)
Lipase	.4 ± .30(7)	.9 ± .40(7)	3.0 ± .00(7)	1.6 ± .43(7)

data does not equal the corresponding datum from the set being compared. x is either the number of times a datum from one set is greater than the corresponding datum from the other set, or the number of times it is less, whichever is smaller.

As an illustration, suppose we wish to know whether amylase activity in the salivary glands (S) is statistically different from the control (K). (See Table 6.2, which is based on data from Table 6.1.) First we use a + to indicate that a datum from S is greater than a datum from K. Since the activity of K is defined as 0 (Zero), and we have not allowed for negative activity, we have no occasion to indicate S < K. For tied data we use an =. N is then the number of + signs. x equals the number of − signs, since there are fewer of them than + signs. Therefore, x = 0 (Zero). P is obtained directly from the table of probabilities. The one-tailed probability is used since S can never be less than K. Now suppose we have carried out this procedure for all sections of the digestive system and found that both S and M have significant amylase activity. We now wish to determine whether the activities of S and M differ. As shown in Table 3, +, −, and = are used to indicate S greater than, less than or equal to M. (Note that the + and − could be interchanged.) N then equals the total number of + and −. x equals the number of − signs, since there are fewer − than +. The table of probabilities is again consulted, but this time we must double the

Table 6.2. An illustration of the use of the sign test to determine whether there is significant activity in a section of the digestive system. Data are from the amylase results in Table 6.1.

S		*K*	
0	=	0	
2	+	0	+ means S > K
3	+	0	= means S = K
3	+	0	N = number of + = 6
3	+	0	x = number of − = 0
3	+	0	P = .016 (from Table D of Siegel 1956)
3	+	0	

Table 6.3. An illustration of the use of the sign test to compare the activities of two sections of the digestive system when both have significant enzyme activity. Data are from the amylase results in Table 6.1.

S		*M*	
0	−	3	+ means S > M
2	−	3	− means S < M
3	+	2	N = number of + and − = 7
3	+	2	x = number of − = 2
3	+	2	P = 2 x .227 = .454 (from Table D of Siegel 1956)
3	+	1	
3	+	2	

given P value since we must use a two-tailed test. (There are now two ways S can differ from M—greater than or less than—so the probability that a difference is due to chance is doubled.) For these two examples our conclusions would be that (1) there was significant amylase activity in both S and M, and (2) there is no evidence that the activity in S differed from that in M.

If we continued in this manner with the data in Table 6.1 we would arrive at the results summarized in Table 6.4. The conclusions based on these results are as follows:

1. Sucrase activity was found within the crop, midgut and hindgut. The mean relative sucrase activity in the midgut was significantly greater than the mean relative activity in either the crop or the hindgut. M > (C,H) > (S,K)
2. Amylase was found to be active in the salivary glands and in the midgut. There was no evidence of a difference in relative activity between these two sections. (S,M) > (C,H,K)
3. Protease was active in the midgut and hindgut, with a significantly greater mean relative activity in the midgut. M > H > (S,C,K)
4. Lipase activity was found in the midgut and hindgut, with the activity in the midgut being greater than that in the hindgut. M > H > (S,C,K)

Table 6.4. A tabular summary of P values for the data in Table 6.1. Example: to find the probability P that for sucrase the activity in M differed from that in H only by chance, read across in row M to column H—P = .008. An asterisk denotes a significant difference ($P < 0.05$). N. S. means not significant; used when $N < 5$.

Sucrase

	C	M	H	K
S				N.S.
C		.016*	N.S.	.016*
M			.008*	.004*
H				.016*

	C	M	H	K
S				N.S.
C				N.S.
M			.032*	.008*
H				.016*

Amylase

	C	M	H	K
S		>.10		.016*
C				N.S.
M				.008*
H				N.S.

	C	M	H	K
S				N.S.
C				N.S.
M			.032*	.008*
H				.031*

Chapter 7

Effects of Fasting and Exercise on the Depletion of the Liver and Muscle Glycogen in the Mouse *(Mus musculus)*

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Introduction

The goals of this laboratory exercise are two-fold. First, students are to discover the differential effects of fasting and exercise on the glycogen content of the liver and skeletal muscle and are to infer the underlying biochemical mechanisms from these data. Second, the exercise serves to introduce students to terminal mammalian experimentation, a useful experience, particularly for those who will do no more, and for those opposed to mammalian studies. The instructor should be prepared to discuss the reasons for such experimentation with students of very divergent and often emotional views.

To achieve the first goal, we have found that including an overview on glycogen metabolism in the student material is very helpful as it stimulates the students to use texts and journal articles; without it, they don't, and their understanding of the results is superficial. A sample overview will be found in the Student Guide Section.

This exercise can be used at any level where the students have had, or are taking, a good biochemistry course that includes a consideration of metabolism. We have successfully used it in a second-year physiology course for nurses, kinesiologists, and other health para-professionals, as well as in a fourth-year course for pre-professional students and in a first-year course for medical/dental students.

Modifications that increase the value of the exercise for specific disciplines are easily made. For example, endocrinologists may wish to infuse glucagon, insulin or epinephrine to study the hormonal control of glycogen metabolism. Infusion can be easily effected through the tail vein (Plager 1972). Similarly, pharmacologists may wish to infuse autonomic mimetics or blocking agents, or hypoglycemic drugs, and so on. A general modification that gives very useful information is to determine blood glucose (Kingsley and Getchell 1960). This necessitates the drawing of blood and in order to minimize the effects of this stress on glycogenolysis decapitation followed by collection of the trunk blood is the preferred method. However, collection via the intra-orbital sinus (Ambrus et al. 1951) from heavily anesthetized animals is also acceptable.

The preparation time (1 instructor, 20 students) for the described exercise is 2 hours. Students will require 2.5 hours to complete the exercise.

Student's Guide

Below is a sample Exercise Introduction, the most important of the information handed out to the students. The students should also receive a detailed set of instructions (which can be drawn from the Instructor's Guide) and a series of questions (Appendix 1).

1. *Exercise Introduction*

The purpose of this experiment is to determine the effects of fasting and exercise on the concentration of glycogen in the liver and skeletal muscle.

All body cells, except those of the brain, can store glucose in the form of glycogen. When necessary, glycogen can be broken down into its constituent glucose molecules which are then used either for ATP synthesis or for maintaining glucose levels in the blood. Physiologically, the most important glycogen stores are in the liver and the skeletal muscles. They are degraded during exercise and/or fasting.

The breakdown of glycogen occurs by the same process in all cells. Specifically, the enzyme *glycogen phosphorylase* attaches a phosphate ion onto the glycogen's terminal glucose and then cleaves the entire complex off:

$$(\text{glucose})_n + P_i \xrightarrow{\text{phosphorylase}} (\text{glucose})_{n-1} + \text{glucose}-6-\text{phosphate}.$$

Similarly, the product, glucose−6−phosphate, can enter the glycolytic pathway in all cells. However, because of the phosphate group, it cannot pass through the cell membrane and enter the blood. Only the liver and the kidneys have the enzyme, *glucose−6−phosphatase,* necessary to produce the free glucose that can diffuse through the membrane and into the blood:

$$\text{glucose}-6-\text{phosphate} \xrightarrow{\text{phosphatase}} \text{glucose} + P_i.$$

During exercise, glycogen is degraded in all cells for use in ATP synthesis. However, it is particularly important in skeletal muscles. There are two reasons for this. First, an actively contracting muscle requires amounts of ATP that can be generated only by the fast reactions of anaerobic glycolysis—the oxidative phosphorylation reactions are too slow to meet the demands for ATP. Since the anaerobic pathways can only use glucose as the substrate and since the blood cannot supply it in adequate quantities, the muscle breaks down its own glycogen. Second, an actively contracting muscle rapidly becomes deficient in O_2 and thus the oxidative phosphorylation reactions become chemically impossible forcing the use of the anaerobic reactions. The use of glycogen during exercise is largely controlled by *epinephrine.* This hormone is released from the adrenal gland and increases the activity of glycogen phosphorylase and several key glycolytic enzymes.

During exercise the blood glucose tends to drop as the sugar is taken up into the active tissues. However, two processes act to maintain it at approximately normal post-absorptive levels. First, epinephrine increases the activity of liver glucose−6−phosphatase so that large quantities of hepatic glycogen are released into the blood as glucose. Second, the end-product of anaerobic glycolysis, pyruvate, is synthesized into glucose and recycled. Actively contracting muscles produce large quantities of pyruvate and convert it into lactic

acid. The blood transports this to the liver and kidneys where it is synthesized into glucose and released into the blood. This synthesis of new glucose is called ***gluconeogenesis.***

During fasting, most body cells switch to the oxidative phosphorylation of fats to generate ATP. However, the brain is an obligate user of glucose and thus the maintenance of adequate blood glucose levels is of crucial importance during fasting. This is achieved by the breakdown and release of liver glycogen during the first 6–36 hours post-feeding, and by gluconeogenesis after that. The kidneys do not store enough glycogen to significantly assist in maintaining blood glucose during the first phase, but their gluconeogenic capacity is important during the second. Thus, as the blood glucose declines, the pancreatic chemoreceptors stimulate the cells of that organ to release ***glucagon.*** This protein hormone specifically stimulates liver phosphorylase and glucose–6–phosphatase causing glycogen degradation and glucose release. Glucagon also stimulates the gluconeogenic enzymes of the liver and kidneys and provides a substrate for them in the form of amino acids (glucagon induces degradation of skeletal muscle proteins). Moreover, glucagon also stimulates the release, from the adipose depots, of the fats used by the non-brain cells.

Instructor's Guide

1. ***Materials, Supplies and Equipment***

Since this exercise is best done by students working in groups of four, most requirements will be expressed on a per group basis. "Class" requirements are adequate for 20 students.

a. ***Group Needs***

- 1 mouse fed ***ad lib***
- 1 mouse fasted 24 hr
- 1 mouse fed ***ad lib*** and forced to swim for 30 min
- ether and an ether jar
- 3 pairs scissors
- 3 pairs forceps
- 1 plastic tub (14″ X 9″ X 5")
- 1 torsion balance (Roller Smith)
- 6 15-ml conical centrifuge tubes and rack
- 8 test tubes, 20 X 150 mm, and rack
- 1 waterproof marking pen
- 50-ml bottle of distilled water
- 100 ml 95% ethanol
- 100 ml 60% ethanol in a 125-ml plastic wash bottle
- 6 glass rods, 150 X 2 mm
- 1 200-ml beaker
- 3 1-ml Mohr long tip pipets
- 6 2-ml Mohr long tip pipets
- 1 10-ml Mohr long tip pipets

b. *Class Needs*

1 water bath at 75° } both to contain racks for 20 × 150 mm
1 water bath at 100° } test tubes and for the conical centrifuge tubes

3 bench top centrifuges (e.g., IEC Model CL) with 15-ml sleeves
2 automatic dispensing pipets (e.g., Repipet)
1 vortex mixer
several spectrophotometers (e.g., Spec 20)
200 ml 30% KOH (w/v)
200 ml 0.2% anthrone (w/v) in 95% sulfuric acid
10 ml glycogen standard (10 μg/ml)
saturated Na_2SO_4 soln.
2 plastic tubs (14″ × 9″ × 5″)
Parafilm
Kimwipes
benzoic acid

2. *Sources of Materials and Supplies*

The sources of the materials used in this exercise are not critical. Chemicals purchased from several different suppliers have behaved identically. Similarly, as long as equipment performs at, or near, the requisite conditions, it may be used; i.e., a water bath that holds 90° but not 100° is still usable.

Healthy mice of any strain and either sex can be used. These can often be purchased from your university's vivarium at lower prices than commercial suppliers charge. Small, local suppliers are often unreliable in terms of quantity and quality of mice supplied and even large suppliers (e.g., Charles River Breeding Laboratories Inc., Wilmington, Mass., or the Jackson Laboratory, Bar Harbor, Maine) may be unable to fulfill your needs on short notice. Moreover, large suppliers will not ship animals after Wednesday. Thus, submit orders well in advance (weeks) of the day they are required.

Anthrone may be purchased from any chemical house. We usually use Fisher Certified ACS (cat. no. A–836).

Glycogen may also be obtained from any supplier. We normally use glycogen from oysters (Sigma cat. no. G8751). However, glycogen can be replaced with dextrose with little loss of accuracy or precision.

3. *Reagent Preparation*

95% H_2SO_4: To 50 ml of distilled water add 1 l of ACS Analytical Reagent conc. H_2SO_4 and cool. This acid is stable indefinitely.

Anthrone reagent: Dissolve 400 mg of anthrone in 200 ml of 95% H_2SO_4 by use of a magnetic stirrer and a teflon-coated stir bar. Place the reagent in

an automatic dispensing pipet for use. *n.b. This reagent is unstable and must be prepared the day of use.*

30% KOH: Place a 500-ml beaker on a magnetic stirrer, add 250 ml of water and a teflon-coated stir bar. Slowly add 75 g of KOH while stirring vigourously. Stir until dissolution is complete, allow to cool, and place in an automatic dispensing pipet for use. This reagent is stable indefinitely.

Glycogen standard: Dissolve 50 mg of glycogen in 20 ml of glass-distilled water. Dilute 4 ml of this solution to 100 ml with a 0.25% benzoic acid solution. The final glycogen concentration will be 10 $\mu g\ ml^{-1}$. The benzoic acid solution, which prevents bacterial growth, is prepared by adding 1.25 g benzoic acid to 500 ml of glass-distilled water and stirring overnight to effect dissolution. The standard is stable indefinitely.

4. *Exercise Procedure*

The goal of this exercise is to illustrate some of the controls on energy metabolism by quantifying tissue glycogen in fed, fasted, and fed-exercised mice. In essence this involves treating the mice, isolating the tissues, and isolating, purifying and quantifying the glycogen. A flow chart of the procedure is presented in Figure 7.1.

a. *Animal Preparation:* The fasted mice should receive no food for 24 hrs preceding the laboratory. The fed animals should have access to food *ad libitum.* Exercise is achieved most reliably by swimming: simply place the mouse in a 14″ × 5″ × 9″ tub containing 4″ of water; as it tires, prod it into activity again. The mouse should be in the water for 30 minutes and sacrificed immediately thereafter. *Caution:* The water temperature should be maintained at about 30° to prevent death from hypothermia.
b. *Tissue Isolation:* Number the mice by putting pen marks on their tails. Kill the animals with an overdose of ether. Make a midventral incision with the scissors and excise a piece of liver. Blot it dry with a kimwipe (it will stick to paper towels) and remove a piece weighing about 100 mg. Weigh the tissue accurately and record the weight. The sample should then be placed *immediately* in a previously prepared glycogen isolation tube (see Glycogen Isolation). Next, remove the skin from one of the hind limbs and remove and weigh about 100 mg of the gastrocnemius muscle. This should also be placed in an isolation tube. *Hint:* Ether causes glycogenolysis and more accurate results, and a faster death for the animal, is obtained by decapitation. However, students do tend to find this procedure objectionable.
c. *Glycogen Isolation:* Six conical 15-ml centrifuge tubes should be numbered. Add to each, by use of the automatic pipet, 1 ml of 30% KOH. Also add, by use of a Pasteur pipet, 1 drop of sat. Na_2SO_4. Place a glass rod in each tube. Transfer of the tissue to the KOH is effected by holding

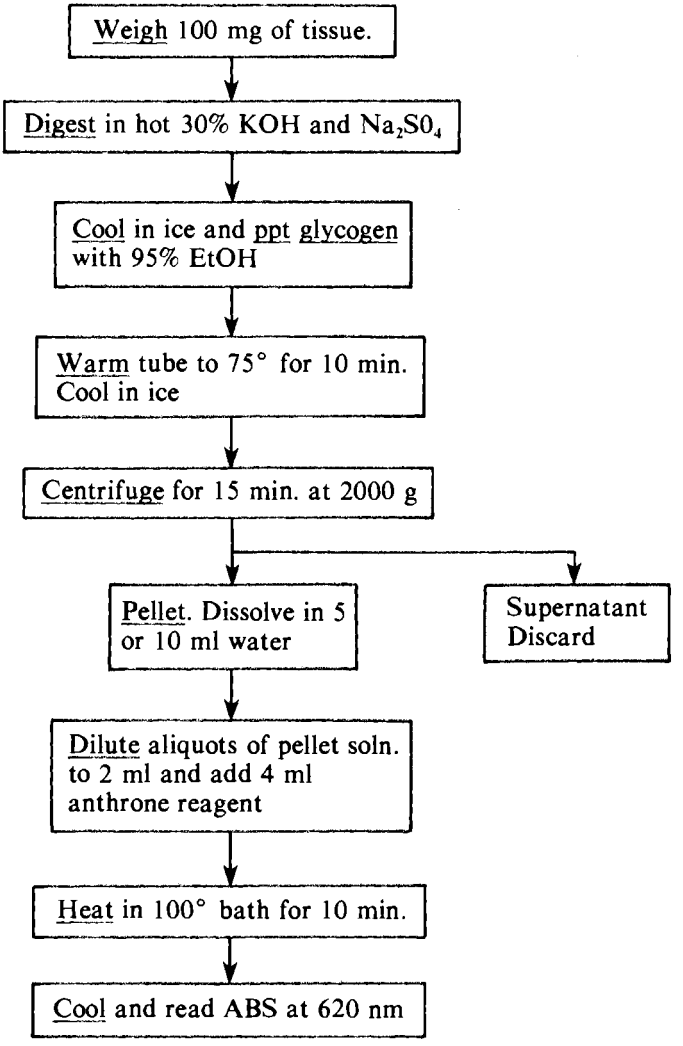

Figure 7.1. Generalized procedure for measuring tissue glycogen.

the rod vertically, placing the sample on the wet end, and carefully replacing the rod, with tissue, into the tube. Care must be taken to ensure that the sample does not become stuck on the tube walls. Digest the tissues by placing the tubes in a boiling water bath for 15 min; periodically remove each tube and shake it to ensure complete digestion. Remove the tubes and cool them in an icebath (14″ × 9″ × 5″ plastic tub filled with water and crushed ice).

Precipitate the glycogen by pipetting 1.5 ml of 95% ethanol into each tube. Stir the precipitate with the glass rod. Next, wash any precipitate from the rod and the sides of the tube with about 4 ml of 60% ethanol by use of a wash bottle. Remove the rod. Recrystallize the glycogen by warming the tubes in a 75° water bath for 10 min and periodically removing them from the bath and shaking them (it is not necessary for the entire precipitate to re-dissolve). Cool the tubes in the icebath.

Equalize the contents of the tubes by adding additional 60% ethanol where required and centrifuge at 2000 g for 15 min to pellet the precipitate. CAREFULLY pour the KOH-ethanol supernatant down the drain and place the tubes upside down on several layers of paper towels to drain. After draining, pipet exactly 10 ml of distilled water into the tubes containing liver glycogen and 5 ml into those with muscle glycogen. These solutions can then be assayed as described below.

Hints:

1. The 30% KOH is HIGHLY CORROSIVE. To prevent accidents, we keep the KOH pipet in a plastic tub lined with absorbant paper.
2. More accurate results are obtained if the tissue samples are placed into hot KOH i.e., the tubes containing the KOH are preincubated at 100°.
3. The NA_2SO_4 is essential for complete precipitation of the glycogen. Students with small pellets will invariably have forgotten it and their results will be in error. The problem is easily corrected by adding Na_2SO_4. Such additions can be made at any time up to the decanting of the supernatant; after the addition of the Na_2SO_4 the sample is reprocessed as above.
4. If very accurate results are desired, the liver pellets should be dissolved and the solution *made up* to 10 ml. Muscle samples should be made up to 5 ml.

d. *Glycogen Quantification:* The glycogen is reacted with anthrone in the presence of acid to yield a blue complex that can be measured spectrophotometrically (Seifter et al. 1950).

Pipet 0.5-ml aliquots of the liver glycogen solutions into 20 × 150 mm tubes and add 1.5 ml of distilled water to bring the volumes to 2 ml; for the muscle glycogen solutions simply take 2.0-ml aliquots; these 2-ml volumes are the reactant solutions. Two ml of the glycogen standard (10 $\mu g\ ml^{-1}$) should be added to the seventh tube and 2 ml of distilled water, to serve as a blank, should be placed in the eighth.

Next, fill the large beaker with ice and water and place one tube in it. CAREFULLY add 4 ml of the anthrone-H_2SO_4 reagent by use of the automatic pipet making sure that all the reagent enters the aqueous phase and that none is splashed onto the tube walls. Transfer the tube to a

previously prepared ice bath (14″ × 9″ × 5″ tub filled with water and crushed ice), vortexing it briefly en route to ensure mixing of the reagent and the sample. Process all remaining tubes similarly. Next, place all tubes in a boiling water bath for exactly 10 min, remove and cool. The colour produced is stable for hours at room temperature.

The absorbance (ABS) of the solutions is measured at 620 nm. Zero the spectrophotometer with distilled water and measure the absorbance of the blank; this will normally be 0.01–0.06 and higher readings suggest caution in the use of the sample values. Next, zero the instrument with the blank and read the samples and the standard against it. The standard will normally have an absorbance value of approximately 0.2. Record all absorbance values.

Hints:

1. The anthrone reagent is *HIGHLY CORROSIVE* and is best kept in the same tub as the 30% KOH.
2. The colour reaction begins as soon as the reactants are heated, and heat is generated as soon as the acid hits the glycogen solution.

   Thus, to ensure equivalency between tubes they *must* be kept in ice until placed in the 100° water bath.
3. If the colour of the final solution is so dense that accurate measurements of absorbance are impossible, the solution may be diluted with the anthrone reagent with little loss of accuracy.

Calculations: Because of its simplicity we usually use the ratios method for calculating glycogen concentrations even though the assumptions underlying the method are not strictly met.

1. (a) The glycogen concentration in the 2 ml of muscle glycogen solution that were reacted with the anthrone is:

$$\mu g\ ml^{-1} = \frac{(\text{ABS unknown})\ (\text{Concentration of Standard})}{\text{ABS standard}}$$

$$= \frac{(\text{ABSu})\ (10\mu g\ ml^{-1})}{\text{ABSs}}$$

   (b) The total glycogen in the muscle sample is obtained from:

   (concentration of muscle glycogen soln.) (volume of soln.) = μg glycogen

   e.g., μg glycogen = $(20\ \mu g\ ml^{-1})\ (5\ ml)$

   $= 100\ \mu g$

   (c) The concentration of glycogen in the muscle tissue sample is obtained from:

$$\frac{(\text{total muscle glycogen})}{\text{wt of muscle sample}} = \mu g\ mg^{-1}$$

$$\text{e.g., } \frac{100\ \mu g}{100\ mg} = 1\ \mu g\ mg^{-1} = 0.1\%$$

2. (a) The calculations for the liver samples are similar, except for the effects of diluting the liver glycogen solution 1 to 4 for reaction with the anthrone. Thus, the glycogen concentration in the liver glycogen solution is given by:

$$\mu g\ ml^{-1} = \frac{(ABSu)(10\mu g\ ml^{-1})\ (4)}{ABSs}$$

Also, the volume of liver glycogen solutions is 10 ml, not 5, as in the case of the muscle samples.

Typical results are given in Table 7.1.

Table 7.1. Representative effects on tissue glycogen from fasting, exercising, and feeding mice. L = Liver, M = muscle.

	Fed		*Fasted*		*Exercised*	
	L	*M*	*L*	*M*	*L*	*M*
Tissue wt. (mg)	102	98	106	89	90	101
ABS	1.75	.35	.24	.25	.04	.04
Glycogen in Tissue Precipitate Solution ($\mu g\ ml^{-1}$)	389	19.6	53	14	9	2
Glycogen in Tissue ($\mu g\ mg^{-1}$)	38	1.0	5.1	0.8	1.0	.1

blank absorbance = 0.01
standard absorbance = 0.18
Normal tissue values for fed mice: liver: 1−9% glycogen; muscle: 0.1−0.3% glycogen.

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APPENDIX I
Questions for Students

1. Calculate the percent decrease in tissue glycogen in the fasted mouse using the values from the fed mouse as control levels. Which tissue lost the most glycogen? Explain the endocrinological and biochemical bases for this result.
2. Repeat question 1 for the exercised mouse.
3. Based on the knowledge you obtained from this exercise, construct a model for the control of tissue glycogen.

Chapter 8

A Rapid and Simple Procedure for Preparing Vertebrate Karyotypes

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Joseph R. Larsen is currently Director of the School of Life Sciences at the University of Illinois. He designed the basic core course in biology required of all preprofessional students in life sciences. He has been involved with this course for over thirteen years and directly responsible for its continued management and has written the laboratory manual for the course. He has also designed the laboratories and written the manual for Biology 104, a service course for the College of Agriculture. His research involves the study of the ultrastructure of the insect central nervous system and chemo-, mechano-, olfactory, and visual receptors in insects. He has had a great deal to do with elucidating ultrastructural morphology of various sensory receptors as it relates to neurophysiology and behavior.

Carl W. Gilbert is currently a Visiting Lecturer in Biology in the Department of Physiology and Biophysics at the University of Illinois. He has coordinated the introductory biology laboratory (Biology 110–111) with over 600 students. Future interests include continued involvement in teaching introductory level biology laboratories. Specific research interests are associated with chloroplast development, in particular, two-dimension polypeptide maps of developing chloroplasts.

Introduction

The goals of the laboratory exercise are threefold: (1) to demonstrate a rapid and relatively simple method of preparing vertebrate chromosomes for microscopic examination, (2) show that chromosomes have varied morphology, depending on the location of the centromere and/or the lengths of the chromosome arms, and (3) demonstrate that different species have different karyotypes. This exercise is designed as an introductory biology laboratory (first-year biology course, for majors) used in conjunction with general genetics topics. This laboratory can be completed in a single three-hour laboratory period with students working in groups of 2–4. This laboratory does require sacrificing (killing) animals, and instructors must prepare animals at least one hour before the laboratory begins. All injected animals must be sacrificed since the mitotic inhibitors will eventually kill the animals.

In recent years, cytologists have been using karyotypes (number of chromosomes and chromosome morphology) to help in delineating species. A species here is defined as "a population of interbreeding organisms." However, it is often impossible to observe if two types of animals interbreed in the wild, and breeding observations in the laboratory are usually not under natural conditions. In the past, taxonomists had to rely on superficial features to delineate between species. Chromosome karyotypes are an additional tool now available to the taxonomists. In some instances, animals that were formerly considered to be one species, and were externally alike, have been found to have different karyotypes. An example is the cotton rat *(Sigmodon hispidus)*. All specimens from the southeastern United States population have a diploid number of 52 chromosomes while specimens from Arizona have a diploid number of only 22 chromosomes (Zimmerman and Lee 1968; Larsen 1979).

Zimmerman and Lee's results are just one example of how karyotypes are used. Other recent uses have included medicine where clinical illnesses have been linked to chromosome abnormalities (i.e., Down's Syndrome with 47 chromosomes) (Keeton 1972).

The laboratory can be directed in two ways: (1) have the students look at several different animal species and compare their karyotypes or (2) have two or more very similar if not identical species with different karyotypes, and let the students determine cytochemically if they are the same species. The laboratory described here used approach #1 using the rat, mouse, and frog. Their karyotypes are sufficiently different so students can compare chromosome morphologies and see that different species have different morphologies. Also, rat, mouse, and frog species are easy to obtain and maintain during the laboratory period. This procedure should work with all vertebrates with little or no modification.

Instructor Materials

Animal Preparation

The karyotyping procedure is adapted from Zimmerman and Lee (1968). The procedure calls for *in vivo* preparation of the chromosomes by injecting the animal with a mitotic inhibitor like colchicine or velban. These inhibitors interfere with microtubule assembly necessary for anaphase, leaving chromosomes in late metaphase. Animals are to be injected intraperitoneally with a solution of 0.025% colchicine or 0.004% velban one to four hours before sacrificing. The intraperitoneal injections should be split with half of the solution being injected into each side of the peritoneal cavity. The dosage is dependent on the animal's weight (Table 8.1).

Table 8.1. Mitotic Inhibitor Dosage Range.

Animal Body Weight (g)	*Dosage (ml/g body wt)*
5–50	0.02
+50	0.01

Tissue Preparation

The key to this procedure is *speed.* The time the animal dies to the time the cells are resuspended in the fixative solution, should be as short as possible.

Rats and mice are sacrificed rapidly by placing them in a closed jar containing cotton saturated with chloroform. Death generally takes less than 2 minutes. The animals are removed from the jar and used immediately. Frogs are sacrificed by either double pithing or decapitation and a spinal pithing, and used immediately.

Rapidly remove the femurs, tibia and fibula from the hindlimbs and if necessary the humerus, ulna, and radius from the fore limbs. Table 8.2 lists the number of animals required per group and the bone required for the karyotyping. Peel the skin and as much of the muscle off the bones as possible. Dry paper towels are useful in helping remove muscle from the bone. Using a strong pair of scissors, splinter the bones into 2–3 longitudinal or oblique fragments. Drop the bone splinters into a round-bottom test tube (18 × 150

Table 8.2. Bones Required for Karyotyping.

# of Animals	*Bones Required for Karyotyping*
1 rat (large 100 g)	femurs, tibiae and fibula
1 rat (small 100 g)	femurs, tibiae and humeri
2 mice	femurs, tibiae and humeri
1 frog	femurs, tibiae and humeri
2–3 immature mice or frogs	femurs, tibiae, humeri and pelvic bones

mm). Add 8 ml of 1% sodium citrate as soon as possible. Do not let the bone chips dry out. Place the ball of your thumb over the mouth of the test tube and shake the solution and bone chips vigorously for 6–8 seconds to wash the marrow from the bone fragments. The solution should be pale red in color. Immediately place the test tube in a 37°C water bath for 10–15 minutes. Gently invert the test tube once or twice every 5 minutes. Do not leave the marrow cells in the citrate solution over 15 minutes, since the hypotonic treatment will lyse large numbers of cells after that time. At the end of the incubation period, the test tube is gently inverted 3–4 times to wash the marrow away from the bone chips. The supernatant, containing the cells, is poured into a clean conical centrifuge tube. The suspension is centrifuged at 400 rpm (IEC Clinical Centrifuge) ($\sim 500 \times g$) for 3–4 minutes. At the end of the centrifugation, there should be a reddish-white cell "button" at the bottom of the tube. Remove the tube from the centrifuge and let it sit undisturbed in ice for 5 minutes.

Washing and Fixing the Cells

Decant the citrate solution off the cell pellet, and immediately add 3 ml of cold fixative solution (3 parts anhydrous methanol, 1 part glacial acetic acid). Resuspend the cell pellet by gentle aspiration with a pasteur pipette. Recentrifuge at $500 \times g$ (400 rpm) for 3–4 minutes, and discard the fixative. The cell pellet should be white in color at this point. Rewash the pellet with 2 ml of the cold fixative, and centrifuge as above. Decant, and resuspend the cell pellet in 1 ml of fresh cold fixative, being sure to disrupt all of the cell clumps by gently sucking the solution up and down in a pasteur pipette. During the fixative procedure, there may be a lipid layer at the top of the centrifuge tube. When present, discard this layer while decanting. In preparations of frog marrow, there are yellowish lipid globules that are often found sitting on top of the cell pellet. These should be discarded if possible. However, they do not appear to interfere with the fixative or staining process (Lee 1969; Larsen 1979).

Preparation of the Slides

Microscope slides of the chromosomes are prepared by the blaze-dry technique of Scherz (1962). Pre-cooled (4°C) microscope slides in distilled water are placed on a paper towel. From a height of 18–30 inches, fixed cells are dropped (3–5 drops/slide) onto the glass and allowed to spread. Each student should make 3–4 slides. The slide is picked up with the forceps and passed through the flame of an alcohol lamp, which ignites the fixative on the slide. Allow the fixative to burn until it extinguishes itself. Residual liquid is shaken from the slide, which is then allowed to completely dry. The slide is then flooded with carbol-fuchsin stain for 5–10 minutes. The slide is rinsed with distilled water to remove excess stain (rinse until no more stain is re-

moved). The slide is air dried, then examined under low and high power of a microscope.

Results

Students will be able to find karyotypes upon examination, and they should be able to count the number of chromosomes in each karyotype (Figure 8.1). In some cells, the chromosomes will be sufficiently spread so students can determine the number of metacentric, submetacentric, and telocentric

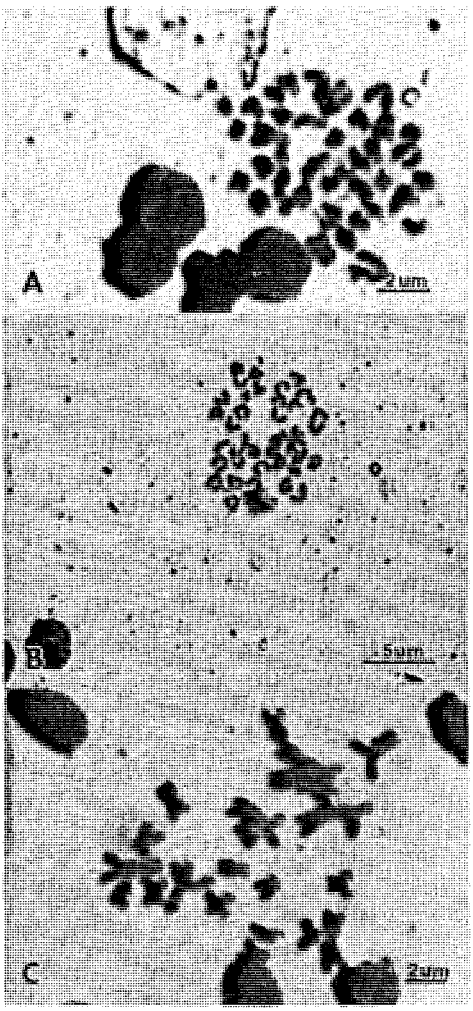

Figure 8.1. Carbol-fuchsin stained vertebrate karyotypes. A. Rat karyotype, scale 2 µm; B. mouse karyotype, scale 5 µm; C. frog karyotype, scale 2 µm.

Table 8.3. Expected Chromosome Numbers

Animal	*# of Chromosomes*	*Chromosome Type*		
		Metacentric	*Telocentric*	*Submetacentric*
Mus musculus (house mouse)	40*	—	40	—
Rattus norvegicus (albino rat)	42*	14	20	8
Sigmodon hispidus (cotton rat—eastern)	52**	3	48	1
Sigmodon arizonae (cotton rat—Arizona)	22**	8	4	10
Rana pipiens (leopard frog)	26*	Not Available		

*Hsu and Benirschke (1967)

**Zimmerman and Lee (1968)

pairs. Table 8.3 lists some expected results. Since a majority of the bone marrow cells are not in metaphase, it will take some time to find karyotypes. Rat and mouse karyotypes have a higher success rate, and every student should be able to find 5–20 karyotypes per slide. Frog bone marrow karyotypes have a lower number of cells in metaphase and therefore a lower number of karyotypes on the slide. It often takes a careful examination of an entire slide before a good karyotype is found.

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APPENDIX I

Supplies Required for 20 Students Working in Pairs

A. *Animals*

Mice	2/pair of students
Rat	1/pair of students
Frog	1/pair of students

B. *Chemicals*

Chloroform—reagent grade	1 pt
Colchicine (0.025%)	50 ml
Sodium Citrate (1%)	1000 ml
Fixative Solution	1000 ml
Anhydrous Methanol	750 ml
Glacial Acetic Acid	250 ml
Carbol-fuchsin Stain	200 ml
Basic Fuchsin	
Absolute Alcohol	
Phenol (88%)	

C. *Equipment*

1 37°C water bath
5 Alcohol Lamps
2 Large Scissors
10 Ice Buckets (1/pair of students)
2–3 Clinical Centrifuges
20 12-ml Conical Glass Centrifuge Tubes (minimum)
20 18-×-150-mm Test Tubes (minimum)
1 box Pre-washed Glass Microscope Slides (100/class)
8–10 Syringes and Needles (disposable)
1 box Disposable Pipettes
1 Large Closed Jar with Cotton
10 Forceps
Ice
Paper Towels
Plastic Bags for Animal Disposal
Dissecting Kits

APPENDIX II

Suppliers for Animals and Chemicals

A. *Animals*

Rats	Murphy Breeding Labs. Inc. Rt. #2, Box 416 Plainfield, Indiana or Your Local Supplier
Frogs	Carolina Biological Supply Co.

B. *Chemicals*

Colchicine	Sigma Chemical Co., St. Louis #C9754
Basic Fuchsin	Sigma Chemical Co., St. Louis #F2002
Chloroform reagent grade	Fisher Scientific Co.
Anhydrous Methanol	Fisher Scientific Co.
Phenol (88%)	Fisher Scientific Co.
Glacial Acetic Acid	J.T. Baker Co.
Sodium Citrate	Fisher Scientific Co.
Velban	Eli Lilly Co.

C. *Equipment*

All equipment can be purchased from the following companies:

1. A.H. Thomas Co.
   Vine Street at Third
   P.O. Box 779
   Philadelphia, PA 19105
2. Fisher Scientific Co.
   711 Forbes Avenue
   Pittsburgh, PA 15219
3. Sargent-Welch Scientific Co.
   1617 E. Ball Road
   Anaheim, CA 92803

APPENDIX III
Solution Preparation

Sodium Citrate (1%):	10 g sodium citrate in 1 liter distilled water and warm to 37°C
Fixative Solution:	Anhydrous methanol 750 ml Glacial acetic acid 250 ml mix and cool to 4°C
Carbol-fuchsin Stain:	(a) 1 g of basic fuchsin in 10 ml absolute alcohol (b) 5 ml of 88% liquid phenol in 100 ml distilled water Solutions a and b are mixed then filtered through #1 filter paper. The stain is stable for 1 week at room temperature.
Colchicine (0.025%):	25 mg in 100 ml distilled water. Solution is stable several days if refrigerated.
Velban (0.004%):	4 mg in 100 ml distilled water. (Stability is unknown.)

Chapter 9

The Elucidation of a Biochemical Pathway

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Introduction

The objectives of this exercise are to elucidate a biochemical pathway and to order a series of mutant alleles in the pathway. The pathway used is the synthesis of prodigiosin in *Serratia marcescens*. This exercise is used in a large class (325 students) at Stanford University. This is the first college-level biology lab the students have, although they have had some chemistry lab and several quarters of biology lecture. This experiment is ideal for someone who is not sure of his or her sterile technique and wants some practice. The experimental procedure is very simple and if a plate becomes contaminated, nothing except that plate is lost.

The experiment takes less than an hour to set up but the students must check the plates several times during the week. This could take one to two more hours. They could even take the plates home with them as they are incubated at room temperature. The preparation time for the instructor is that time required to pour 16 peptone-glycerol agar plates per group of students. We work in groups of four students but smaller groups would work well. The instructor needs to start work on the experiments several weeks in advance in order to provide one set of cultures per two groups of students (see Instructor's Materials).

Student Materials

One of the most widely used methods of elucidating the sequence of reactions (pathway) leading to the synthesis or degradation of a given compound by a given organism utilizes mutants of that organism which do not have the ability to synthesize or utilize that compound. The method is simple and elegant. If the compound in question is an essential one, such as the amino acids or nucleotides are, the mutation is lethal—that is, the organism soon ceases to reproduce—unless the compound in question is supplied to it in its growth medium. Mutants of this kind are called *auxotrophs* (for the compound in question) and their normal or wild-type counterparts are called *prototrophs*. More generally, an auxotroph is any mutant which is unable to grow (or grows poorly) on a medium on which the wild-type can grow.

Auxotrophs can be produced from prototrophs as a result of treatment with mutagenic agents such as UV and X-ray irradiation or the chemicals nitrous acid and hydroxylamine, etc. After treatment, many organisms die and many are unchanged, but a few organisms will be found that require a particular compound for growth that they could formerly synthesize for themselves. That the alteration in nutritional requirements is genetic in nature is illustrated by the observation that progeny of the new auxotrophs have the same nutritional needs as their parent cells.

Various selective techniques have been developed to enable one to isolate a very few auxotrophic mutants from the vast excess of prototrophs with which they will be found after mutagenic treatment. Most of these techniques depend on the fact that, in a particular medium, the prototrophs will be able to grow, while the auxotrophs, because they are unable to synthesize a compound necessary for growth, a compound not supplied in the medium, will not grow. (They do not die, at least not for some time.) Various agents, such as penicillin, are lethal only to growing cells and have no effect on non-growing ones. Thus, by incubating a mixture of auxotrophs and prototrophs—in a medium in which the prototrophs can grow but the auxotrophs cannot—in the presence of an appropriate concentration of penicillin for several hours, one can selectively kill the prototrophs. If one then washes the incubation mixture free of penicillin and incubates portions of it in a medium which now *does* contain the compound which is necessary for growth, the auxotrophs will now grow and can thus be selected for.

When one actually performs a selection experiment of this kind and isolates a number of auxotrophs of independent origin (ones which did not arise from an identical mutational event) for a particular compound, let us say the amino acid tryptophan, one almost invariably finds that the mutants isolated can be separated into groups. That is, though all the mutants have in common the inability to synthesize tryptophan, one may find that upon genetic analysis the mutational sites will map in several different but linked regions of the chromosome (in the case of *E. coli)* or even in unlinked regions (in the case of *Neurospora).* Furthermore, if one supplements the medium in which the tryptophan auxotrophs are incubated with various compounds which one might suspect of being involved in the biosynthesis of tryptophan, one can demonstrate biochemical differences among the mutants. Some auxotrophs will be found which are capable of growing on indole, for instance, as well as tryptophan, while others will not grow on indole. Still others will be found which will grow on anthranilic acid, indole, or tryptophan.

The fact that indole and anthranilic acid can, in some tryptophan auxotrophs, satisfy the requirement for tryptophan (see following formulas), suggests that these compounds may play a rather direct role in the biosynthesis of tryptophan, that they may in fact be intermediates in the biosynthesis of this amino acid from simple nitrogen and carbon sources, such as NH_4^+ and glucose. Such intermediates are termed precursors of the final compound in the biosynthetic pathway. (One must be careful not to conclude that because a compound can be utilized to overcome an auxotrophic deficiency, it is therefore a normal intermediate in the biosynthesis of the final compound. The cell could simply be converting it to a normal intermediate. This process in fact appears to be what is happening in the case of indole utilization in tryptophan biosynthesis.)

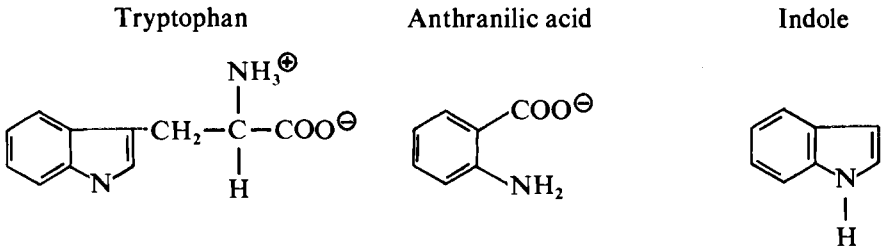
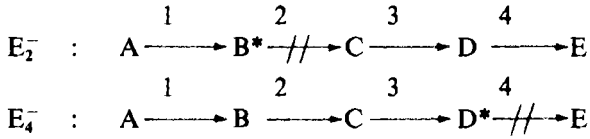


Figure 9.1. The amino acid tryptophan and two related compounds.

Consider now the following generalized scheme for a biosynthetic pathway: $A \xrightarrow{1} B \xrightarrow{2} C \xrightarrow{3} D \xrightarrow{4} E$ where A is the initial compound in the pathway, E is the final compound or end-product, and B, C, and D are intermediates. Enzyme 1 catalyzes the conversion of A to B, 2 catalyzes the conversion of B to C, and so forth. In this pathway, E, the end-product, normally accumulates in the cell where, if it is an amino acid, it is used for protein synthesis. In line with the one-gene—one-enzyme hypothesis, one would expect that many mutants which required E for growth (E^- auxotrophs) would have a *single* reaction in the pathway which was blocked due either to a missing or to a non-functional enzyme. Moreover, one would expect that a mutation which caused any one of the enzymes 1 through 4 to be non-functional would result in what would be a phenotypically E^- auxotroph. However, it is important to note that at least four biochemically distinct classes of E^- auxotrophs can exist, one blocked in each of the reactions 1 through 4. Furthermore, since E normally accumulates, one might expect that the intermediate which immediately precedes any reaction in the pathway which is blocked would also accumulate and, since it in general will not be used up as E would be, one might expect extremely large excesses of such intermediates to accumulate.

This line of reasoning, then, suggests a way in which (1) a minimum number of steps in a biosynthetic pathway can be established, (2) a group of mutants can be at least partially separated into biochemically distinct types, (3) the groups of mutants can be used to establish the order in which the steps in the biosynthetic pathway occur, (4) with the addition of biochemical techniques, the chemical nature of the intermediates can be established. That all of these things should be possible simply because of the nature of the pathway is suggested by the following line of reasoning: Consider two E^- auxotrophs, E_2^- and E_4^-. Suppose that E_2^- is blocked in reaction 2 and E_4^- in reaction 4. In light of the previous discussion, one would expect E_2^- to accumulate B and E_4^- to accumulate D. Their pathways would look as follows: in which $-//-$ indicates a blocked enzyme and D* indicates that the asterisked compound is accumulated in large excess and very probably excreted into the medium.



Comparison of these pathways reveals the following very useful fact: E_2^- is functional in step 4, exactly the one in which E_4^- is blocked. (The converse is also true with respect to step 2.) Since E_2^- can clearly convert D to E and since E_4^- accumulates and excretes D, one would predict that a cell-free filtrate of E_4^-, grown on sufficient but limiting E, would be able to support the growth of E_2^- *without* any addition of E, since it contains D which E_2^- can convert to E. In fact, such filtrates do in many cases support the growth of another biochemically distinct auxotroph. Note, however, that not every auxotroph will support the growth of every other biochemically distinct auxotroph. A cell-free filtrate of E_2^- will *not* support the growth of E_4^- because, although E_4^- is quite capable of performing the reaction in which E_2^- is deficient, it is itself blocked in a subsequent reaction in the pathway. This property of the system allows one to order the biochemically distinct auxotrophs in the same order as the enzymic steps in which they are deficient, that is, in the same order as the biosynthetic pathway. Close examination of the system will convince you of the generality of the following statement: in a linear pathway in which only single enzymic blocks exist, if a mutant X is able to feed (has a cell-free filtrate which supports the growth of) a mutant Y, then X is blocked in a reaction in the pathway which is subsequent to the reaction in which Y is blocked. The converse of this statement (that if X is unable to feed Y it is blocked in a reaction prior to that of Y) is not necessarily true. Why not?

Thus, without any knowledge of the detailed biochemical nature of the steps involved, one can, by the procedure described above, order a series of mutants for the production or degradation of a given compound in the same order as the reactions in which the mutants are blocked occur in the normal pathway.

The sort of investigation described above generally involves a moderate amount of technical manipulation, particularly in the case of auxotrophs where cell-free filtrates are often required. Several systems exist, however, in which the technical aspects are extremely simple and the procedures are very fast. One such system, involving the production of the bright red pigment prodigiosin by the bacterium *Serratia marcescens*, will be used in this experiment.

Prodigiosin is not a compound essential for survival of the organism. Mutants defective at various steps in the biosynthetic pathway are recognized, not by their inability to grow on minimal medium (as auxotrophs are recognized), but rather by their lack of the organism's normal red color. Similarly, feeding in this system is not the ability of an extract of one mutant strain to

support the growth of another. In this system, feeding is indicated by the ability of one mutant strain to synthesize prodigiosin (and thus look red) when grown in the presence of another. (The second mutant strain is said to feed the first.) However, the theoretical considerations developed earlier apply to this system as they do to biosynthetic pathways producing compounds necessary for cell growth. Feeding tests can therefore be used to order steps in the pathway.

Instructor's Materials

In order to elucidate the relationship of each mutant to the other a series of feeding tests are done on peptone-glycerol agar plates. The feeding test consists of streaking each of two strains on the surface of the medium in a petri dish so that they form a V not quite closed at the bottom. It should be a V with a narrow opening and wide arms, so as to insure maximum precursor production by each strain (see Figure 9.2). A positive feeding test consists of the production of red (not orange) pigment. Basically a positive test makes the mutant look like the wild type. After preparation the plates should be incubated at room temperature and examined every few days, i.e., after 2, 4, and 7 days. Some feeding patterns are slow to develop. The plates may be incubated, if desired, at temperatures up to 33°C. Above that temperature the color does not develop.

The plates should be incubated right-side up. This is contrary to normal microbiological practice and should be emphasized. The reason for this means of incubation is that some of the mutants (XII–114, 9–3–3, XII–20) produce a volatile product which is heavier than air. If the plates are incubated upside down the precursor collects in the lid of the petri plate and is not as effective in feeding auxotrophic strains.

The mutants which produce a volatile product cause the mutant streaked opposite it to turn red all along its length. The mutants which produce a soluble product (WF, 3–14, OF) cause the opposite mutant to change color at the bottom of the V. An observant student will note these differences. A less observant student will still be able to deduce the pathway but won't realize some products are volatile, some soluble.

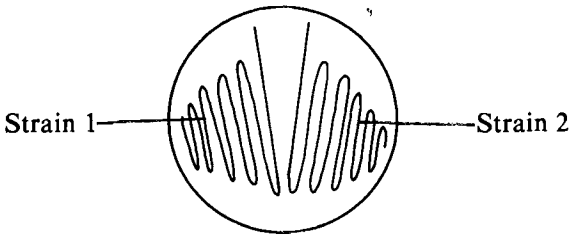


Figure 9.2. Streaking pattern.

Streaking the plates is done with a loop held parallel to the surface of the plate. Be sure to flame the loop before picking up a bit of the stock culture and again after streaking it. A set of new stock plates should be made. Mutants develop more color as they age. The students will need these stock plates for comparison. Only then can they tell if 9–3–3, for example, is darker when streaked against WF than when it's on its own.

The strains we have and use in this experiment and their phenotype are as follows:

Strain	*Color*
OF	dark orange
WF	light orange
C–11	purplish
XII–20	light purple
9–3–3	dark purple
Hy (wild-type)	dark red

Pour plates at least one week before the experiment, because color development is best when plates at least this old are used. The biochemical pathway for prodigiosin production and the feeding results are shown in Figure 9.3.

The table is read in the following manner. If the numbers of two mutants appear in the box they both feed each other e.g., 9–3–3 and WF, but if just one number appears then the number appearing is *fed,* e.g., C–11 feeds 9–3–3.

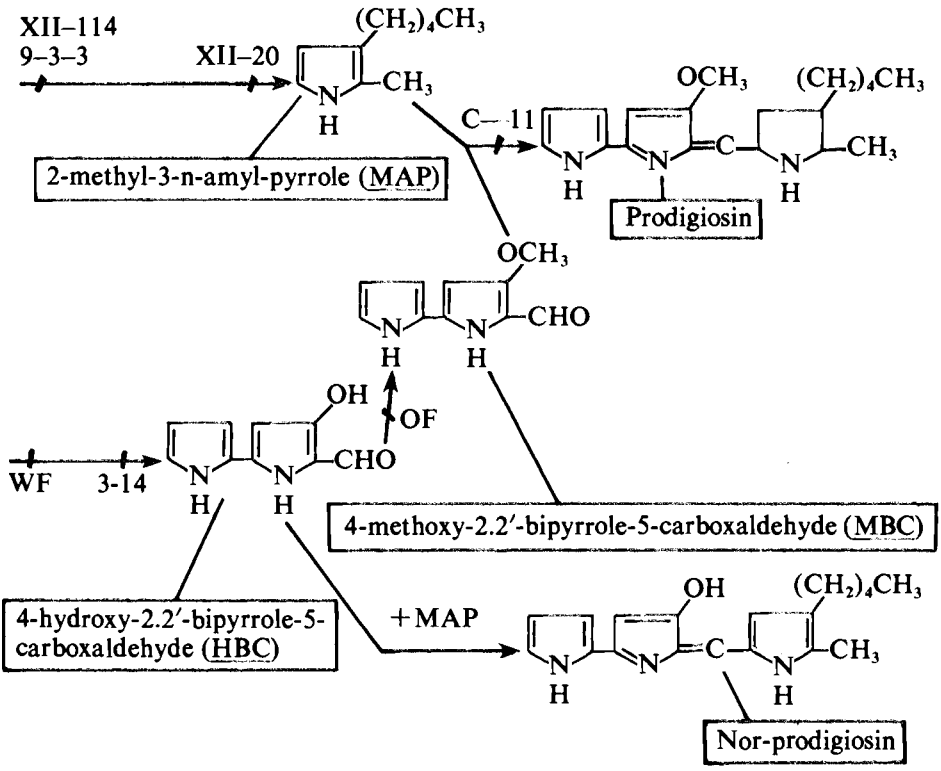
Materials and supplies needed for a class of 20 (if working in groups of four):

Bunsen burners and streakers
Transfer loops
100 peptone-glycerol agar plates
1 set of cultures for every two groups.

The only place I know to obtain the cultures is from us at Stanford University. Other individuals may have them but none of the biological supply houses seem to have more than one or two mutant strains. To obtain cultures please write to:

Ms. Nancy van Zwol, Department of Biological Sciences, Stanford University, Stanford, California 94305. Please include $15.00.

To keep the strains from year to year, slants (fill sterile tube half full of sterile medium and place at angle) should be made of the same media listed in the appendix in 16 × 75-mm screw cap tubes. The tubes should be inoculated and allowed to grow overnight at 25°C. Pipette in sterile mineral oil (heated in oven 160°C for two hours in beaker covered in aluminum foil) to fill tube. Store in refrigerator.



	9–3–3	*WF*	*C–11*	*3–14*	*OF*	*XII–20*	*XII–114*
9–3–3	--						
WF	9–3–3 WF	--					
C–11	9–3–3	WF	--				
3–14	9–3–3 3–14	WF	3–14	--			
OF	9–3–3 OF	WF	OF	3–14	--		
XII–20	9–3–3	XII–20 WF	XII–20	XII–20 (3–14)*	OF XII–20	--	
XII–114	--	XII–114 (WF)	XII–114	XII–114 (3–14)	XII–114 (OF)	XII–114	--

*( ) Indicates a weak reaction.

Figure 9.3. Biochemical pathway and expected feeding results for *Serratia* mutants.

References

Hayes, W. The genetics of bacteria and their viruses. 2nd ed. New York: John Wiley and Sons; 1968: 87–92, A restatement of the theory of biochemical genetics and 108–124, a survey of cases to which these methods have been applied.

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Srb, A.M. Genes and metabolic pathways. Editor, Vincent G. Dethier. Topics, the study of life; New York: Harper and Row; 1971: 265–272. An excellent introduction to the material covered in this experiment.

APPENDIX

Preparation of Peptone-Glycerol Agar Plates

Peptone-glycerol	1 liter
Bacto-peptone	5 g
Glycerol	10 ml
Bacto-agar	15 g
H_2O	1 liter

Place all of the above in a 1.5- to 2-liter flask and sterilize at 15 lbs. pressure for 30 minutes. Allow to cool until you can hold the flask comfortably, and pour 25 ml per plate into sterile glass or plastic petri plates. Store the plates upside down for one week to one month before use.

You will need 3 liters of media to make 100 plates, but 1 liter is about all you can pour conveniently by hand. If you have an automatic pipetter you can make a larger quantity in a carbuoy, but then you must increase the time for sterilization to 90 minutes for 10 liters.

This would be a good time to make some slants for storing the strains (see Instructor's Materials) as only 50 ml of media is needed and it's inconvenient to make such a small amount.

Chapter 10

Brassica campestris L., a New Plant for Teaching Genetics*

L.V. Crowder, J.A. Hawk, T.M. Gradziel, S.E. Fast

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J.A. Hawk is Assistant Professor of Plant Science at the University of Delaware, where he teaches genetics and plant breeding and is the leader of a corn breeding project. He holds B.S. and M.S. degrees in biology from Purdue University and the Ph.D. from Cornell University. He taught biology in high school and at a community college in Vermont. As a graduate teaching assistant at Cornell his interests focused on developing a higher plant organism suitable for teaching genetics, namely *Brassica campestris* L.

T.M. Gradziel is a graduate teaching assistant in the Department of Plant Breeding and Biometry at Cornell University. He holds the B.S. degree in agriculture from the University of Massachusetts. Undergraduate research interest was related to aspects of overcoming genetic incompatibility. At Cornell he has studied seed development and maturity as a means of further shortening the generational cycle of *Brassica campestris* L.

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S.E. Fast is an experimentalist in the Department of Plant Breeding at Cornell, having direct responsibility for the germplasm collection of the *Brassica campestris* genetic program. After undergraduate training at the University of Cincinnati, she obtained a M.S. degree in geology at the University of Michigan. She became curator in the Geology Department at Cornell and was a teaching assistant. Two years were spent with the Teachers for West Africa Program in Ghana. In 1972 she joined the Department of Plant Breeding at Cornell as a technician and researches the *Brassica* mutants used in teaching introductory plant genetics.

Introduction

Brassica campestris L. is a higher plant laboratory organism available for teaching plant genetics (Hawk and Crowder 1978a). Mutants isolated in the S_1 generation (Hawk and Crowder 1978b) have been used for inheritance studies by students enrolled in an introductory course at Cornell University. This species is normally outcrossed because of sporophytic incompatibility but can be self-fertilized by bud pollination. The incompatibility factor eliminates the need for emasculation and reduces the likelihood of selfed seed set when hand-pollinating fully opened flowers. A main raceme develops 10 or more flowers that are easily manipulated. Several side branches emerge later and permit additional crosses. Each pod produces 10 or more seeds that can be germinated immediately or stored in coin envelopes for several years.

This paper outlines procedures for carrying out inheritance studies with the *Brassica*; describes genetic materials, cultural practices and pollinating techniques; reviews record keeping, harvesting and seed processing; suggests an outline for the project report; and discusses the maintenance of mutant stocks.

Procedure for Conduct of Experiment

15-week Semester—At the first laboratory (week 1) flowering plants of several mutant forms, as well as the normal or wild-type, are made available. After practice pollinations, using the wild-type plants, students select two mutants for reciprocal crosses whenever possible. There is a review of techniques and procedures for sowing seeds and culturing *Brassica* plants, keeping records, harvesting and processing seeds.

Each student is responsible for preparing a project design to be handed in prior to harvesting F_1 seeds. A handout is provided to assist students in developing the project design. The student outline sets forth questions (hypotheses) to be investigated, notes techniques (crosses) to distinguish among alternative hypotheses and predicts results for each hypothesis. It is important to include a schedule indicating times to plant, pollinate, make observations and harvest seeds. The student must closely adhere to the schedule so as to be able to finish the project.

Parent Plants—The mutant plants should be true-breeding for the characters being studied. They may be heterozygous for other traits. The student will need to make observations of plant characteristics and prepare a written description as an aid for identification in subsequent generations and to include in the final report.

F_1 Progenies—Within 25 to 30 days seeds on the parental plants will be mature and ready for harvest. From 10 to 12 plump seeds are selected (Hanson 1959) from each of the four parents for planting (two parents if male sterile

is used). There is no dormancy period so that planting can be made as soon as pods are harvested and seeds are shelled. F_1 seedlings emerge in about three days and periodic observations of plants should be made thereafter.

Flowering begins in 13 to 16 days after planting. From 8 to 10 F_1 progenies are selected for making intercrosses, self-pollinations (bud stage) or backcrosses to the mutant type (testcrosses). Parental genotypes, planted by the instructor, are available to students for making testcrosses.

F_2 Progenies—The seeds of F_1 progenies can be planted immediately after harvest. A maximum of 144 plants (two flats) is sufficient to determine the mono- and dihybrid inheritance patterns. The experiment should be designed to obtain the most information with the smallest number of plants, at least 18 from each F_1 plant if available. Both test-cross and F_2 data are desirable for confirmation of a hypothesis. Remember that data from different lines that trace to the original parents can be combined to further substantiate results, but they must comprise a homogenous population. The homogeneity test (Little and Hills 1978) shows whether this is feasible.

10-week Semester—Wild-type and mutant flowering plants are made available at the first-week laboratory for practice pollinations and crosses, respectively. Observations will be made on parental plants and the F_1 may be grown-out for study.

F_1 seeds corresponding to the parental crosses of each student are provided for planting at the first-week laboratory. An alternative that reduces the time interval is to provide F_1 flowering plants during the first week. The students make intercrosses, backcrosses, and self-pollinations and then decide which of these to use in the F_2 and test-cross analyses in accordance with their independent project designs.

Admittedly, this modified version is less desirable than the full sequence of events. Students are involved in a "hands-on" operation, however, as contrasted to the usual demonstration-type laboratory exercise with higher plants.

Genetic Materials

Four mutants were originally isolated at Cornell University (Hawk and Crowder 1978b). Seeds of these and the wild-type can be obtained from the Department of Plant Breeding and Biometry, Cornell University, Ithaca, New York 14853.

Tucked Petal (tu)—Petals are curled downward and to some extent inward as opposed to being expanded and unfurled in the wild-type. In addition, the buds are swollen near the base, giving a Japanese lantern-like appearance. This is a pleiotropic effect which allows classification of the trait prior to flowering. Mutant plants are normal in regard to seedling and plant development.

Jagged Petal Margin (jp)—Petal margins are cut (serrated) as opposed to the normally smooth condition. The mutant can be detected at the first true leaf stage due to a pleiotropic effect that causes a wrinkled and thickened leaf characteristic. In combination with tucked petal this trait is hidden in the double recessive condition, i.e., an epistatic effect.

Dwarf Plant (dw)—Mutant plants attain a height 12 to 15 cm at maturity as contrasted to 40 to 45 cm for normal plants. They are darker green in color which permits classification in the seedling stage.

Male Sterile (ms) —Anthers are small, pointed, whitish, and devoid of pollen. Buds are not as plump and usually are lighter green than normal ones. Petals may be smaller and lighter in color than the normal gold type.

Wild-type—An early-flowering, short life-cycle population (B-1) was developed at Cornell University by selecting among accessions P.I. 175054 and 175079 obtained from the North Central Regional Plant Introduction Station at Ames, Iowa (Hawk and Crowder 1978a). It is used for outcrossing so as to maintain mutant types and for practice pollinations. The population is not devoid of recessive alleles in the heterozygous state and is a source of other mutant types. In actuality, this is not an undesirable feature but adds interest for student observation of F_2 segregating populations.

Practice Seeds—Seeds from the student F_1 and F_2 populations can be bulked and used to grow plants for practice pollinations. This material will contain various mutants due to segregation and recombination of recessive alleles carried in the heterozygous condition. Students will be fascinated in searching for and identifying the mutants.

Source of Additional Mutants—Outcrossing in *Brassica campestris* is by wind and insects and is reinforced by self-incompatibility alleles. Recessive mutants are therefore not frequently observed. The incompatibility effect does not appear until flowers open. Thus, self-fertilization is attained by means of bud pollination. Mutants are usually exposed among S_1 progenies of the wild-type population. They occasionally appear spontaneously among progenies and segregating populations of student material. The mutant types can be maintained by sib-pollination or outcrossing to wild-type plants.

Additional mutants are also available from the Department of Plant Breeding and Biometry at Cornell University.

Procedures for Working with *Brassica campestris*

Flats and Soil Mix

1. Flats. We find Todd Planter flats that are 67.6 x 34.5 x 9.0 cm and contain 72 cavities each 25 cm^2 to be of convenient size. The model 200 is available from Speedling, Inc., P.O. Box 7098, Sun City, Florida 33586.

Use of large-size containers will increase axillary branching and tend to delay pod maturity.

2. Soil mix. An artificial soil mix (Cornell mix) containing osmocote provides a suitable medium for growing plants but other types can be adapted.

3. Filling flats. Flats are filled with artificial mix and packed down with the fingers. They are refilled and repacked with a board or palms of the hands. The mix is leveled to the top of the flat by brushing one hand over the top of the flat. This prevents subsequent spillage over the sides when watering. The packing and leveling are important to avoid excessive settling of the mix and to provide a firm base for seedlings.

4. Wetting flats with the mix. After the flats have been filled, they are soaked thoroughly with a *gentle* water spray. The mix may be difficult to saturate and must be spot checked to assure thorough wetting at the bottom. Be sure to spray any excess soil mix off the outside of the flats.

Note: Always soak the filled flats prior to planting as watering after sowing may wash the seeds out of cavities.

5. Washing flats after use. After seed harvest remove soil mix wedges, scrub flats with a stiff brush, and wash thoroughly with a strong spray of water. Then dip entire flat into a chlorine solution prepared by mixing 500 ml of household bleach (Clorox) with 40 *l* of water. This reduces the build-up of mineral deposits and algae. Allow flats to dry thoroughly before reusing.

Planting

1. Holes. Make planting holes about 3.0 mm deep in the center of each cavity using the eraser end of a pencil. Poor emergence results from planting too deeply.

2. Density. Plant *only* one seed per hole since mutants may be selected against by overcrowding and by thinning. Do not hold seeds over the flat during planting as they may be dropped and are difficult to find in the dark soil mix.

3. Covering. When planting is finished squeeze the holes shut with the fingers or cover lightly with vermiculite.

4. Fungicide treatment. A captan solution should be added to the surface of the flats by a technician. It is prepared by adding one tablespoon of wettable captan per gallon (approximately 4.5 *l*) of water in a sprinkling can with a fine nozzle. The captan solution is applied to each flat as an aid in control of fungi that cause damping off. One gallon is sufficient to treat 6 to 8 flats.

Labelling Flats

Plant seeds in rows from left to right and front to back, following the short dimension of the flat. In this fashion there are 12 rows and 6 cavities per row. Always start at the front of a row to plant a progeny group; do not put 2 progeny groups in the same row.

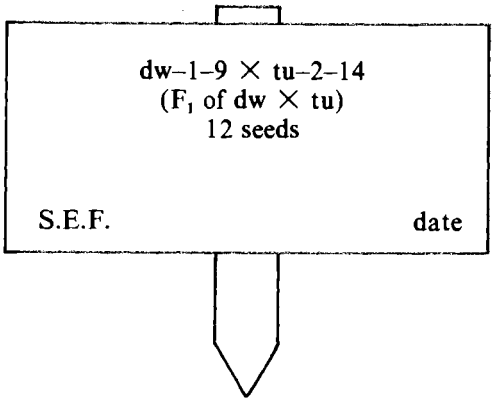

Figure 10.1. Planting label.

For example, if there are 9 seeds of one group, plant 6 seeds in the first row and 3 in the second row, leaving the last 3 cavities empty. Start a new group in row 3. As plants grow they obscure planting labels in the flat. Use a small piece of stiff paper to label the planting, as illustrated in Figure 10.1. The card is then stapled onto a stake and placed in the first cavity of the first row of a planting.

The card contains the following information:

1. Planting identification. Letters identify the mutant, e.g., dw-1 denotes a particular dwarf plant line and tu-2 denotes a particular tucked petal line.

   a. The final number designates a specific plant of the given trait. This is important in recording ancestry.
   b. The cross is depicted with the *female* parent always written first.

2. A brief description of the planting is given in parentheses. In this example, the planting is an F_1 of the cross dwarf (dw) by tucked (tu). The student may also wish to note what is to be done with the plants. For example, intercross to make F_2.
3. Below the description is given the number of seeds planted.
4. In the lower left corner are the student's initials.
5. In the lower right corner is the date of the planting.

Growing the Plants

1. Lighting. Flats are placed on the greenhouse bench without additional lights. After seedling emergence in two or three days the plants are grown under a combination of continuous fluorescent and incandescent lighting in order to induce early flowering. Over a bench of 1.2 x 2.4 m size we use 8 fluorescent bulbs and 8 60-watt incandescent bulbs about 1.0 m above the

plants. Two of the four fluorescent fixtures have been wired to hold 4 incandescent bulbs. After pollination, plants are placed on another bench under continuous fluorescent light to reduce heat and enhance seed set.

2. Temperature. The greenhouse is maintained at 21 to 25°C. High summer temperature (above 35°C) will cause pollen abortion.

3. Watering. The technician waters as needed using water held in a tank and tempered to greenhouse temperature. *Students should not add water* as too little or too much can injure plants. Watering is done with a flared water-beaker nozzle and pressure controlled. A heavy spray damages seedlings. For a day or so after pollination, water should be applied at the plant base to avoid washing pollen from the stigmas. As the plants mature, water is cut back and they are only spot-watered. The plants are no longer watered once the lower pods turn completely yellow.

Good drainage is important. Placing two narrow bamboo sticks under the flats creates an air space and aids drainage.

4. Insect control. Aphids are a primary greenhouse pest and are controlled by use of Temik, a systemic insecticide. When plants are two weeks old they should be treated by a *registered technician*, using 10 to 15 particles per cavity of the tray. *Students should not ask for or apply insecticide.* They must *not* munch on the leaves. After working with the plants *hands should be thoroughly washed.* As an additional insect control the greenhouse may need to be regularly sprayed with an appropriate insecticide.

5. Spacing. Provide space for plants to be pollinated by placing them in alternate cavities, checkerboard fashion. Wider spacing is an aid to avoid accidental crossing with neighboring plants. Plants can be moved by sliding a wooden pot label downward into a cavity and gently lifting the entire wedge of soil mix upward. A light watering before transplanting will prevent the soil wedges from crumbling. Transplanting should be done just prior to flowering, when the plants have grown sturdy enough to be moved without injury. After moving plants, press soil firmly against the edges of the cavity.

Note: These are the conditions we have found to favor rapid and vigorous plant growth. It will be necessary for instructors at other institutions to make appropriate adjustments and modifications in culturing plants.

Pollinating

1. A convenient pollinating kit can be assembled by placing straight-tipped forceps, a small bottle of 95 percent alcohol, and tags in a small plastic container.

2. Cross pollinations. The *Brassica* under study is normally cross-pollinated and most plants are self-incompatible. A typical flower is illustrated in Figure 10.2.

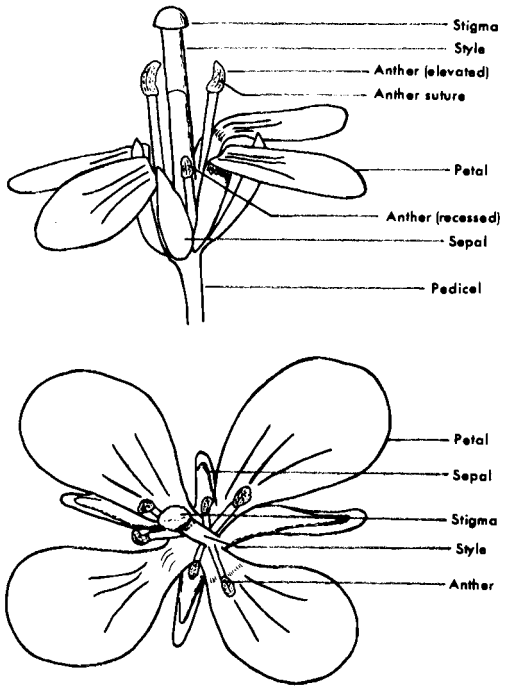

Figure 10.2. Enlarged flowers of *Brassica campestris* L.

a. Precautions to avoid accidental cross- or self-pollination can be accomplished by proper spacing, isolation of individual plants and by careful pollinating techniques. The self-incompatible nature helps guard against accidental self-pollination but occasionally it may occur. Even one selfed seed in an F_1 progeny could distort ratios.
b. Making crosses. A successful cross places only the desired pollen on a particular stigma. This is accomplished via forceps dipped in alcohol solution (kept in a small bottle) prior to making different crosses or selfs. Hands are kept free of pollen by dipping fingers in alcohol. The forceps and fingers are *allowed to dry* and then a dehisced anther (when pollen is shed) from the selected male parent is plucked off towards the base and rubbed onto the stigmatic surface of the female parent. Following pollination one can observe a coating of pollen on the stigmatic surface. Be careful not to knock pollen from the mother plant onto the stigma, as some plants may be self-fertile. The suture of an immature anther faces toward the style (introrse) but turns outward (extrorse) when mature (except for an introrse mutant in which the mature anthers are introrse). The pollen on a mature ex-

trorse anther is shed away from the style. There are six anthers per flower, of which four are elevated and two recessed.

It is important to pollinate only young flowers since older flowers are not receptive. As the flowers age, petals tend to lose their color and offer little resistance to being pulled off by forceps. To avoid futile pollinations snip off old flowers with the forceps, leaving a short stub of the pedicel as a marker where the first pollination begins. Viable pollen will be yellow. Pollen becomes greyish with age and loses its viability. After pollinating, exercise care in watering. Place water at base of plant to avoid washing pollen from the stigmas.

Note: Pollinate 4 or 5 of the youngest flowers and 4 or 5 of the oldest buds. This is usually accomplished without aid of magnification, except those persons having poor eyesight may need magnifying glasses of 10X.

c. Recording and tagging crosses. Small jewelry tags (about 2.0 cm long) are used to record crosses. To prevent deterioration with age, tags are dipped momentarily in a full-strength cuprinol solution, a wood preservative, then dried for 24 hours. Cuprinol is manufactured by the Dareworth Co., Avon, CT 06001.

Each tag should include the following information:

— Identification of female and male parents.
— The number of flowers and buds pollinated.
— Date may be placed on the reverse side of tag if space is limited.
— Student's initials.

An example of a properly labelled tag is shown in Figure 10.3.

The sample tag indicates that plant number 3 of a particular mutant (the mutant phenotype of plant number 3 is found on the planting stake and need not be repeated on the tag) was crossed to plant number 14 of the tucked (tu-2) mutant. The symbols 5F6B mean that 5 flowers and 6 buds were pollinated. The pollination date was 1/7 and the pollinator was Susie E. Fast.

Note: Always use a pencil to record information on tags since ink (including ballpoint) will be washed off when plants are watered.

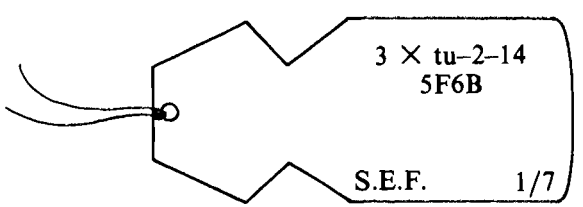

Figure 10.3. Properly labelled tag for a cross.

Carefully loop the tag directly below the first pollinated flower, being careful not to break the plant stem.

d. Numbering plants. In order to keep accurate records it is important to number each plant and include this number with the mutant line abbreviation whenever making reference to a given plant. Information on the tag should also be entered on a record sheet.

3. Self-pollinations. A self is made by placing pollen from young open flowers onto the stigmas of buds of the same plant. This technique is termed bud pollination. The buds are used since self-incompatibility is not fully expressed in the bud stage. There is variation among plants with regard to level of self-incompatibility. We have found some plants that set selfed seeds on open flowers whereas others produce only a few seeds even after bud pollination.

The stigmas of buds are exposed by gently slicing along the edge of the sepals with forceps to open the bud, and trimming away the sepal tips. This exposes the stigma for reception of pollen transferred from a dehisced anther of the same plant. A few flowers are also pollinated to check incompatibility and self-fertility.

Note: Self-incompatibility is determined by self-incompatibility genes. Seed set may be low even on certain cross pollinations. This is probably due to a common-type self-incompatibility allele and can be overcome by bud pollinating.

Self-pollinations are labelled the same as for crosses except that the plant number is circled (Figure 10.4). Selfings should also be entered on the record sheet.

Note: All the above techniques will be demonstrated in the first lab and students will have ample opportunity to ask questions and practice techniques prior to making crosses or selfs for the project.

4. Checking pollinations for seed set. Check pollinations about 3 days after a cross and 5 days after a self to determine if they were successful. If so, one can observe pod elongation and swellings along the pod where seeds are developing.

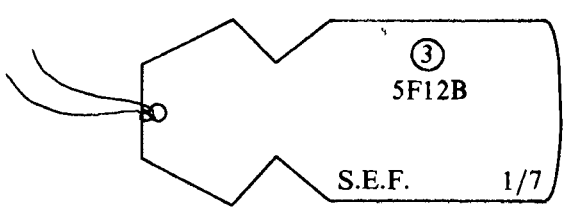

Figure 10.4. Properly labelled tag for a self.

Harvesting Seeds and Handling Progenies

1. Maturity. The pods mature 20 to 30 days following pollination. They should be uniformly yellow and beginning to dry before harvesting. The seed color is usually reddish-brown when ripe. It is not necessary for the pods to be completely dry before harvesting, but seeds harvested prematurely may fail to germinate or germinate slowly. Some seeds may germinate in the pod as maturity progresses.

2. Harvesting. Before harvesting, check the number of pollinations recorded on the tag and count the number of pods set as well as any intervening unsuccessful pollinations. *Be careful not to harvest either below or above the recorded pollinations.* Remove pods and tag and place in a coin envelope.

Note: The progeny resulting from a cross between two parents belong to a *progeny group.* Be careful not to mix seeds from different progeny groups, even if the type of cross was identical (for example, dw x tu). Each progeny group should be planted separately and data collected from each group. Statistical tests are required before pooling data is justified. The chi-square test indicates goodness-of-fit to predicted ratios and the homogeneity test determines whether lines within a progeny group can be combined. Both tests should be explained in a handout and discussed during a laboratory period.

3. Labelling harvest envelopes. Write a description on the envelope of the cross or self using the plant identification and other pertinent information. If the tag is not readable consult the record sheet. Note the date of pollination and number of pods harvested. The approximate number of seeds may be noted. Place initials on the lower left corner of the envelope. A properly labelled envelope is shown in Figure 10.5.

4. Shelling Pods. Pans and screens are made available to separate seeds from pods. Be sure to shell over a pan rather than into an envelope to avoid seed loss. Discard any diseased, shriveled or germinated seeds. Place in an envelope and secure with a paper clip or staple. Store the seeds in a box provided for the laboratory section. It is important that corners of the envelope are tightly folded.

5. Handling progenies. As noted previously, keep each progeny group separate. Also, keep each cross between F_1 sibs separate since this permits the identification of pollination accidents that may distort segregation patterns.

Records of the Experiment

Keeping detailed, accurate records is important to success of the project. It is important to take time to transfer each pollination noted on the tag to record sheets that are available.

1. Record ancestry. It is essential to keep a full record of the pedigree. This information should already be on the harvest envelope and thus transferred to the record sheet at the time of planting.

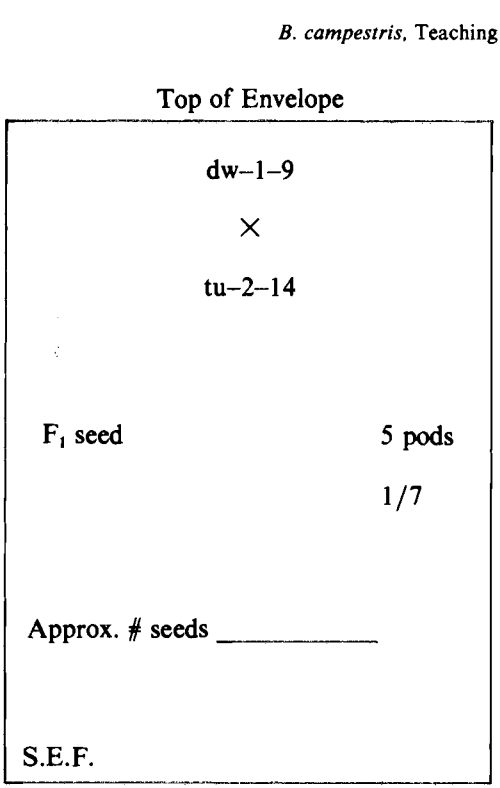

Figure 10.5. Harvest envelope with pedigree information.

2. Record sheets. In addition to the information found on the pollination tags, the record sheet will show planting date, number of seeds planted and seedlings emerged at 7 days, detailed descriptions of the characters under study, and other pertinent observations such as new mutants. It is important to date observations since some characters may change during plant growth. Duplicate records are advisable to avoid chances of loss. A sample record sheet is shown in Table 10.1.

Table 10.1. Sample record sheet.

Pedigree:		Page:	
Generation:		Name:	
No. of planted seeds:		Date Planted:	
Seedlings emerged by 7 days:			
Plant #	*Description*	*Pollination*	*Date*
1.			
2.			
etc. . . .			

Note: Procedures for working with the *Brassica*, along with any other pertinent material, can be given to students in a handout at the first class or laboratory session.

Final Report

A report on the inheritance study is due at the end of the semester (quarter). The following outline should be used as a guide:

1. Introduction—Set forth the purpose of the study, noting the traits under study and concepts being considered.
2. Materials and Methods—Give a brief resumé of methodology (the student handout may be cited) along with a description of the mutants used.
3. Results and Discussion—Present data in tabular form, listing both mono- and dihybrid results; show chi-square and homogeneity tests. Discuss the data from the viewpoint of proposed hypotheses and postulate why discrepancies might have occurred.
4. Summary and Conclusions—Write a brief resumé of the experiment and make a statement of conclusions.
5. Bibliography—Cite references.

References

1. Hawk, J.A.; Crowder, L.V. *Brassica campestris* L., a higher plant for teaching genetics. J. Hered. 69:121–124; 1978a. (A discussion of methodology used to select short life cycle *Brassica* wild-type and mutant populations and their use in teaching genetic principles.)
2. Hawk, J.A.; Crowder, L.V. The inheritance of four mutants in early flowering *Brassica campestris* L. J. Hered. 69:125–127; 1978b. (A description of techniques in development of mutant types and observed inheritance patterns.)
3. Hanson, W.D. Minimum family sizes for the planning of genetic experiments. Agron. J. 51:711–715; 1959. (A review of the technique used to determine the number of plants needed to evaluate inheritance patterns.)
4. Little, T.M.; Hills, F.J. Agricultural experimentation: design and analysis. New York: John Wiley and Sons, Inc. 1978. (Contains detailed procedures for performing chi-square and homogeneity tests in evaluating inheritance ratios.)

APPENDIX

Planting Time

Days to flowering vary with genetic material and growing conditions. At Cornell, seeds of practice-pollinating material are planted 18 days prior to use. The tucked mutant can be planted at the same time, but jagged petal margin, dwarf and male sterile mutants should be planted three days earlier to coordinate flowering. The instructor will need a time schedule to assure that mutant-type plants are ready for testcrossing F_1 progenies.

Maintaining Genetic Material

The wild-type population is maintained by intercrossing normal plants of B-1. This is accomplished by mass-pollinating:

1. A small web made from cheesecloth is used to collect pollen from plant 1; plant 2 stigmas are then lightly brushed while simultaneously collecting pollen; plant 3 is brushed; then plant 4, plant 5, etc.

2. Forceps are used to collect a mass of anthers and pollen from random plants. Then stigmas are rubbed in a systematic fashion, the pollen source being renewed from time to time.

3. Plants in full flower can be enclosed in a cage with honey bees for a period of two or three days.

Tucked petal and dwarf mutant types can be maintained in a similar manner. It is advisable, however, to outcross mutants to wild-type plants every second or third generation. This assures that plant vigor and the number of incompatibility alleles are kept at near maximal. To recover the mutant trait, F_1 seedlings are sib-pollinated (plant 1 x 2, 2 x 3, etc.) and F_2 segregants selected. Pollen viability of dwarf plants is drastically reduced when the temperature approaches or exceeds 35°C.

Plants of the jagged petal are less vigorous than the wild-type and some other mutants. Seed set is generally low but pollen viability is good. Thus, the mutant must be maintained by outcrossing to the wild-type, sib-pollinating F_1 plants and holding F_2 seeds in reserve. Mutants for student use are recovered among the F_2 segregating population. The male sterile mutant can only be maintained in the heterozygous condition by outcrossing to the wild-type and sibbing F_1 progenies or crossing the homozygous recessive to the heterozygote (a testcross).

Chapter 11

Uses of the Honey Bee *(Apis mellifera)* in Teaching Introductory Biology Laboratory Courses

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Introduction

The honey bee (*Apis mellifera*) (Figure 11.1) forms a colony that is easy to maintain within the laboratory environment, easy to observe, and incredibly complex and interesting. Observation honey bee colonies make it possible for students to observe and study many aspects of the sociobiology of this insect. They can give the student direct experience with important ideas and concepts, including caste and caste determination, division of labor, colony homeostasis and the superorganism concept, pheromonal communication, and foraging recruitment as shown by the dances of the honey bee. The queen, worker, and

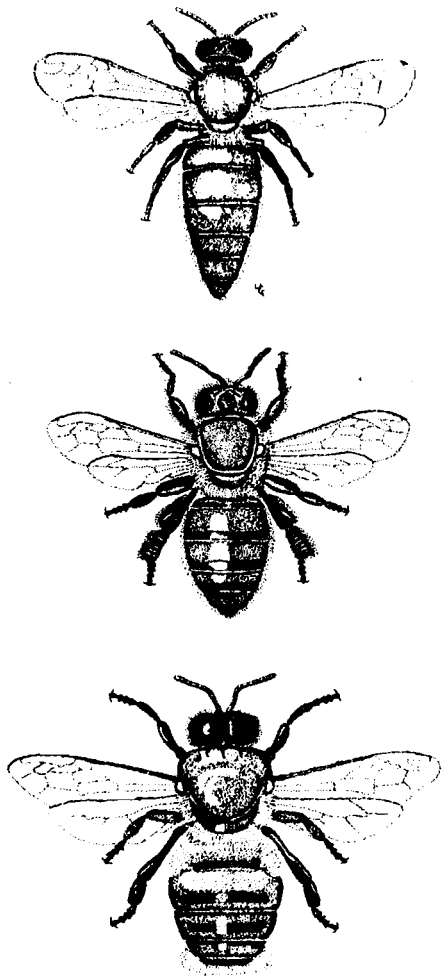


Figure 11.1. Queen (top), worker (center), and drone (bottom) honey bees (*Apis mellifera*).

drone honey bees show complex morphological adaptations that well illustrate the structure-function concept. It is also relatively easy to do electrophysiology on the antenna of the worker. Even the bee's most objectionable attribute, the stinger, offers students an opportunity to study one of the most effective individual and colony defense systems known. Considering the many virtues of this organism, I think that every science and biology department should have at least one observation colony available for their students to experience. This chapter describes how to establish and maintain observation colonies of honey bees, as well as their use in various biology courses.

Observation honey bee colonies can be used intensively or only occasionally in a course. In the introductory biology laboratory course at Cornell University, we have found that the honey bee colony is an excellent system for introducing students to scientific methodology. The observation bee hive is ideal for an investigative laboratory since there is much to observe and most of it is both interesting and unfamiliar to students. These observations lead to question asking, the formulation of a research hypothesis, and design of the study. In our course, students spend about two to three hours in this phase of the investigative sequence. They then have the entire next laboratory period to conduct the study. An additional week could also be devoted to a follow-up study to give students a really sound introduction to the investigative process. Alternatively, benefit can also be gained by using the observation colony simply as course enrichment to demonstrate specific phenomena within the course's subject matter. Some use could be made of observation honey bee colonies in any of the following courses: introductory biology and zoology, behavior, sociobiology, and entomology. Simply having an observation hive available in the laboratory can create student interest for biology and lead to much learning even if never used as a formal part of any course.

In courses concerned with electrophysiology, the electroantennogram technique can easily be performed with the honey bee. An observation colony not only makes workers readily available as antenna donors but also allows a combination of electroantennogram and behavioral bioassays in pheromone studies. The pheromone communication system of the honey bee has been well studied, and synthetic forms of many of its pheromones are readily available.

The first part of this chapter describes methods for obtaining, establishing, and maintaining observation colonies within the laboratory. The second part presents the various uses of observation colonies in biology laboratory courses, including morphological studies of worker bees. The final section presents a simplified electroantennogram method and its application for pheromone studies with the honey bee. Appendix I contains sources of equipment and supplies useful in working with honey bees, and Appendix II consists of preparatory information on chemicals mentioned in this article.

Establishment of the Colony and Equipment

When Can Observation Colonies Be Used?

Local weather conditions will determine when observation colonies can be maintained within the laboratory. Honey bees require precipitation-free days with an air temperature in excess of about 15°C for flight. Although a closed observation colony can be set up on a short-term basis (one to two days), the most effective use of the honey bee colony requires hives that provide the colony access to the outside. Although sugar syrup and pollen substitute can be supplied within the hive to satisfy the colony's nutritional needs, the bees do require periodic flights to defecate, which they will not do within the colony. The minimum requirements for long-term use (months +) of observation colonies of bees are for outside temperatures to exceed 15°C for at least several daylight hours every one to three weeks. In warmer parts of the country where this requirement is met year-round, hives can be maintained continuously. In areas with prolonged winter weather, their use is restricted to the warmer months. For example, they can be established in the spring, maintained throughout the summer, and disbanded in late fall. In Ithaca, NY, we can set up colonies in March and use them continuously until late November. In warmer months, with access to natural food sources, colonies will grow and colony reproduction or swarming will occur within the observation hives, which are continuously adjusting colony size to the space available.

The difficulty in successfully overwintering observation colonies results from their small size and the warm in-lab environment, which causes them to remain relatively active. Because of this activity, they consume much food and accumulate excessive fecal material, which usually leads to disease and dysentery unless cleansing flights are possible. With the onset of dysentery, social organization breaks down and the colony perishes. However, because a small colony cannot produce sufficient heat to survive outside, outdoor overwintering is not advisable. Occasionally we have been successful in overwintering observation colonies indoors in Ithaca, usually due to a fortuitously even distribution of warm periods for cleansing flights throughout the winter. Overall, for areas with winters similar to those in upstate New York or more severe, I would recommend disbanding the colonies with the onset of cold weather and reestablishment the following spring.

Obtaining the Observation Hive

Plans to build a simple observation hive are shown in Figure 11.2. Figure 11.3 shows the version of this hive currently used in the introductory biology laboratory course at Cornell University. Only a single honeycomb should exist between the hive's two transparent sides. This construction allows the observer to see all that is occurring within the colony. Several biological supply houses

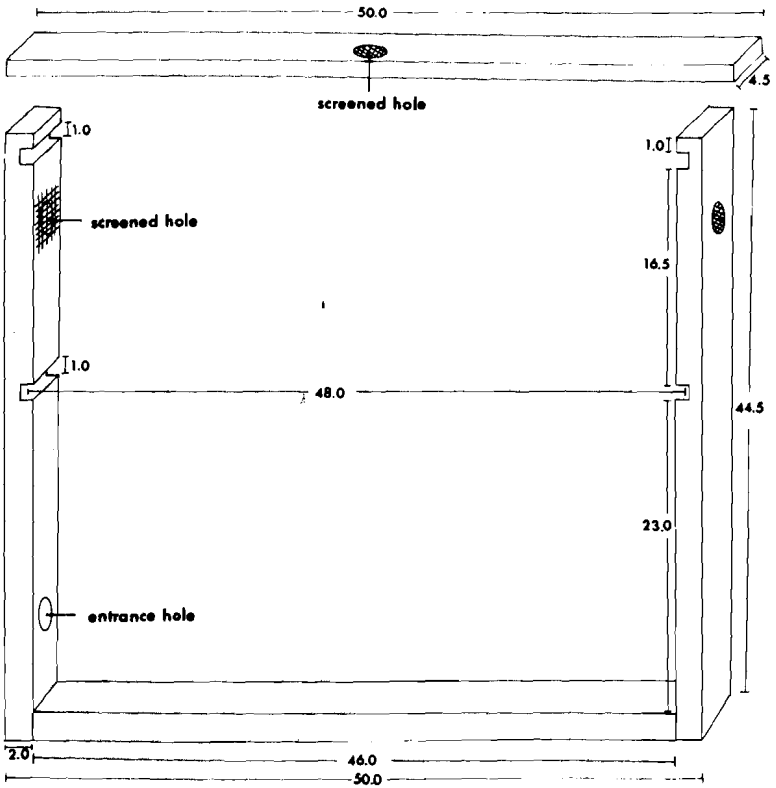


Figure 11.2. Plans for the construction of an observation bee hive (all measurements are in cm). Glass or plexiglass hive covers should measure 50 × 46.5 cm.

sell observation colonies with multiple combs. Since many of the biologically interesting events occur at the center of the hive, this is a most inappropriate arrangement. Our hives include a full-size comb below with a shallow-depth comb above. A larger hive could be built to accommodate two full-size combs. The inside dimensions of the hive are important and are designed to maintain the so-called bee space, about 1.0 cm, between all internal surfaces. Bees require this much space for free movement within the colony and will build bur comb in spaces that exceed this dimension. Covers can be either plexiglass or glass. Plexiglass is light and can be attached to the frame of the hive directly with screws. Unfortunately, plexiglass scratches and we have found that after several years of use must be replaced. Glass is heavier but it is easier to see through than plexiglass and need not be replaced. Plate, safety, or wired glass reduces the risk of breakage. A cloth cover of some sort should be used to exclude light when the colony is not being observed. The basic hive design can be modified in various ways. Holes in the top of the hive can be

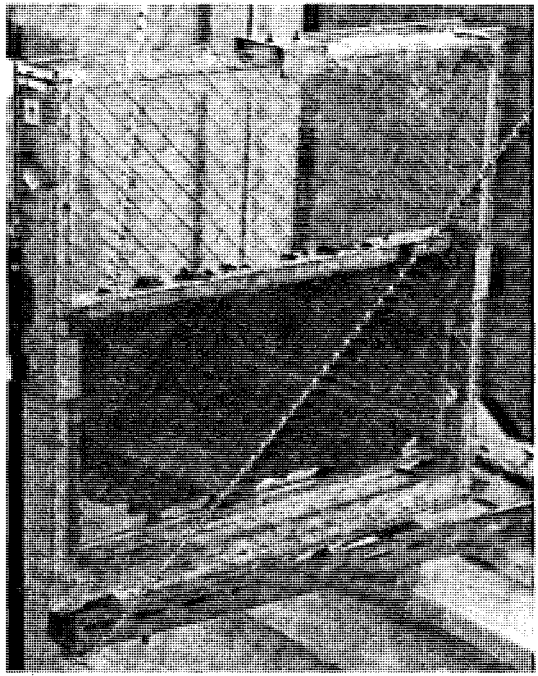


Figure 11.3. Observation hive used in the introductory biology laboratory course at Cornell University to better show construction. Covers are plate glass reinforced with wire. Note sliding access port on top for adding and removing bees and velcro-covered hole on back side to permit introduction of thermometer or CO_2 tube (see text).

used to add things or to remove bees. As shown in Figure 11.3, we have a short piece of rigid plastic tubing glued to a small section of plexiglass (dichloroethane is an excellent plexiglass glue) that slides over the top access hole. This makes it easy to remove workers walking by on the top comb with the bee aspirator (described later) without releasing additional bees to the laboratory room. Workers can be added to the hive by placing them into the tube, corking it, and sliding the device so that the tube is now over the access hole. We use a plexiglass top to facilitate observations in the upper part of the hive when things are added to the colony. Holes can be drilled in the hive sides so that pheromones can be tested within the colony, as described later.

The number of observation hives you should obtain will depend in part on how intensively you plan to use the honey bee in your teaching situation. If students are to do investigative studies, we have found that about one colony for every four students is adequate.

During inclement weather you should provide your bees with sugar water (1:1 sucrose to water). Inverted plastic catsup dispensers are ideal; drill holes in the top of the hive to fit the delivery end of the dispenser.

Getting the Bees

Unless you keep bees yourself, we suggest that you contact a local beekeeper to obtain the bees for your observation hives. The local agricultural extension agent can help you locate a beekeeper. If you are to build your own hives it might be good to locate the beekeeper first and determine the size of the comb-holding frames he uses, since these vary somewhat. The so-called full-depth frames are 9⅛ inches deep (23.2 cm), but if the equipment is homemade this can vary. Also, the shallow-depth frames come in a variety of sizes. Most beekeepers are fairly knowledgeable, friendly people, and you should have little difficulty in locating one that would be agreeable to setting up hives for you. You will need an adequate number of bees (about 0.5 kg or 3000 workers) and one queen per hive. If the beekeeper puts one full-sized frame of brood with the bees that are on it into the hive and also shakes the bees from another frame into the hive, an adequate supply of workers will be obtained. Queens can be obtained in several ways, depending in part on the beekeeper's wishes. The queen from the colony that provided the workers can be introduced directly. If the beekeeper raises his own queens, the observation hives can be most efficiently set up from the small colonies called nucleus colonies (nucs), which are used to house the virgin queen until she has completed her mating flights. Alternatively, you could buy the beekeeper replacement queens from a commercial queen producer that the beekeeper recommends. The old queens can be put directly into the observation hives with the appropriate group of workers.

Foreign queens can be put into observation colonies with a special cage called a queen, or introduction, cage. Queens are shipped in these cages through the mail from queen producers. If the beekeeper prefers to keep his own queens, you could order queens from a commercial queen producer and use the cages to introduce them to the colonies of workers obtained from the beekeeper. The beekeeper can help you with the details of how to use the queen cages, which can be left in the hive after the queens have been released. A less attractive alternative to obtaining a queen is to use a queen cell containing a pupa. This is riskier because the queen must go on mating flights five to six days after she emerges, and a small percentage are lost on these excursions outside the hive. In the unlikely situation that you cannot find a beekeeper who is interested in providing you with the bees or the queens, you could order packages of bees and set up the entire hive from the packages. A three-pound package of bees (about 10,000 workers and one queen in an introduction cage) could be used to set up two colonies if you purchase an

additional queen. This is a somewhat more complex matter, and, because the colony will be without brood, it will take longer for the colony to grow and become marginally interesting from an educational viewpoint. Also, queen acceptance in small package colonies that start out with no brood is notoriously poor. This argues against using this arrangement to establish the colony if the services of a local beekeeper are available.

Reimbursement of the beekeeper should relate to the extent of services rendered and material provided. Assuming the beekeeper does all the work, including setting up the hives and providing all material, we would suggest an upper limit of about $30–35 per colony ($5–6 for the queens, $10–15 for the bees and combs, and the rest for labor). If you buy the queens and you do a significant amount of the work, what you pay should be reduced appropriately. Also, if after you are through with the hives the same beekeeper is to get back the colonies, you should pay less. For example, if you buy new replacement queens and the beekeeper gets the colonies back at a time when they will do him some good, perhaps just the cost of the queens plus labor would be adequate. Clearly, some dickering is in order.

If the colonies are not to be set up permanently (for example, if you are in an area with a significant winter), the beekeeper will probably be interested in getting back the bees and equipment. He may combine several hives to form a full-sized colony or may set up nucleus colonies.

Installation of the Observation Hive

Regarding the selection of a location for the observation colony, take the following concerns into account. The in-building environment should be appropriate for the colonies' well-being; avoid areas exposed to extremes of temperatures (i.e., do not locate the colonies over heating or cooling ducts or radiators; provide shade from excessive sunlight). Select an appropriate location so that adverse interactions with people are avoided. In this respect, locate your observation hives so that flight away from the colony is not across areas that people frequent. Bees tend to fly up as they leave the colony. A second-floor location is ideal; if on the ground floor, a location away from walkways is necessary. Alternatively a hedge or fence that extends a meter or more above the colony's exit would provide safety for an adjacent area with intensive human activity.

Mounting the hive to the building will vary with the details of your local conditions. The important thing is stability. A broken observation hive within a classroom is most embarrassing! Providing access to the outside can be accomplished with polyethylene tubing (rigid or flexible). To avoid drilling holes in buildings, if windows can be opened, a plywood spacer with a hole can be installed immediately above the sill.

Immediately after installing the hive, leave it covered and undisturbed for several days. After the bees have learned where the flight hole is located, they

will not be confused by the light when the cover is removed, and normal activity will be observed within the colony.

Movement of Established Observation Colonies

Once a colony has become established at a specific site, even for only a few hours, most of the foragers will have learned the colony's location relative to local landmarks. It is not advisable to move an established colony a short distance (for example, to another side of the building or a different building on the same campus) because most of the foragers will return to the old location and be lost to the colony. Colonies must be moved at least 4 km from the old colony location in order to avoid this problem. This is also an important consideration when originally setting up the observation colonies; the donor colonies must be at least this distance from the proposed site of the observation colonies or the foragers will return to the donor colonies.

Additional Useful Equipment and Techniques

Removal of bees from a colony will be necessary in certain types of studies and is easy to do. A simple corked hole in the hive's top is suitable, but the device shown in Figure 11.3 allows a more controlled means for both removing and adding bees. Figure 11.4 shows a simple aspirator for removing workers

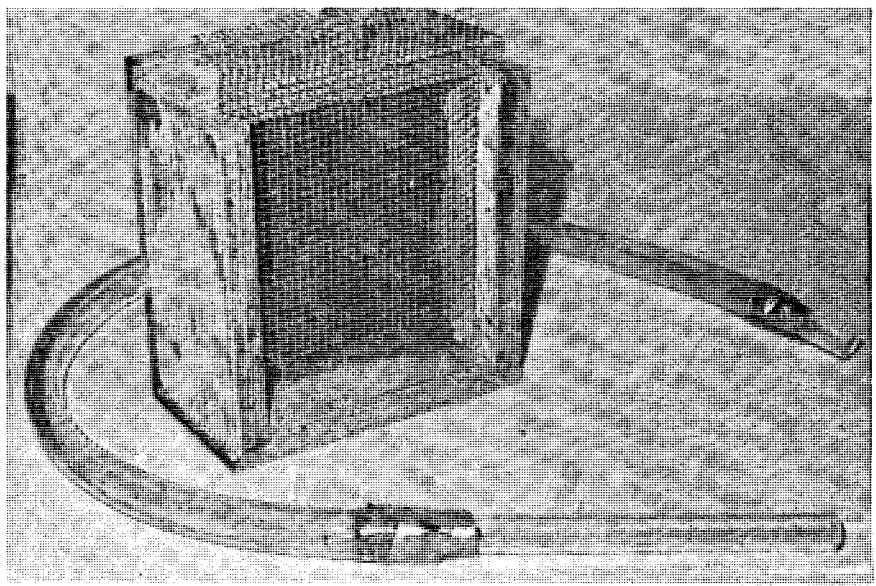


Figure 11.4. Bee aspirator and holding cage (construction of both is described in the text).

from around the access hole within the hive. It consists of a short length of rigid plastic tubing with the proper outside diameter so that it fits snugly into the access hole. A small piece of plastic screening is placed over the end of the tube, and this screened end is inserted into a length of flexible polyethylene tubing, of the proper diameter, and about 40 cm in length. Bees can now be pulled into and retained inside the rigid plastic tube by inhaling sharply. If a number of students will be using the same aspirator it would be good to have some germicidal solution available to clean the mouth end of the device.

Bees should be anesthetized with CO_2 before marking. Gas can be delivered directly into the aspirator tube. Usually an exposure of 15–20 sec of pure CO_2 will anesthetize the bee for at least several minutes. Usually 10 minutes are required for complete recovery. Bees can be marked individually with various combinations of colors of model airplane paint. A small paintbrush should be used to put just a spot of paint on the dorsal surface of the thorax and/or abdomen. Alternatively, you can purchase (see Appendix I) numbered, plastic discs of various colors that are designed to be glued on the bee's thorax. Canadian balsam is the preferred glue and is included with the marking kit. Handle bees with insect forceps during the marking procedure.

In some of the studies to be described, a holding cage is useful for retaining workers for from several hours to days away from the colony. For short periods of time, a simple covered plastic container, with holes to provide ventilation, is adequate. For longer time periods, a holding cage similar to that shown in Figure 11.4 is better. This is a five-sided wooden box with a sliding screen door in front. A piece of honeycomb is cut to fit tightly in the box's back. Retained bees will usually quietly rest on the comb and remain healthier for longer in a cage like this. A pipette can be used to fill up some of the cells with sugar solution to provide a food and water source for the bees.

For studies where newly emerged adult workers (or drones) are required, a special cage designed to enclose a framed honeycomb is useful. This can be easily made (see Figure 11.5 for an example). The comb containing mature brood cells is placed within the cage and kept in a moist incubator at a temperature of about 35°C.

Basic Observations and Studies on Observation Colonies*

This section contains a description of the basic phenomena that can be observed within observation colonies. The suggested studies can be used in several ways by the instructor; students can independently work on these projects in an investigative lab setting, or the instructor can select those studies

*Some of the material in this section has been adapted from the laboratory text *Investigative Biology*, Glase et al. 1979.

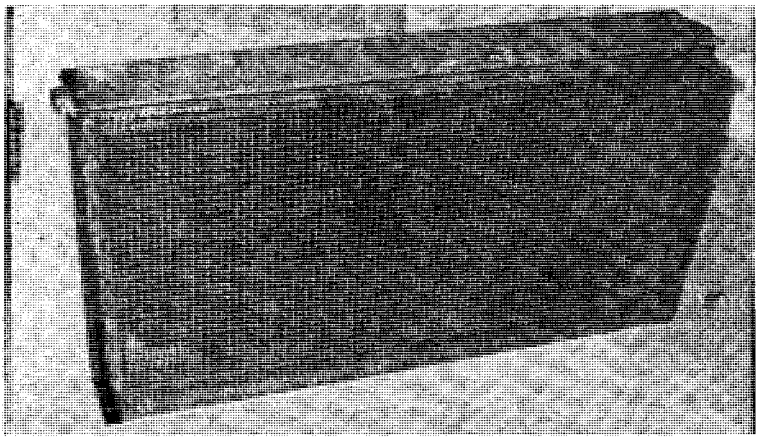


Figure 11.5. Cage for holding a frame of brood in order to obtain newly emerged bees. Cage should be constructed to provide a bee space over the comb surface so that bees can eclose freely.

most appropriate to the course's objectives and either present them in a demonstration format or have the class collect data in instructor-designed studies. The bibliography section of this chapter contains a number of sources of additional information on most of these topics.

Caste Identification and Structure of the Hive

In addition to workers and the queen, your observation colonies may also contain some drones; drone production is dependent on the season and colony strength. We have found pinned specimens of workers, queens, and drones useful in helping students identify the honey bee castes. We also have available larval and pupal workers in ethanol (70%). If your colony has a laying queen, eggs and larvae can be observed within the colony. Students should be able to find brood cells with emerging workers, providing the queen has been laying for at least 21 days.

You will have to help students in identifying the contents of the honeycomb cells. Open honey cells are obvious. Full honey cells are capped with pure white wax, which may become travel-stained but is always whiter than the cappings on brood cells. Cells containing pollen are usually only partly filled, seldom capped, and the pollen is usually packed firmly into the cell's bottom. Cells with brood contain eggs, larvae, or pupae. Cells with eggs and larvae are open while those with pupae are capped. The egg is 1.0 mm in length, elongate, rounded at the ends, and placed so that it is attached by one end to the cell base and protrudes into the cell's cavity. Larvae hatch from

the egg on the third day after oviposition and remain curled up on one side until the cell is sealed on the eighth day. After the cell is sealed, the larva stretches out and spins its cocoon (this makes the brood cappings considerably darker than those on honey cells), becoming the pupa. The adult worker emerges on the 21st day. High-intensity spotlights and magnifying lenses are useful to see into the bases of cells.

In honey bees, three innate tendencies determine the pattern of comb usage in a colony. First, the queen begins laying eggs in the lower center area of a comb. Second, workers prefer to store honey in the upper, peripheral parts of combs. Third, workers store pollen next to the brood area in a given comb. Thus, the brood nest is usually elliptical in shape, having the oldest brood in its centermost cells (capped mature pupae) and the youngest (eggs, young larvae) in its peripheral areas. Honey storage occurs in cells found in the corners of combs and along its edges. Capped honey cells are in the most peripheral parts of the upper comb. Between the honey and brood areas is a band of cells, 2 to 6 centimeters wide, containing pollen.

Worker Activities within the Hive

In addition to those workers seen walking about within the hive, many will be seen head-down in cells with only their abdominal tips showing. These individuals are either cleaning cells in preparation for oviposition by the queen, feeding larvae, or loafing. Cell cleaners usually rotate within the cell as they lick and scrape the cell walls. Nursing can be verified by observing the larva after the worker departs. Loafing workers are motionless and may remain within the cell for extended periods of time. Workers head-down in honey cells are either feeding or depositing nectar. In addition, during periods of intense nectar flow, you may see workers standing in the honey storage areas of the hive manipulating nectar with their mouthparts, in a process that promotes evaporation of water and nectar concentration. See Gary (1975) for a description of this process.

You will most likely observe wax secretion in crowded colonies, where little space is available for incoming nectar. Nectar is automatically metabolized and converted into wax so that workers unable to empty their honey stomachs will be producing wax scales. A pair of wax glands is located on each side of the midventral line of the four major overlapping abdominal segments. Only in 12- to 18-day-old workers are wax glands fully developed and actively producing wax. Workers standing motionless on honeycombs or hanging from the hive's side covers may be secreting wax. Look for the small, transparent, oval wax scales projecting from between the abdominal segments. Wax scales will drop off and accumulate along the bottom of the hive if wax production exceeds usage. Comb building should be evident within your hive. In addition to the main combs, bur combs are actively constructed as braces

between the main comb and the hive's sides and between the frames. The capping of brood and honey cells also allows study of wax usage. Patient, careful observation of single, partly capped cells will show that bees often appear to work in opposition when building, one bee sticking a masticated piece of wax to a partly capped cell only to have it removed several minutes later by another bee. Many bees are involved in the capping of a single cell.

Propolis is a yellow, sticky, resinous material that is collected by workers from certain plants. It is used as a glue within the hive to anchor the combs to the hive's surface and to fill cracks and crevices. During the warmer parts of the day, you can see foragers returning with propolis, which is carried, as is pollen, on their specially modified hind legs. Within the hive, propolis will be especially used to fill in the cracks where the wooden members are joined and between the hive covers and sides. Morse (1972) provides additional information on the characteristics and uses of propolis.

In addition to nectar, pollen, and propolis, bees also collect water, both to dilute the stored honey prior to use and in cooling the hive. On very warm days, bees can be seen depositing water on the comb surfaces to promote evaporative cooling. Fanning workers are commonly observed at such times. However, because fanning is used not only in colony thermoregulation but also in general ventilation and honey production (one important step in converting nectar to honey is the evaporation of moisture to reduce the water content from about 80%, typical of most nectars, to about 15%), fanning workers can be seen at almost any time. Some of the investigations described later present studies that can be done on the functions of fanning in the honey bee colony.

Worker-Brood Interactions

Quantification of the amount of care received from workers by larvae is both easy and instructive, particularly if larval age is also determined. Relative age classes can be based on larva size or by daily marking the location of cells containing eggs in order to obtain a sample of larvae of known ages. Larval care can be quantified by recording the number of worker visits and the duration of each visit per time unit. As reported by Gary (1975), larvae received more than 1300 visits per day during the eight-day period from egg laying until the cell is capped preceding the pupal stage.

Hive-Cleaning Activities

The absence of dead bees or detritus within the colony suggests that some mechanism is available to the colony for quickly removing these objects. Dead bees and pieces of straw can be added to the hive through the top access holes. How many workers are involved in the removal of detritus? How direct is the path to the exit hole? Students can time the events that occur and trace the path of removal with wax pencils on the hive cover for more detailed analysis.

Map measurers (see Appendix I) are useful for determining the length of these paths. See the article by Morse (1972) for additional information on this topic.

Colony Division of Labor

Division of labor within the honey bee colony is based principally on the age of the worker (Lindauer 1961). With some preparation, it is possible for students to study this most important phenomenon. One must add marked workers of known age to the hive and then make observations in order to determine with what tasks the various age groups are mainly involved. About four weeks before this study is to be done, we obtain a comb with emerging brood (see *Additional Useful Equipment and Techniques*). This is kept in a 35°C incubator, and each day, the newly emerged workers are removed, marked with a dab of model airplane paint on the thorax dorsal surface, and added to the observation hive. Day-old bees are easy to mark because they cannot fly or extrude their stingers. Because of this it is not necessary to anesthetize them, and they can be handled with insect forceps. Also, young workers such as this are readily accepted by the colony even if they are not colony members. We usually add about 20 marked workers to each observation colony. We then wait two days before we change colors and add 20 more bees, obtaining additional brood combs as necessary. The procedure is continued for about four weeks. By then we have added about 180 marked workers to the hive whose ages can be placed with nine or ten three-day age classes.

Before this study can be done it is necessary to define criteria so that worker behavior can be classified into well-defined tasks such as cell cleaning, comb building, brood care, fanning, foraging, loafing, etc. A good approach is to have students make the necessary observations and develop these criteria in one laboratory period and collect data during the following period. Individual marked bees can be observed and their activities classified into these task groups.

An alternative approach would be to initially mark a larger number of newly emerged workers (at least 50) and collect data on their changing activities as they age during the following weeks. Either approach could also provide some information on the age-specific mortality/survivorship of the honey bee worker, and this could be an interesting separate study for those concerned with this area. Worker mortality is low and uniform until the bees begin leaving the nest as foragers (Wilson 1971).

Worker-Worker Interactions

A large variety of worker-worker social interactions will be evident to the careful observer. Productive studies can be done on the more obvious behaviors associated with grooming and food exchange between workers. For example, workers solicit grooming by performing several stereotyped shaking move-

ments that are easily observed. It is important to remind students that bees do not normally use visual cues while within the hive.

Queen Behavior and Queen-Worker Interactions

The egg-laying behavior of the queen can be a basis for several studies. Is the queen's search for suitable cells in which to oviposit systematic or random? Marking pens and map measurers are useful in plotting her course during egg-laying and to determine the number of cells that she re-examines. Does the queen always examine a cell before ovipositing in it? Are there any temporal patterns in her egg-laying activity?

The queen's court is an excellent behavioral system to study. Is its membership constant or changing? What do individual workers do after they have been exposed to the queen for a period of time? Is the size of the court influenced by the queen's activities (when she is actively egg-laying or simply moving over the combs), by her rate of movement, or by her location within the colony? Does the court size for queens from different colonies differ significantly? Can differences be correlated with colony characteristics (e.g., size of the worker population)?

Early in the semester we involve our students in a group project to collect data on the court sizes of the queens in the four observation colonies in each room. The objectives of this project are to show the degree of variability that biological phenomena typically exhibit, to illustrate how statistics can be used to describe and better understand a group of data, and to show how a consideration of the sources of sample variability (both intra- and inter-hive) can lead to investigations of specific hypotheses.

Colony Response to Smoke

If you are familiar with beekeeping, you know that before a beekeeper opens a hive to manipulate and manage the bees, he burns leaves, straw, burlap, etc., in a special incinerator called a bee smoker and blows the smoke into the hive. After the bees have been exposed to smoke, they are much more manageable and less likely to sting.

Bees respond to smoke by moving to areas of honey storage and filling their honey stomachs. This behavior probably evolved to insure that the colony could carry as much honey with it should fire necessitate abandonment of the hive site. Bees with full honey stomachs are less likely to sting. Another clear response to smoke is an increase in the number of bees fanning. Fanning workers can almost always be seen within a colony, since fanning is their main means of ventilating the hive for both thermoregulation, honey ripening, regulation of CO_2-O_2 levels, and removal of in-hive pollutants, such as smoke. If students want to examine the effect of smoke on either of these two phenomena (engorgement and fanning) they should get good pretreatment estimates and then monitor the number of bees fanning and feeding from honey cells through

time after smoking the hive. A well-defined sampling procedure should be developed. Students could also determine if this response is learned or innate by systematically collecting data on the responses of newly emerged bees. Smoke can be added to the hives with aerosol cans containing hardwood smoke concentrate (see Appendix I). Spray for only three seconds through the top-central access hole.

Colony Thermoregulation

Bees are able to maintain the hive temperature within fairly strict limits despite outside temperature fluctuations. Bees respond to an increase in hive temperature in a number of ways. Fanning increases markedly, and, again, students should have pretreatment data to observe this. Also, bees start to leave the brood nest, retreating to the edges of the combs, to the top bars, the bottom of the hive and onto the landing platform. Also, bees carry water into the hives and deposit it over the combs to facilitate evaporative cooling. This, however, is difficult to observe since nectar and water carriers show similar behavior. However, quantitative data can be gathered on fanning and heat-induced movement of bees.

Standard infrared heat lamps are suitable for heating the colony, but care should be taken to increase the temperature gradually and not to exceed an in-hive temperature of 40°C. Holes can be drilled in the side of the hive to allow for the placement of a thermometer within the hive between the two combs. An additional thermometer can be taped to the outside of the hive in the same location. One 250-watt heat lamp at a distance of 60 cm from the hive will increase the temperature within the hive at a reasonable rate above 30°C, but it may be necessary to move the lamp closer to reach 40°C. However, it is relatively easy to overheat a small colony and kill bees, especially brood, so do not exceed 40°C. Sampling areas 6 cm x 6 cm are appropriate for collecting data on the total number of bees and the number fanning as the inside and outside temperature varies.

Colony Response to CO_2

Adequate ventilation within the hive is necessary to both facilitate evaporation of water in honey ripening, and to insure a sufficient oxygen supply to replace that which the colony members are constantly using. It has been found that bees possess receptor organs on the antennae allowing them to monitor CO_2 content in the air (Seeley 1974). When the CO_2 level rises above a certain concentration, the colony responds by increasing the number of fanners. CO_2 can be added to a colony by first filling a balloon to a moderate size with CO_2 from a tank. Twist and pinch the balloon's neck to prevent gas leakage and fit the open end over a glass tube. The glass tube should fit into a hole drilled in the hive's wooden side and be long enough so that the gas

will be delivered to the center of the hive. Release the CO_2 slowly (over a period of about 30 seconds) by releasing finger pressure on the balloon's neck, and observe the response in the lower part of the hive. Pretreatment measurements of the % fanners per unit area should be made and monitored through time after CO_2 has been added. See the article by Seeley for more sophisticated methods and comparative results.

Colony Recognition of Members

Bees can be removed from a hive and marked individually with numbered, plastic discs or paint as mentioned previously. After the marked workers recover from the anesthesia, they can be placed into another colony via the top access holes or returned to the parent colony as a control to determine the possible effects of handling and marking on colony response to the bee. The behavior that colony members exhibit toward marked foreign and nonforeign workers can be quantitatively compared. Specifically, the numbers of workers attracted to the introduced bee and the degree and nature of contact should be monitored through time. Because the age of the introduced bee influences colony reaction rather markedly (see Free 1977 for a discussion of this), it is best to provide students with bees of known age. It is easy to collect foragers from the landing boards of several hives and to introduce these bees. Because only the older bees become foragers, you are using at least the same general age class of workers if foragers are employed in studies of colony member recognition. An alternative study would be to compare colony response to foreign bees of known age starting with newly emerged bees, which will be readily accepted by the recipient colony.

Forager Bee Behavior

During daylight hours when the weather is appropriate, you will be able to observe foragers returning with nectar and pollen. Waggle-dancing foragers will be particularly common during the warmer periods of the day. The interactions between foragers and the younger, hive bees are particularly interesting, as is the behavior of pollen-laden foragers. These individuals will actively move about within the hive until they find a pollen-deposition area. After examining several pollen-containing cells, they will straddle a selected cell with their middle and hind legs thrust into the cell. Specialized movements of the legs are used to dislodge the pollen pellets from the pollen baskets of the hind legs. When the forager moves on, the pellets can be seen resting on the compressed pollen mass in the bottom of the cell. A hive bee will usually very quickly enter the cell and with her mandibles break up and work these pellets into the pre-existing pollen mass. Careful observation is required to see all these events. One interesting observation that students may make while examining the hive is that frequently the pollen areas of the comb consist of

cells containing differently colored pollen. If bees are bringing in pollen of different colors, one observes that bees seek out cells with pollen of the same color (and floral source) as that of the pollen they bear. This can be studied in detail (see Morse 1972).

The waggle dance of foragers will be of interest to your students and can be studied in a number of ways. To directly verify the hypotheses concerning the communicative function of the waggle dance, one would need various feeding stations at known directions and distances from the observation hive, marked bees that had been trained to visit one of the several feeders, a device for measuring the angle from the feeder to the hive to a line from the sun perpendicular to the horizon, and a protractor to measure the angle of the waggle run relative to gravity. Studies of this nature are not feasible in a regular lab, although you might suggest that interested students consider them as potential field projects (see von Frisch 1967 for methods). Several aspects of the waggle dance can be practically studied in the laboratory.

a. What effect does the waggle dance have on workers exposed to it? Students could monitor the activities of bees that have followed a dancing bee for a timed interval to determine the percentage that actually leave the hive. Is there a relationship between the exposure time to a dance and the probability that the attending bee will become a recruit?

b. How do potential recruits gain access to the information content of the dance? Close observation will show that a dancing bee usually has several workers closely following her. These potential recruits usually keep their antennae in continuous contact with the dancer. She effectively leads them through the dance, including the waggle run, and it is in this manner that they learn the angle at which to fly, relative to the sun, upon leaving the hive. The rate of the dance (number of waggle runs per unit time) communicates the distance (effort) required to reach the food source. Relative to this question, what can students study? Again, visual cues cannot be used by the potential recruits. If recruits gain access to the waggle dance's information by tactile cues, what fraction of bees exposed to a dancing bee actually touch the dancer?

c. Does the dance occur before or after unloading of nectar or pollen? This can be answered by simple direct observation, particularly in reference to pollen bearers. The only criterion we can use to determine if a field bee has collected nectar is to see if she feeds hive bees upon her return. Generally, students should find that dancing is done both before, during, and after unloading of nectar, depending on the quality of the nectar.

d. Is there a relationship between the distance from the hive to the food source and the distance from the hive entrance to the position on the comb where the dance is executed? It has been found that the further away the food source from the hive, the greater the distance the field bee travels from the

entrance into the nest before dancing. Thus, slower dances should occur at greater distances from the entrance hole.

e. Is the angle of the waggle run relative to gravity, as has been suggested? Our observation hives can be tilted to an extent. If the hypothesis is correct, a known tilt should cause a predictable deviation in the angle of the waggle run in comparison to pre-tilt dances. Measure the angle of the waggle run of a dancing bee several times, tilt the hive by a known angle, and measure the waggle runs of the same bee under the new condition. Protractors and plumb bobs are useful in this study.

Guarding and Scent Fanning

On days with a good bee flight, guard bees can be observed stationed on the end of the flight tube and landing board. (If an outside window ledge is not available, you may want to install a simple landing platform below the tube to facilitate observations of guard bee activities and scent fanning.) As incoming foragers land they are examined by guard bees, presumably to determine that they are colony members. Scent fanning is also observed on the landing platform. Foragers who have just landed will sometimes scent-fan, particularly if they experience difficulty in finding the hive entrance. A scent-fanning bee stands facing the hive and, while exposing the Nasanov gland (see the section *Studies Using the EAG Technique*) on its elevated abdomen, rapidly beats its wings, creating an air current containing Nasanov pheromones. These pheromones help orient incoming foragers. The frequency of scent-fanning workers will increase after temporarily covering the entrance with a board to disorient foragers, or by similar treatments.

Pheromone Studies

Studies involving several different pheromones of the honey bee are described below. Note: the pheromones mentioned should be made in ether at a concentration of 1.0 $\mu g/\mu l$ (see Appendix II). Microcapillary tubes can be used to accurately measure microliter quantities of these solutions. To do these studies it will be necessary to drill some small holes ($\approx$ 3-mm diameter) through the hive covers so that the pheromone can be applied. One hole through each side opposite the center of the main comb is adequate. If your covers are plexiglass, drilling the hole is easy. For glass covers, we have used carbide-tipped masonry drills on a drill press to make holes. Drill halfway through one side, turn the cover over, and complete the hole through the opposite side.

a. Beekeepers have known since antiquity that the probability of being stung while manipulating bee colonies increases exponentially after the first sting is received. Huber, the famous Swiss student of the bee, discovered in 1814 that freshly excised stingers or the odor of stingers placed near the hive

elicit aggressive attacks by worker bees. It is now known that an alarm pheromone is produced by two masses of exocrine cells associated with the stinger. The pheromone, identified recently as *isopentyl acetate (IPA)*, is released by partial extrusion of the stinger when the worker assumes a defensive posture and/or after the stinger has been anchored in the victim and detached from the bee. Within the hive, workers exposed to IPA orient toward the source of the pheromone and investigate it aggressively with their mandibles and forelegs. Under stimulation by the pheromone, workers move about rapidly and show erratic, jerky movements (these movements may serve to alert additional bees). Extracts of stingers show that the amount of IPA varies somewhat with the age of the worker: young adults (0–5 days of age) contain little or no IPA, while forager bees (15−30+ days old) average about 5 μg per stinger (Boch et al. 1970).

Filter paper strips (3 x 25 mm) that have been marked with pheromone can be inserted into the colony via the holes through the covers. The attraction of the workers to the IPA-marked filter paper and the increase in their movement are the most easily observed behavioral responses to the pheromone. The following approach can be used to quantify these responses. With marking pen and cm ruler, draw a square (about 5 x 5 cm) centered on the hole. Upon application of the treatment, count the number of bees touching the filter paper, and also the number within the square, at 10- to 15-second intervals until the response wanes (about 2–3 minutes). Pretreatment measurements with clean, unmarked paper should also be made. Allow the colony to recover for a couple of additional minutes and test again. This should be repeated with additional pheromone and stingers until adequate data have been collected. In determining if a relationship exists between the amount of pheromone applied and the response of the colony, we suggest using 5-, 10-, 15-, and 20-μg levels. Increase in mobility due to IPA can be measured by connecting the sides of the square with two 5-cm lines that intersect at the application hole. Count the number of bees crossing the line at regular intervals before and during application of the pheromone. *Important:* Allow the solvent to evaporate *before* inserting the paper strip into the hive. This will take only about 5 seconds.

To determine how a detached stinger compares with the synthetic pheromone in eliciting a response, use the following procedure:

1. Obtain a bee and lightly anesthetize it with CO_2.
2. With a fine forceps grasp the stinger by its base and detach it from the bee. (Kill the bee by crushing its thorax.)
3. Place the stinger on a piece of filter paper (3 x 25 mm) and mash it thoroughly with the forceps. Remove any chitinous remains of the structure.
4. Test immediately in the observation hive.

b. In addition to IPA, workers possess an additional alarm pheromone. The mandibular glands of older workers produce the substance *2-heptanone*, which is delivered to the bee's oral area via a short duct. Evidence suggests that alarmed bees can use this pheromone to mark intruders that they encounter near or within the nest. As the worker grapples with the intruder, perhaps holding it with its mandibles, it may secrete some 2-heptanone onto the individual, and the pheromone could then release aggression toward the marked object on the part of colony members. The mandibular glands of guard and forager bees have been shown to contain about 25 μg of 2-heptanone although it is not known how much of this pheromone would normally be released by the worker. The behavioral response of workers toward 2-heptanone is qualitatively like their response to IPA as discussed in the preceding study.

Dissection of the mandibular gland is not too difficult with practice. The whole gland could then be tested and compared with the synthetic pheromone. Figure 11.6 shows the location of the mandibular gland on the head of the worker. Use the following procedure to dissect it.

1. Obtain a worker from a holding cage and anesthetize it with CO_2. Kill the bee by crushing its thorax, and cut off its head with a pair of fine scissors.
2. Pin the head onto a bit of wax in a small petri dish positioned with the lateral surface of the head up. Adjust a binocular stereoscopic microscope for best viewing.

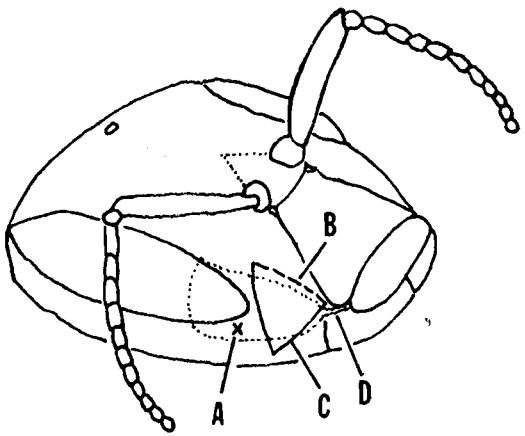
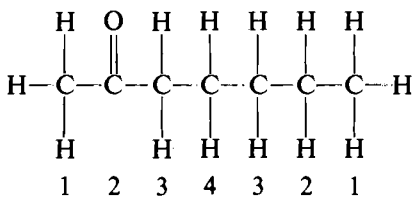


Figure 11.6. Drawing of the head of a worker honey bee to show the location of the mandibular gland: A, mandibular gland; B, cuticle "flex-line"; C, triangular cuticle flap; D, mandibular gland duct.

3. Use a sharp insect pin to make a row of very small puncture wounds through the cuticle along the two lines of an imaginary triangle (labeled "C" in Figure 11.6) located between the bee's compound eye and mandible. Use fine forceps to flex this triangular flap back and expose the underlying mandibular gland. The gland is whitish and fits into a small depression located immediately below the cuticle in this area.
4. With fine forceps grasp the gland and remove it to a small strip of filter paper (3 x 25 mm). Thoroughly mash it with forceps tips and test it immediately in the observation colony. Some practice may be needed before you can successfully remove this small exocrine gland.

c. The pheromone 2-heptanone is one of four isomers having the same molecular formula ($C_7H_{14}O$) and weight (114.18) but differing in the location of the single oxygen atom. The following structural formula is for 2-heptanone, which has the oxygen atom bound to the second carbon atom:



The isomers 1-, 3-, and 4-heptanone have their oxygen in the first, third, and fourth positions, respectively. To determine how specific the receptor is for the alarm pheromone 2-heptanone (the only isomer produced by the worker) students could test 10-μg quantities of the other isomers and perhaps also the hydrocarbon heptane, using the procedures described in the previous study.

d. As mentioned, IPA is only present in significant quantities within the stingers of older workers. Newly emerged bees have no IPA. It has also been found that during the early stages of the worker's adult life the mandibular glands produce no 2-heptanone. In the first 12 days of the average worker's life, she spends most of her time feeding larvae. During this phase the mandibular glands produce other substances that in conjunction with secretions from the other salivary glands serve, in part, as the diet of developing larvae. It isn't until after the worker ceases in her role as a "brood nurse" that her mandibular glands start producing 2-heptanone. If newly emerged workers are available, their stingers and/or mandibular glands can be tested and compared with those of older adults to determine if they differ in their pheromonal activity.

Morphological Study of the Honey Bee*

In addition to pinned specimens of workers, drones, and the queen honey bee, it is instructive to provide students with prepared microscope slides of the head, wings, legs, and stinger of the worker for examination with the binocular stereoscopic microscope. These slides can be purchased from several biological supply houses, but with a little practice it is easy to prepare your own slides (see Morholt et al. 1966). An examination of these slides is best after students have made the basic observations described in the previous section and should help them develop an appreciation for the subtle ways in which the structures of organisms are adapted for their functions. In this section I will describe only the most important, more easily observed features on the external surface of the worker, but see Snodgrass (1975) for an excellent, comprehensive treatment of the anatomy of the honey bee.

Wings

In addition to the general structure of the wings, the row of small hooks along the leading edge of the hind wing and the fold in the rear margin of the fore wing opposite the hooks are particularly interesting. Students can observe how bees, prior to take-off from the landing platform, latch the hooks into the fold, allowing both wings to function as a single unit.

Head

This slide is usually prepared so that the various mouthparts are spread apart and can be studied. One can then watch how these parts are extended and combined by the bee to produce the proboscis by observing workers feeding on drops of sugar water within the hive.

The bee's mouthparts allow the animal to both chew materials and imbibe liquids straw-style. The paired mandibles, or jaws, are used to masticate pollen and, during comb building, wax, as well as to scrape surfaces, as in cell cleaning. They are located in a folded condition at the tip of the head proper. Immediately below the mandibles and projecting forward are the components of the bee's proboscis. The proboscis is composed of three parts: a central labium and two maxillae located at each side of the labium. The labium has its origin beneath the mandibles. The central part of the labium forms the bee's tongue, a hairy-ringed structure. Also, two lateral branches of the labium, called palpi, are evident. The two maxillae are the outermost components of the proboscis.

*Some of the material in this section has been adapted from the laboratory text *Investigative Biology*, Glase et al. 1979.

When not in use, these parts are separate and drawn back under the head. To commence feeding, the parts are simultaneously drawn forward and combined to form the food canal, the maxillae and labial palps being brought together around the tongue. This food canal leads up into the mouth. A large muscular sac in the head, called a "sucking pump," creates the negative pressure within this canal needed to draw up nectar as the bee visits flowers.

Legs

This slide should contain a fore, middle, and hind leg from the worker and can be used to illustrate exoskeleton appendages in general. Each leg is formed by six main segments connected to each other and movable at joints. The last segment of each leg, the so-called "foot," shows adaptations allowing the bee to cling effectively to both smooth and rough surfaces. Two double-pointed claws projecting from the foot readily attach to rough surface features, whereas the oval, central lobe-like structure, located between the claws, can be extended into a suction-cup device for maintaining a hold on smooth surfaces.

Although the general function of the legs is locomotion, several interesting adaptations have evolved for more specific functions. We will consider two of these: the antenna cleaners of the fore legs and the pollen baskets of the hind legs.

Antenna Cleaners. This structure is found near the joint between the fourth and fifth fore-leg segments and consists of a deep semicircular notch on the proximal end of the fifth segment and a clasp-like lobe projecting from the fourth segment across the notch. Note the row of comb-like stiff bristles bordering this notch. In the cleaning procedure the bee bends its head down, raises its fore leg, inserts the antenna into the open cleaner, closes the notch with the clasp, and draws the antenna through the notch. The stiff hairs within the notch scrape any foreign material off the antenna.

Pollen Baskets. The fourth and fifth segments of the hind leg are considerably broader and more flattened than those segments on the other two leg pairs. The pollen basket is the area bordered by long, curved hairs located on the outer surface of the fourth segment. As a bee visits flowers, the hairs covering its body become covered with pollen. During flight the bee collects this pollen from its body with its fore and middle legs; the stiff hairs on these legs are used to comb the pollen off the body's hair. The bee then rubs these pollen-covered legs across ten transverse rows of bristles located on the fifth segment of the hind legs. The pollen that accumulates on the fifth segment of one hind leg is now pushed into the pollen basket of the other leg. Large quantities of pollen can be packed into the depression and bordering curved hairs of each pollen basket. As the bee prepares to deposit the pollen into a cell within the hive, she uses the conspicuous spine on the middle leg to pry the pollen pellet out of the basket.

The Honey Bee Stinger

The ovipositor of the worker has become modified from a structure for transmitting eggs from the ovaries to the outside to a device for injecting poison in defense of the colony. The stinger is composed of three main parts, which should all be visible on the slide: a shaft, a poison sac, and a motor apparatus—a collection of muscles and skeletal parts involved in moving the shaft, anchoring it in the victim, and expelling the poison. The shaft itself is formed of three components: a dorsal stylet and two barbed ventral lancets. A poison canal runs the length of the shaft from the poison sac duct to an opening at the shaft's pointed end. The poison sac is seen as a thin-walled structure behind the shaft. The motor apparatus, including two sheaths that fit around the shaft, is located on both sides of the shaft's base, between it and the poison sac.

When the stinger is at rest, it is retracted within the body, with the bulk of the shaft surrounded by the sheaths, and only the shaft's tip exposed. A stinging bee bends the end of its abdomen downward and, with a sudden jab, inserts the shaft's tip into the victim. The barbs on the lancets anchor the stinger and the struggling bee rips itself loose from the stinger. Most workers soon die from the wounds incurred in this process. The motor apparatus moves each lancet independently, and the alternating motion of the lancets, the barbs of one holding while the other moves forward, serves to drive the stinger more deeply into the victim. At the same time, other muscles continue to pump venom through the poison canal into the victim. A potent alarm pheromone, isopentyl acetate, is released from glands found within the stinger and this serves to "mark" the victim for further aggression from other workers.

To observe the action of a live stinger, obtain a worker from the observation colony and anesthetize it with CO_2. With thumb and index finger, grasp the worker by its two pairs of wings and allow it to recover from the anesthesia. It is not possible to be stung by a bee held in this manner. The bee can be made to anchor its stinger into rough cloth or leather by pressing its abdominal tip against the material. The detached, pulsating stinger can now be studied in detail. It is frequently possible to smell the banana-like odor of isopentyl acetate being released by the detached stinger. Stinger movement may continue for five to ten minutes or longer.

Electroantennogram Studies with the Honey Bee

General Procedure

Recently a bioassay has been developed that uses the electrophysiological response of an insect's whole antenna as a measure of the presence or absence of pheromones. The electroantennogram (EAG) technique is an extremely sensitive tool for measuring electrical changes produced by receptor cells

within the insect's antenna as the antenna is exposed to various chemicals in gaseous phase. The EAG technique is particularly instructive if used in conjunction with a behavioral bioassay, such as those described in the *Pheromone Studies* section. The basic technique is as follows (details of the procedure are given in a subsequent section): *first,* the antenna is carefully removed from the insect. The antennal base is pressed into some wax in the bottom of a small dish, which is filled with a physiological saline solution. The saline solution should make good contact with the antennal base. The ground electrode from an oscilloscope is positioned in the saline solution, and the instrument's input electrode positioned so that it makes contact with the tip of the antenna. Thus, the antenna is now part of the electrical circuit connecting the two electrodes, and the oscilloscope can measure and record antennal response amplitudes. Filtered air is continuously blown over the antenna at the rate of about 1 m/sec. Pheromones, extracted insect products, or fractions collected from a gas chromatogram column can now be introduced into the air stream, which will deliver them to the antenna, and the response is recorded by the oscilloscope. With very small insects the whole head can be mounted on wax in the petri dish (see Roelofs 1975 for discussion of the general use and limitations of the EAG technique and more information on methods).

The Antenna of the Honey Bee

The antennae of the honey bee are a pair of freely movable appendages that, together with the two large compound and the three small simple eyes, are the main organs whereby the bee perceives the world. In the worker (see Figure 11.7), each antenna consists of 12 segments; the *scape* (which artic-

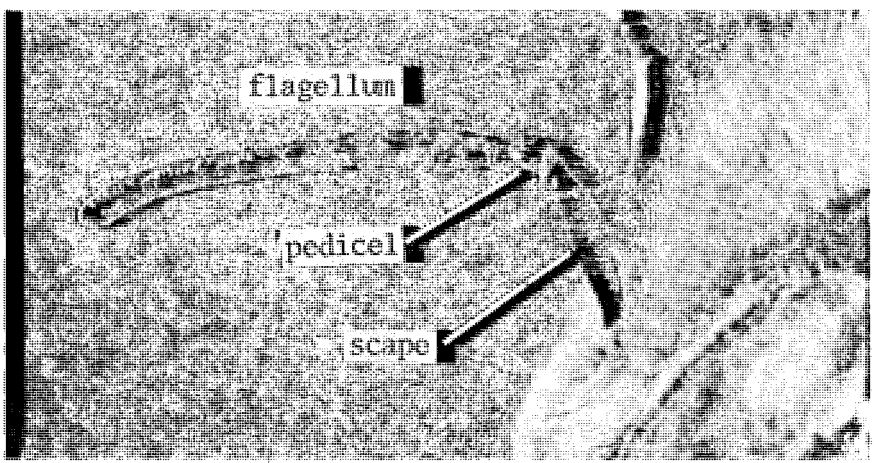


Figure 11.7. Photomicrograph of the antenna of the worker honey bee (*Apis mellifera*) showing the scape, pedicel, and flagellum.

ulates via a socket joint with the exoskeleton of the head), a short *pedicel,* and 10 distal segments collectively called the *flagellum.* The bee's antennae are its main organs of chemoreception (although some of the mouthparts also contain chemoreceptors) and touch. In addition, the antennae contain sensory receptors for humidity, CO_2 concentration, and temperature. When the electron microscope was used to study antennal structure it became evident that the exoskeletal surface was highly modified into a diversity of sensory structures called *sensilla.* Figure 11.8 shows two scanning electron micrographs of

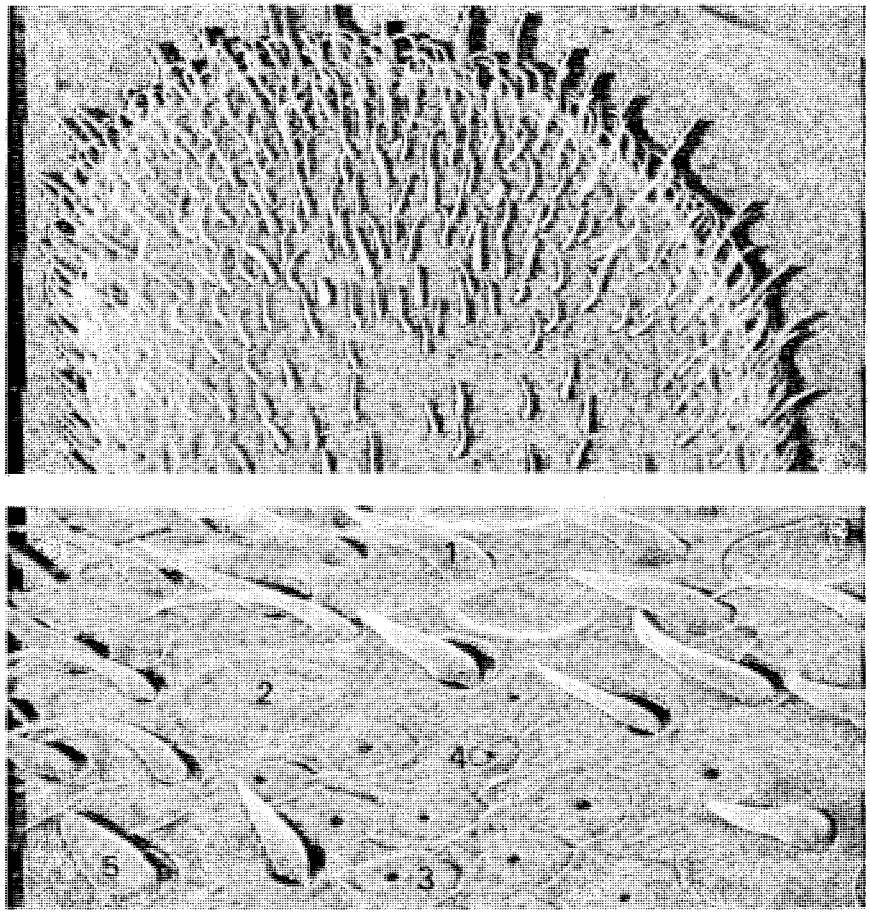


Figure 11.8. Scanning electron micrographs of worker honey bee (*Apis mellifera*) antennal structures. *Above* shows a portion of the antennal tip segment at 500x magnification. *Below* shows five different sensillum types found in a portion of the second antennal segment from the tip at 1980x magnification. (Note: 1 = *sensillum trichodeum*; 2 = *s. placodeum*; 3 = *s. coeloconicum* or *s. ampullaceum*; 4 = *s. campaniformium*; 5 = *s. basiconicum.*) EMs courtesy of Dr. Richard Fell.

portions of honey bee antennae with several of the more prominent sensilla indicated. The basic tactile receptor found abundantly on the antenna as well as on the rest of the body surface is called the sensitive hair, or *sensillum trichodeum.* This receptor type varies considerably in structure, and evidence suggests that some sensitive hair types may be involved in olfaction. Some 8000 to 9000 *sensilla trichodea* are found on each worker antenna. The main odor detector is the plate organ, or *sensillum placodeum,* of which there exist 3000 per antenna in the worker. It is interesting that the drone honey bee has 15,000 plate organs per antenna, an increase that may help him to better locate the queen via her sex pheromones during the mating flights. These two sensilla occur rather uniformly on the flagellar segments, particularly on the upper and median surfaces. Small clusters of less common sensilla are also commonly seen (Figure 11.8). Pit cones (*sensilla coeloconica* and *sensilla ampullacea*) are small, recessed plates with a central pit. Some evidence suggests that these are the CO_2, humidity, and temperature receptors in the honey bee. The *sensillum campaniformium* is like a pit cone except it has a small, raised area in the center instead of a pit. Its function is unknown. Finally, one can also commonly observe large, thin-walled pegs called olfactory cones (*sensilla basiconica*) that together with the plate organs are involved in chemoreception.

The dendrites of from one to twenty (depending on the sensillum type) neurons lead away from each of the sensilla and are discharged more or less directly by the stimuli impinging on the surface of the sensillum. The neurons join together within the antennae to form large, double nerves that carry information directly to the insect's CNS. In the EAG, one measures the change in the electrical output of the whole antenna resulting as a summation of all the depolarizations of individual neurons that respond to the substance being tested. During the EAG, the antenna is held in a fixed position in a stable environment, so that the antenna is responding only to olfactory stimuli. However, as stated earlier, because the EAG only measures the overall response of the antenna to the stimulus, it is impossible to know without a behavioral bioassay what action the CNS will initiate based on the information coming to it from the antennal nerves.

The Antennal Preparation (see Figure 11.9)

The apparatus includes a petri dish with a central mound of tacky wax. The dish should be filled with EAG Ringer's solution so that most of the tacky wax is covered but some of it projects above the Ringer's solution. A ring stand supports a mechanical manipulator for positioning the input electrode and a holder for the ground electrode. Each electrode consists of a length of silver wire inserted into a pasteur pipette that is then partially filled with Ringer's solution. Both these electrodes are wrapped with aluminum foil to

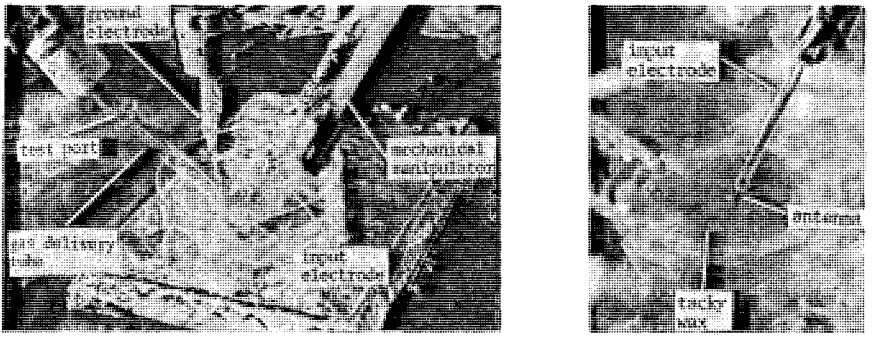


Figure 11.9. Arrangement of the apparatus for the electroantennogram bioassay. *Left* gives overview of main components for presenting test substances and measuring antennal responses. *Right* shows close-up of antenna and input electrode.

shield them from extraneous electrical fields and grounded. The base of the ring stand is also grounded. A second ring stand holds a gas delivery tube for presentation of the substance being tested.

1. Antenna donors can be removed directly from a holding cage by grasping the bee firmly with a pair of medium forceps. Kill the bee by crushing its thorax with the blunt end of a pencil or similar object.

2. While holding the bee, use a fine pair of forceps to detach an antenna by carefully grasping the scape (first segment, see Figure 11.7) and gently pulling the antenna loose at its point of articulation with the head.

3. Transfer the antenna to the petri dish and stick it into the tacky wax where it emerges from the Ringer's solution. The antennal base should be submerged in the Ringer's solution and firmly anchored into the tacky wax, but the entire flagellum must stick above the Ringer's solution. The best way to anchor the antenna in the tacky wax is to push the tip of the fine forceps, where the antenna is held, directly into the wax, just below the point where the wax emerges from the Ringer's solution. Open the forceps slightly, to release the antenna, and remove them. The antenna should now be firmly held and make good contact with the Ringer's solution. *Do not wet the flagellum with Ringer's.*

4. By rotating the petri dish, position the antenna near the end of the input electrode. Use the mechanical manipulator to move the input electrode so the Ringer's solution in the pipette's tip makes contact with the tip of the antenna (see Figure 11.9). To insure sound electrical contact between the input electrode and the antennal tip, manipulate the electrode so the upper end of the flagellum sticks into the Ringer's solution about *one millimeter.* Now carefully withdraw the pipette so only the very tip of the antenna's last segment makes contact with the Ringer's solution. Careful use of the mechanical manipulator is necessary for success.

5. Check to be sure that the ground electrode is in contact with the pool of Ringer's solution in the petri dish. The silver wire within the ground electrode should make contact with Ringer's solution within the pipette which should now be continuous with the solution within the petri dish. If the antennal base is in good contact with the Ringer's in the petri dish and the antennal tip with the input electrode, a complete circuit should be achieved, with the antenna the only link between ground and input probe. A resting antennal output trace on the oscilloscope will indicate that the circuit is complete.

Preparatory Notes

A mechanical manipulator is essential for the precise movement of the electrode required in this preparation. We have successfully adapted Nikon mechanical stages to perform this function. The stage is clamped to a metal bar which is attached to the ring stand with a standard clamp holder. The input electrode fits into two short sections of polyethylene tubing that have been cut lengthwise and glued to the mechanical stage with silicone seal (General Electric MR 30). The ground electrode fits through a cork that is also attached to the ring stand via a utility clamp. See Figure 11.9. We chloridize our silver wire electrodes by immersing both the input and ground electrodes in a 10% (w/v) solution of KCl and connecting these electrodes to a 6-V, 600-μA circuit for 30 seconds. We then reverse the position of the electrodes in the circuit and continue chloridizing for another 30 seconds. Another method for chloridizing electrodes is to dip them into molten silver chloride for 1 sec (Roelofs 1975). Electrodes should be chloridized initially and every several days. The recipe for Kaissling's EAG Ringer's solution is found in Appendix II.

Oscilloscope

We use a Tektronic type 564 storage oscilloscope, with a type 2A61 differential amplifier, and a type 2B67 time base. An oscilloscope with storage is essential in the EAG technique. Stimulated antennal outputs for the honey bee range from 0.5 to 6.0 mV (see Table 11.1). The resting antennal output for the honey bee is about 30 mV. An appropriate amplifier or pre-amplifier will be required to obtain this sensitivity range. Time base should be set at 1.0 sec per division. Coaxial leads connect the ground electrode to the instrument's ground, and the input electrode to A. The electrode shields and ring stand bases are all connected to chassis ground by alligator clips and light hook-up wire (24 gauge).

If you have successfully prepared the antenna-oscilloscope system, you should observe a trace showing some small-scale vertical deflections, with an amplitude not exceeding 0.2 mV, moving across the cathode ray tube (CRT).

Two problems can be diagnosed at this time:

a. If the trace shows *no* small-scale vertical deflections, you have probably wetted the surface of the flagellum excessively, allowing a direct circuit from ground to input electrode that does not include the antenna. Obtain a second antenna and try again.

b. If the trace shows small-scale vertical deflections greater than 0.2 mV, you have either not made a good contact between the tip of the input electrode and the antennal tip, *or* the ground electrode is not contacting the EAG Ringer's in the petri plate, *or* the antennal base is not in adequate contact with the Ringer's. Also be sure that the Ringer's within each electrode is actually touching the silver wire.

The Pheromone Delivery System (refer to Figure 11.9)

In EAG systems a continuous air stream is directed at the antenna, and substances being tested for pheromonal activity are puffed into the air stream and carried to the antenna. The following procedure should be used.

1. The gas delivery tube with an input port for the test substances should be directed precisely toward the antenna and be at a distance of about 2.0 cm from it. The air stream velocity should be sufficient to quickly deliver substances to the antenna, but not so intense as to cause response from the antennal tactile receptors.

2. Test material to be delivered to the antenna is placed in a cartridge composed of a piece of filter paper (3 × 25 mm) in a pasteur pipette. Cut the paper with *clean* scissors and use only *clean* forceps to handle it. The test substance is usually added to the filter paper with a microcapillary tube of a specified capacity. Position the paper in the upper end of the pipette for adding the test substance to it.

3. To test the material, first withdraw the plunger of a 5-ml glass syringe to the 3.0-ml mark. Insert the cartridge into the syringe. Figure 11.10 shows one way of adapting a syringe for receiving a pasteur pipette. Be sure the filter paper piece is in the tip end of the pipette. Hold the syringe in one hand with your thumb on the plunger and your fingers grasping the syringe barrel. Position the tip of the cartridge just within the port of the glass delivery tube. Depress the plunger firmly and quickly to "puff" 3.0 ml of air containing the substance being tested into the system's air stream. Be careful to direct the puff into the port, but do not bump the glass delivery tube with the cartridge.

4. If the antenna is sensitive to the substance being tested, an immediate response should be observed on the CRT screen. By carefully coordinating the delivery of the puff, and using the storage feature of the scope, one or two antennal responses can be recorded per sweep. The rapid change in the resting antennal output, due to the simultaneous firing of the neurons sensitive to the substance, can now be measured.

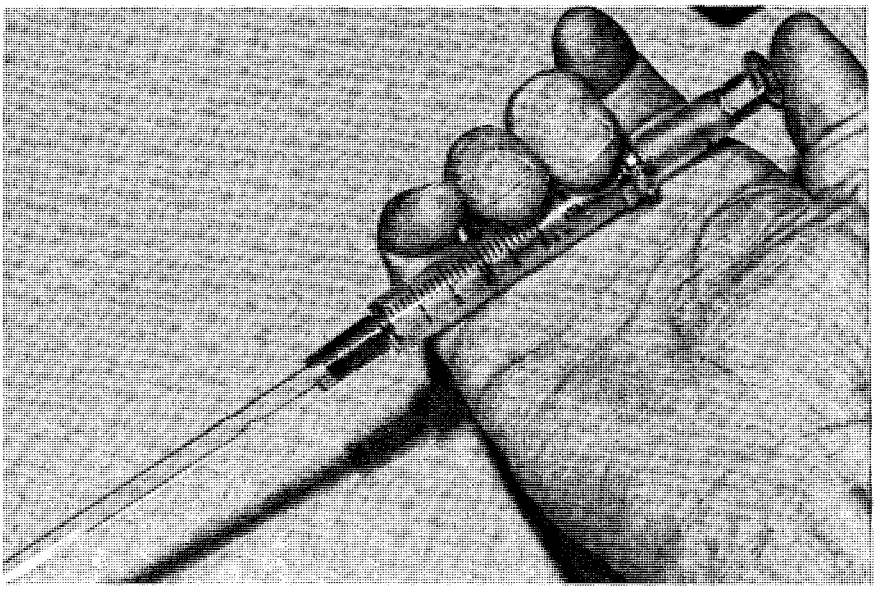


Figure 11.10. Shows syringe adapted to hold pasteur pipette and method for holding syringe to depress plunger quickly in order to deliver discrete "puff" of pheromone-saturated air into the air stream.

5. Since the increase in air flow past the antenna due to the depression of the syringe may cause some firing of the tactile receptors (*sensilla trichodea*) of the antenna, you will want to record several traces obtained by puffing a cartridge containing a piece of plain filter paper into the air stream with the same procedures used in the pheromone tests. These measurements must be subtracted from those obtained in pheromone tests in order to reflect changes in resting potential due only to olfaction.

Preparatory Notes

You can use compressed or bottled air, but it should be filtered first. We use a 1000-ml flask with four alternate layers of glass wool and activated charcoal. Polyethylene tubing carries the filtered air to a short section of 1-cm-diameter glass tubing terminating about 2–3 cm from the antenna. Pheromone-saturated air is injected into the air stream through a 0.5-cm-diameter hole located about 3 cm from the end of the glass tube. If the glass air tube is inserted through a cork, it can be conveniently manipulated by attaching it to a ring stand with an adjustable utility clamp.

Cartridges must be prepared with *clean* pasteur pipettes and *clean* filter paper. Use only freshly washed scissors for cutting, and forceps for handling the paper. Microcapillary tubes fill automatically to the proper volume, and

the liquid is dispersed by placing a finger over the hole in the bulb and squeezing it. Pheromone should be added to the filter paper piece while it is positioned within the upper end of the pipette. Now position the paper in the tip end of the pipette by simply jiggling the cartridge, or push the paper there with a clean probe. A cartridge can be used to deliver at least five puffs before it is necessary to replace the paper and apply new pheromone. The same pasteur pipette can be used for all trials with the same pheromone.

Use of the syringe to deliver pheromone to the system will require practice. Several things are important: first, the plunger must be set to the 3.0-ml mark *before* the cartridge has been inserted. Otherwise the pheromone-saturated air will be drawn into the barrel and clean air moved into the pipette. The reverse should happen (clean air in barrel should push pheromone-saturated air out of the cartridge and into the EAG system). Therefore, when several puffs are delivered in succession, be sure to break the syringe-cartridge connection each time before the syringe plunger is repositioned to 3.0 ml. Also, the plunger should be quickly and firmly depressed so all the pheromone-saturated air is delivered to the EAG air stream and the antenna in a single burst. This will cause a clean, discrete response on the CRT screen. However, bumping the gas delivery tube with the pipette's tip should be avoided since it creates noise which is picked up by the antenna's tactile receptors.

If the trace shows the appropriate small-scale deflections indicative of a "good" antenna prep, but the antenna responds little or not at all to known pheromones, two possible problems may be involved: the antenna may be inserted into the input electrode too far (only the tip of the last segment should be within the Ringer's of the input electrode), masking many of the olfactory sensilla; or the antenna is dead. If adjusting the position of the input electrode does not improve the situation, obtain a new antenna and try again. *Note:* Antennae are usually active for at least *30* minutes after being removed from the insect, and some respond for hours without showing a decrease in sensitivity. However, if treated roughly during mounting they may "give out" early.

Studies Using the EAG Technique

Consult the section *Pheromone Studies* for ways in which the EAG technique can be used, either in conjunction with the behavioral bioassays described there or separately. In addition to the synthetic pheromones IPA and 2-heptanone and the stinger and the mandibular glands, the pheromones of the Nasanov, or scent, gland can also be studied with the EAG technique.

The Nasanov, or scent, gland is another important pheromone-producing structure found in the worker. It is located in a pouch on the dorsal surface of the abdomen between the last two abdominal segments. The worker can flex its abdomen upward and expose this gland. It usually fans its wings at

the same time to help dispense the pheromone better. The Nasanov secretion is attractive to worker, queen, and drones. A number of different situations are associated with the use of the Nasanov gland. Workers frequently scent near the entrance to the hive and this may help inexperienced bees locate the nest site. Forager bees that are disoriented in trying to locate the hive site will scent vigorously upon finding it. When a swarm of bees leaves the old hive site to found a new colony, they will scent at the interim site the swarm selects. When the swarm leaves the interim site and moves to its new nest location, the first bees that arrive also scent-fan vigorously. Scenting occurs if the queen is removed from a colony, a situation that occurs when the virgin queen leaves on her nuptial flights. This may help the queen relocate the colony upon her return. Finally, foraging workers will sometimes mark particularly good food sources with Nasanov exudate and this may help recruits find the food source.

At least four different substances are produced by the Nasanov gland. *Geraniol* and *citral* are considered to be the main pheromones of the gland, although debate exists as to which substance is most attractive to workers. To obtain information on this question, both substances can be tested in an EAG bioassay to determine if the two pheromones differ in the response they elicit from the antenna. About 5 μg of each substance would be a biologically relevant amount to test in the EAG bioassay.

Table 11.1 shows data obtained with the EAG technique from worker honey bees' antennae for all pheromones mentioned in this chapter.

Table 11.1. Mean electroantennogram responses in mV from antennae of 12-day-old worker honey bees (*Apis mellifera*), for a control and 10 μg of four pheromone treatments. Note: Each of ten antennae was tested six times with the control and all pheromones.

	EAG Response in Millivolts				
	Control (Air Only)	*Citral*	*Geraniol*	*Isopentyl Acetate*	*2-Heptanone*
mean	1.13	2.43	1.91	3.58	5.20
standard deviation	0.26	0.35	0.38	0.45	0.60

References

Boch, R.; D.A. Shearer; A. Petrasovits. Efficacies of two alarm substances of the honey bee. Journal of Insect Physiology 16:17–24; 1970.

Good source of information on behavioral bioassays of IPA and 2-heptanone in the honey bee.

Dadant and Sons, eds. The hive and the honey bee. Hamilton, IL: Dadant and Sons, Inc.; 1975.

Best source of both practical and technical information on bee biology and beekeeping in general.

Free, J.B. The social organization of honey bees. Studies in biology no. 81. London: Edward Arnold Publ., Ltd.; 1977.

Up-to-date general reference source on the honey bee.

Gary, N.E. Activities and behavior of honey bees. Dadant and Sons, eds. The hive and the honey bee. Hamilton, IL: Dadant and Sons, Inc.; 1975: 185–264.

Excellent discussion of behavioral biology of the honey bee with comprehensive bibliography.

Glase, J.C. Introduction to the methods of science: the honey bee. Glase, J.C.; Ecklund, P.R.; Zimmerman, M.C.; Greco, J.L.; Essig, F.B. Investigative biology. Denver, CO: Morton Publishing Co.; 1979: 1–27.

Introductory biology laboratory text with an investigative approach.

Lindauer, M. Communication among social bees. Cambridge, MA: Harvard University Press; 1961.

Contains a particularly good discussion of colony division of labor, swarming, and nest-site selection behavior in honey bees.

Michener, C.D. The social behavior of the bees. Cambridge, MA: Belknap Press of Harvard University Press; 1974.

Major information source on the behavioral biology and ecology of Apoidea, including extensive discussion of *Apis mellifera*.

Morholt, E.; Brandwein, P.F.; Alexander, J. A sourcebook for the biological sciences. New York: Harcourt, Brace and World, Inc.; 1966.

Contains useful information on techniques, procedures, demonstrations, sources of materials, etc., of interest to all teachers of laboratory biology courses.

Morse, R.A. Environmental control in the beehive. Scientific American 226(4): 93–98; 1972.

Good discussion of colony defense and homeostasis.

Roelofs, W.L. The scope and limitations of the electroantennogram technique in identifying pheromone components. N.R. McFarlane. ed. Crop protection agents—their biological evaluation. New York: Academic Press; 1977: 147–165.

Good discussion of the EAG technique and its research applications.

Seeley, T.D. Atmospheric carbon dioxide regulation in honey bee (*Apis mellifera*) colonies. Journal of Insect Physiology 20:2301–2305; 1974.

Research report on colony response to variation in carbon dioxide.

Snodgrass, R.E. The anatomy of the honey bee. Dadant and Sons. eds. The hive and the honey bee. Hamilton, IL: Dadant and Sons, Inc.; 1975: 75–124.

Description of the morphology of the honey bee by a world authority on insect morphology.

von Frisch, K. The dance language and orientation of bees. Cambridge, MA: Belknap Press of Harvard University Press; 1967.

The best reference for information on orientation behavior in the honey bee by the premier student of the bee.

Wilson, E.O. The insect societies. Cambridge, MA: Belknap Press of Harvard University Press; 1971.

Wilson, E.O. Sociobiology. Cambridge, MA: Belknap Press of Harvard University Press; 1975.

These two books should be a part of every biologist's library!

APPENDIX I
Sources of Materials

Bees (Package Bees and Queens)

There are many reliable producers of package bees and queens; I have listed only two here. Consult the classified section of a copy of one of the beekeeping magazines listed below for more information, or talk with an experienced local beekeeper for recommendations.

1. York Bee Company
   P.O. Box 307
   Jesup, Georgia 31545
   (912–427–7311)
2. Homer E. Park
   P.O. Box 38
   Palo Cedro, California 96073
   (916–547–3391)

Beekeeping Equipment and Supplies

Two of the biggest suppliers of beekeeping equipment are listed here. Write them for catalogues.

1. Dadant and Sons, Inc.
   Hamilton, Illinois 62341
   (217–847–3324)
2. A.I. Root Company
   Medina, Ohio 44256
   (216–725–6677)

Beekeeping Periodicals

Two of the best known North American periodicals are listed below.

1. The American Bee Journal
   c/o Dadant and Sons, Inc.
   Hamilton, Illinois 62341
   (217–847–3324)
2. Gleanings in Bee Culture
   c/o A.I. Root Company
   Medina, Ohio 44256
   (216–725–6677)

Bee Morphology Microscope Slides

Carolina Biological Supply Company
Burlington, North Carolina 27215
(800–334–5551)

Hive Bomb (Aerosol Smoke Concentrate)

Dadant and Sons, Inc.
Hamilton, Illinois 62341
(217–847–3324)

Insect Forceps

These must be ordered from Austria; ask for "No. 53 Pinzette nach Leonhard." 1976 price was $1.00/each.

H. Winkler
Dittergasse 11
A–1180 Wien, Austria

Insect Pins (and Other Entomological Supplies)

Carolina Biological Supply Company

Map Measurers

Forestry Suppliers, Inc.
205 West Rankin Street
Box 8397
Jackson, Mississippi 39204
(800–647–5368)

Marking Kits

Write for their current prices. They require prepayment in deutsche marks with an international money order.

Chr. Graze K.G.
Postfach 2107
D-7056 Weinstadt-2
Bei Stuttgart, West Germany

Microcapillary Tubes (Pipettes, Microcaps)

VWR
P.O. Box 1050
Rochester, NY 14603
(800–462–3151)

Pheromones (and All Other Chemicals Mentioned)

Fisher Scientific
15 Jet View Dr.
Rochester, NY 14624
(Enterprise 9954)

Tacki-wax (Cenco Softseal Tacki-wax)

Central Scientific Company
2600 S. Kostner St.
Chicago, Illinois 60623

APPENDIX II
Chemical Preparation

Pheromones—1 $\mu g/\mu l$

Note: The following volumes of pure chemical when added to 25.0 ml of ether will produce a pheromone concentration of approximately 1 $\mu g/\mu l$. (Determinations based on pheromone density.) These pheromone solutions can be kept refrigerated for several weeks with no loss of activity.

Pheromone or Related Chemical	*ml*
2-heptanone	.03
3-heptanone	.03
4-heptanone	.03
heptane	.04
isopentyl acetate	.03
citral	.03
geraniol	.03

Kaissling's EAG Ringer's Solution

NaCl—7.5 g
$CaCl_2$—0.21 g
KCl—0.35 g
$NaHCO_3$—0.20 g
distilled water—1.0 liter

Chapter 12

Dual Purpose (Process and Content) Laboratory Exercises: Animal Behavior Experiments Using the Hermit Crab, *Pagurus longicarpus*, the Land Snail, *Polygyra* sp., and the Isopod, *Armidillidium vulgare*

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Introduction

A tradition of descriptive introductory biology laboratory exercises was established in the United States at the beginning of this century. Early laboratory curricula concentrated on anatomical comparisons between organisms on a phylogenetic level. The descriptive aspects of the original approach remain entrenched in contemporary introductory biology courses. Emphasis remains on dissections and phylogenetic comparisons, with rote or "cookbook" exercises provided to suggest an accommodation to the shift towards experimental biology characteristic of our era. Rarely do these exercises present the student with a true approximation of laboratory science as it is currently practised. Opportunities to develop hypotheses, evaluate data, perform experiments and derive conclusions are rare. A review of 16 randomly chosen laboratory manuals published since 1960 reveals that half were purely descriptive, featuring instructions for dissecting and comparing, with no experiments. The other half were predominantly descriptive, but provided opportunities to collect and interpret data. In all cases these data were not used towards the solution of a well-defined problem; they were provided simply to make descriptions of phenomena more realistic. Several manuals emphasized the use of more or less sophisticated equipment as a means of training the student to be an efficient data collector.

None of the manuals offered an opportunity for the student to construct his own hypothesis. None required him to devise an experiment *de novo*. Some presented a series of procedures which were called an experiment, but none specified what was the hypothesis being tested and none required that the data elicited be used towards the validation of an hypothesis. One manual called each exercise an experiment, e.g., "Experiment 9, Higher Plants. The Problem: To study the plants at the organ and organ system levels of development."

The teaching of biology as an experimental science has become sophisticated at pre-college levels since the Sputnik trauma forced a revaluation of American science teaching practices. An important contribution was the input of learning specialists and developmental psychologists into the construction of laboratory exercises. The information they provided permitted the scientist-educator to establish a base-line target population (using parameters describing the limitations and capabilities of the student) and goals for raising the level of performance in process-oriented exercises. In short, it became an important purpose of laboratory instruction to provide opportunities for the student to make the transition between learning couched exclusively in concrete (descriptive) experiences and the epistemological (formal operational) aspects of science. The student was now expected to learn both content and process; he was to master that body of facts constituting biology and, in addition, *be able to think and perform in the manner of an experimental*

scientist. This latter objective, certainly a noble one, has only recently been addressed in college teaching laboratories.

The following exercises have been used in introductory biology laboratory courses for both majors and non-majors since 1972. They are part of a series of 39, each of which has dual objectives: to teach some facet of biological content and, using this information, to introduce an aspect of the problem-solving process which characterizes the activities of the scientist. Each exercise treats both process and product with equal respect. The student obtains a well-rounded picture. He seeks the facts which are the rewards of the scientific endeavor, but he is required to derive these facts from unrelated (to him) information in the same manner as did the biologists who actually made the discovery. Each exercise has a dual title as: "Deduction I/Classification of Living Things . . . ," "Development of a Hypothesis/Origin of Life . . . ," "Mechanism vs. Vitalism/Host Specificity in Viruses," "Teleology/Cells and Cellular Differentiation," "The Model/Physical and Chemical Properties of the Protoplast."

Evaluation of student progress becomes complex. It is more difficult to determine growth in understanding of the epistemological aspects of science when compared to the simple mastery of facts. (See Kaplan 1963, 1967 for a discussion of this problem).

Exercise I. The Role of Statistics in Decision-Making
Animal Behavior 1: Habituation in Hermit Crabs

The primary objective of this exercise is to acquaint the student with the role of statistics in decision-making. This does not mean that any attempt is being made to teach statistical techniques in depth– there will be numerous opportunities for this later in his career. At this stage it is likely that the student will benefit from an experience which will provide some idea of the limitations of statistical procedures and the relatively narrow confines governing their use. We are all subjected to a culture that makes something of a fetish of statistics. It is hoped that the student will have the veil lifted from his eyes by the simple and clearcut statistical test used in the following exercise.

A comparison is made between the use of the graph versus the chi-square test to determine the significance of data.

The biological concept is a fundamental problem in ethology. How much of the behavior of lower animals is governed by "instinct," that is, unlearned, automatic, unvarying responses to stimuli? While the answer to this question is not resolved in this exercise, the student begins to think in ethological terms. This fascinating area of biology is opened to him early in his career. The non-science major, too, is fascinated by studies in animal behavior, since this area

is "alive" to him and it is likely that he has pondered, however briefly, the question of the "level of consciousness" of lower animals, such as insects.

Begin the period with a brief discussion of statistics and do an example incorporating the use of chi-square analysis. Place special emphasis on explaining the use of the null hypothesis and on levels of confidence. A good example is to ask the class to determine whether or not you have an illegally "fixed" quarter. To add drama to your illustration take an ordinary quarter out of a special envelope, thus implying that it is unique in some way. Flip it 10 times and analyze the results to see if the number of heads and tails comes out according to chance. Another example would be to determine whether or not there was any bias in placing men and women in the class. If there are 24 students the expected ratio would be 12 men:12 women. One hour of a three-hour period may be utilized for your introduction to statistics.

After finishing your discussion of statistics, explain that the aquarium(s) at the front of the room contains hermit crabs. Each pair of students should send up a representative to obtain two crabs, one in each of two beakers. The procedure should be explained and demonstrated as follows:

1. Each pair of students has three 250-ml beakers and a liter of salt water identical in salinity and temperature to that in the holding tank. Pour about 200 ml of water into each beaker. Now pour the water from each beaker into the empty beaker and back and forth five times to oxygenate it. Label each beaker with the team's initials.
2. Come to the front table with both beakers and place a crab in each beaker.
3. Place one beaker at the back or side of the room as far away from the lab tables as possible. These are the control crabs. Bring the other crab back to the lab table.
4. Allow the crab to adjust to its new environment for about five minutes.
5. The stimulus is administered in the form of a sharp rap on the top of the beaker by a thin book, such as students take notes in. The book is slammed down on the beaker in such a manner as to cover the beaker and cast a shadow on the crab. (Interested students might wish to go beyond the requirements of this exercise and determine whether it is the vibration from the blow of the book or its shadow which causes the response.)

Administering the stimulus becomes a problem in full sections. Twelve students will be pounding on the beakers in what appears to be a random pattern. This may disturb the sensibilities of their colleagues (or their instructor). It may be desirable in such classes to regiment the administration of stimuli by giving a signal, at one-minute intervals, at which time all stimuli are administered by the students at the same time. The virtue of this approach

is that nearby crabs will not be subjected to less audible, but still significant, vibrations emanating from neighboring experiments, since all stimuli are administered together. In small classes the students can move far enough away from one another so that this need not be a problem.

A positive response is the withdrawal of the crab into its shell. After a number of stimuli it no longer withdraws, since it has become habituated to the stimulus. This often happens consistently at about the fifteenth trial.

6. As soon as 30 stimuli are applied and the data recorded in the student's book, one partner will get the beaker with the control crab and gently deposit it about three inches from the experimental crab. If the book is wide enough the stimuli can be applied to both beakers at the same time. Otherwise, both partners must coordinate their efforts. Five stimuli are applied. Data are collected from three neighboring pairs of students and recorded.

7. At this point, but not before, the instructor accumulates the data from the first 30 trials on the chalkboard. (The second set of stimuli, applied to the control and experimental crabs at the same time, must be presented as soon as possible after the first set, lest the habituated crabs "forget.")

When the students have completed 30 trials with the experimental crab and an additional 5 trials with both the experimental and control crabs, call the class's attention to a chart which you have drawn on the chalkboard, as depicted below:

	1	2	3	4	5	6	7	8	9	10	11	12	
Number of trial													*Total pluses*
1.	+	+											1
2.	+	+											2
3.	+	−											3
4.	−	+											4
5.	+	+											5
(Continue numbering to 30)													(Continue to 30)

One student of each pair in the class should record a + or − next to each trial number, so that teams numbered 1 and 2 (the first and second to finish) might record their data as indicated in rows 1 and 2 above.

Notice that the total number of pluses for each trial number is to be recorded on the right-hand side of the chart. This is the data which should be transferred to Table 12.4.

Time: One three-hour period or two two-hour periods.

Student Materials

For the class:

One or several 20–50-gallon all-glass aquaria, plus a few six-quart all-glass tanks.
8 lb. of Instant Ocean salts (Ward's #21W7354, Carolina #67–1422, Turtox #75–61–2) for each 25 gallons of water.

For each pair of students:

Three 250-ml beakers
1 liter of sea water at room temperature
Extra graph paper (5 lines per cm)
Two hermit crabs, *Pagurus longicarpus.*

Instructor's Materials

At the front of the room:

Several small tanks or one large one containing hermit crabs. A large tank of salt water should be at the front of the room for depositing of habituated crabs at the end of the period.
Six small aquarium nets for transfer of crabs.

It is desirable that the laboratory have a clock with a sweep-second hand to obviate the need for students to borrow watches from you or their colleagues.

Ordering and maintaining hermit crabs:

Pagurus longicarpus, the common East-coast hermit crab, is, with the isopod and mud snail, a biologist's boon. It is hardy, harmless, quick-moving, present in large numbers on protected beaches, and exciting to the students. It is a taxonomic challenge to them, since it is an arthropod (class Crustacea) living in a mollusk (class Gastropoda) shell. The crabs are comical as they scuttle towards and away from each other in mock combat. Occasionally they come to grips with one another for a few moments of fierce, harmless battle. They are extremely territorial and will immediately attempt to protect a small area around themselves. Their normal habitat, tide pools and crevices between rocks, is subject to severe environmental variation, from sudden rain showers, which sharply lower salinity, to intense heat as the sun raises the temperature of the pool.

While one sings the praises of the hermit crab, an occasional flaw must be acknowledged. Unfortunately it appears to become "neurotic" when exposed to too many environmental changes at the same time. The crabs should acclimate themselves to their environment for about two weeks. Otherwise, when stressed, they might withdraw into their shells and remain inside, rendering them useless for habituation experiments.

There are several sources of hermit crabs which advertise their availability all year round. I recommend obtaining them either in late October or April-May. If ordered in October, the crabs can be kept in holding tanks indefinitely. Refrigeration is not necessary, although it is desirable if you overload the capacity of the tank. For every degree you can reduce the temperature below 21°C you may add 10 crabs to a maximum of 5 per gallon or 250 per 50-gallon tank at 15°C. Three crabs per gallon of water at 21°C or 150 per 50-gallon tank is a safe load. Feed the crabs ordinary fish food flakes or pieces of clam or meat. In informal tests, I have concluded that they prefer plant to animal food, so seaweed (e.g., *Ulva*) or other plant material may also be tried if available. For short-term storage not exceeding a month, it is not necessary to add sand; a slate or glass bottom will do. For longer periods add crushed shell or calcareous gravel (dolomite) to help maintain proper pH and to act as a substratum for denitrifying, and nitrogen-fixing bacteria which detoxify the ammonia wastes excreted by the crabs.

Instant Ocean salts should be ordered ahead of time and mixed with tap water according to directions. Add about a gallon of tap water to every 50 gallons after the tank is set up to reduce the salinity to a closer approximation of the estuarine habitat from which the crabs were taken. Let the salt water stand for a few days before adding the crabs. Replace about 25% of the water with newly mixed salt water every month. If it is not too impractical, put a handful of crabs or a few fish into the tank a month before adding the full burden of crabs to build up a colony of denitrifying bacteria in the gravel. Use an air pump and several air stones.

The Carolina Biological Supply Company (Burlington, North Carolina) produces a booklet on setting up marine aquaria which they have been distributing free of charge.

Where to order:

Most biological supply houses offer *Pagurus longicarpus* or various species of *Clibanarius* (tropical forms) at a cost which makes them impractical for use in experiments. The following sources will give volume discounts or have much lower prices:

Supply Department
Marine Biological Laboratory
Woods Hole, MA 02543

NEMSCO
P.O. Box 1
Woods Hole, MA 02543

Nasco
Fort Atkinson, Wisconsin 53538

Marino's Aquatic Specimens
301 Evergreen Ave.
Daly City, CA 94015
(Order *Pagurus samuelis*)

If you cannot obtain the crabs from any of the above-mentioned suppliers, write to me and I will endeavor to send you some.

The Role of Statistics in Decision Making*
Animal Behavior 1: Habituation in Hermit Crabs

The most important events in your life will be the results of certain decisions—who to marry, what job to take, etc. But these decisions are the most obvious of the thousands of smaller decisions which punctuate your daily life. If you are really good at decision making, you might become an executive and direct the fortunes of a company. Or you may become a scientist and spend your time deciding what investigations to make and evaluating your own decisions, or a purchasing agent and decide what to buy for a department store.

All decisions are based on data. Sometimes the data are such that they can be organized and subjected to tests to determine their significance. Statistical analysis is a way of showing relationships among data and thereby simplifying the decision-making process.

Virtually all statistical tests are designed to determine whether or not the difference between two sets of data is significant. If the population being dealt with is as large as your class or larger, and if the members of the population are randomly chosen, there are relatively few statistical instruments needed to analyze data drawn from this population. One of the most useful tests of significance is the chi-square test.

The Chi-Square Test

The chi-square test allows you to determine the likelihood that groups vary from one another significantly on a dichotomous or "left-right," "yes-no," "up-down," "tall-short" level. For example, a recent paper in journal *Animal Behavior*** describes the investigations of two ethologists (biologists who study animal behavior) who attempted to determine if a snail deposits a substance on the surface as it travels which is detected and followed by other snails. A Y-shaped tube was constructed and the snails, of a type called "pulmonate" or air breathing, were placed in a closed chamber as depicted in Figure 12.1. About every 20 minutes it was necessary for the snails to travel up the tube to obtain air. When they reached the fork in the Y, they had to choose which tube to take.

In the first set of trials, all those snails which turned in the direction of the first snail were recorded as plus (+). All those turning in the opposite direction were scored as minus (−). Table 12.1 shows the data obtained.

1. How many snails followed the first snail? _____ How many did not? _____

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**M.J. Wells and S.K.L. Buckley, "Snails and Trails," *Animal Behavior*, 20:345–55 (1972).

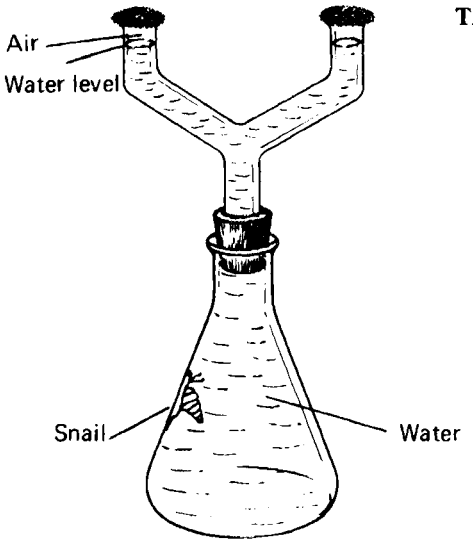


Figure 12.1. Apparatus for testing pulmonate snails.

TABLE 12.1. Tube Choices of 10 Snails

(A plus signifies that the snail chose the tube used by the first snail.)

Snail	*Choice*
1	
2	+
3	+
4	+
5	–
6	+
7	–
8	–
9	+
10	+
11	+

2. On the basis of these data, would you hypothesize that snails have a tendency to follow in the path produced by previous snails? _____ Are you sure? _____
3. What are two possible explanations which could account for the data, other than the tendency of snails to follow trails previously laid down by other snails?

   a. _____

   _____

   b. _____

   _____

4. How can these variables be eliminated? _____

   _____

   _____

   _____

The arms of the Y were oriented toward the light, away from the light, and so that both received the same amount of light. There were always more plus scores than minus scores, although the results were variable. The scien-

tists, after further testing and analysis, concluded that the direction of the light source was probably not a relevant variable.

Having eliminated all the variables they could think of which might have caused the snails to favor one arm over the other, they decided to test the hypothesis that snails *do* follow their predecessors' trails. They scrubbed out each Y-tube after every snail so that there was no trace of the passage of the previous snail. Table 12.2 contains the data from two such trails.

5. For trial 1 how many pluses? _____ How many minuses?

For trial 2 how many pluses? _____ How many minuses?

What do you conclude after examining the data? _____

Clearly, further experimentation is necessary to determine if the responses of the snails are due to chance or if there is really a factor related to trail following at work.

We can subject the data to a chi-square test to see what the chances are that they are distributed at random or that they represent a significant behavior pattern. The following steps are followed in order to use the chi-square test.

TABLE 12.2. Tube Choices of Snails After the Tubes Have Been Scrubbed

(A plus signifies that the snail chose the tube used by the first snail.)

Trial 1		*Trial 2*	
Snail	*Response*	*Snail*	*Response*
1		1	
2	+	2	–
3	+	3	+
4	+	4	+
5	–	5	–
6	+	6	–
7	+	7	–
8	+	8	–
9	+	9	+
10	–	10	+
11	+	11	–

1. The null hypothesis. The conventional method of evaluating the relationship between two sets of data is to state that they do *not* differ and then test this hypothesis to see if it is valid. Thus the null hypothesis states "Sample 1 = Sample 2."

We must now specify what are the odds that, given an unlimited number of trials, Sample 1 = Sample 2. We know that, if you flipped a coin 10 times, there is a good chance of getting 6 heads and 4 tails. There is less chance of getting 7 heads and 3 tails, and even less of getting 8 heads and 2 tails. If we turned up 6 heads and 4 tails in 10 flips, we would not suspect that the coin is "fixed." But if we consistently obtained 9 heads and 1 tail, we would become suspicious. We could calculate the odds that this would happen accidentally. If the odds that the 9:1 distribution was a chance occurrence were sufficiently remote, we would reject the null hypothesis and slug the owner of the coin.

All of us have a "level of confidence" which allows us to accept an event as a chance occurrence or reject it. The level of confidence used by scientists is usually .05 or .01. This means that if an event normally can be expected to occur only 5% of the time by chance we are willing to accept the hypothesis that more frequent occurrence is due to the experimental treatment and is not just an accident. We reject the null hypothesis that it has occurred by chance.

Rejecting the null hypothesis means accepting the hypothesis that there is some factor (other than chance) at work causing the two samples to be different.

2. Testing the null hypothesis. The data of Table 12.1 are that 7 snails used the same fork as snail 1 (the plus fork) and 3 snails used the minus fork. For the chi-square test of significance we will use the formula:

$$X^2 = \frac{\left(\text{Actual observation}_1 - \text{Expected observation}_1\right)^2}{\text{Expected observation}_1} + \frac{\left(\text{Actual observation}_2 - \text{Expected observation}_2\right)^2}{\text{Expected observation}_2}$$

If the snails were not affected by any factor in their choice of either the left or the right fork of the Y-tube, the odds would be 50:50 that each would go right or left, or .5. The expected observation is the proportion if *chance were operating.* In the case of 10 snails we would expect the snails to turn left 5 times and right 5 times. We will compare the expected responses with the actually observed responses.

The snails turned to the plus fork 7 times and the minus fork 3 times. The expected ratio is 5:5. Is the difference between the actual number and the expected number significant at the .05 level of confidence? We now use our data in the chi-square formula.

$$X^2 = \frac{\left(\begin{matrix}\text{Actual}\\ \text{observation}_1\end{matrix} - \begin{matrix}\text{Expected}\\ \text{observation}_1\end{matrix}\right)^2}{\text{Expected observation}_1} + \frac{\left(\begin{matrix}\text{Actual}\\ \text{observation}_2\end{matrix} - \begin{matrix}\text{Expected}\\ \text{observation}_2\end{matrix}\right)^2}{\text{Expected observation}_2}$$

$$X^2 = \frac{(7-5)^2}{5} + \frac{(3-5)^2}{5}$$

$$X^2 = \frac{(2)^2}{5} + \frac{(-2)^2}{5}$$

$$X^2 = \frac{4}{5} + \frac{4}{5} = \frac{8}{5} \qquad 5\overline{)8.000}\ 1.600$$

$$X^2 = 1.600$$

3. Determining level of confidence. We have calculated a chi-square of 1.600. Now we must look up this chi-square in Table 12.3 to determine whether or not it falls beyond the .05 level. In other words, does a chi-square of 1.600 occur more than 95% of the time? If so, we can reject the null hypothesis that the number of plus choices made by the snails is more or less equal to the number of minus choices. Remember: level of confidence indicates the odds that there is a real difference between the actual observation and the expected observation. A chi-square beyond the .05 level of confidence occurs by accident (chance) less than 5% of the time.

TABLE 12.3. Chi-square Values at One Degree of Freedom

Level of Confidence	.50	.30	.20	.10	.05	.02	.01
Minimum Value of Chi-square at DF1	.455	1.074	1.642	2.706	3.841	5.412	6.635

On Table 12.3 we see that the chi-square we obtained of 1.600 exceeds the value at the .30 level but does not exceed the .20 level of confidence. This means that 30% of the time we would expect that the difference between the two samples would not be caused by chance. But we have set the .05 level as our standard; we are not willing to accept a 30% chance. We will accept only a level where less than 5% of the time the obtained difference could be a chance occurrence.

Since the data (7 plus turns, 3 minus turns) could occur by chance more than 5% of the time, we must accept the null hypothesis and say that the odds are that the choice made by the snails was at random.

Using the chi-square test, analyze the data in Table 12.2 to see whether the experiments with scrubbed tubes corroborate the data we just analyzed.

Use the space below for your calculations.

Chi-square for trial 1, Table 12.2 _____ Level of confidence _____

Chi-square for trial 2, Table 12.2 _____ Level of confidence _____

6. Consider all the results of the three chi-square tests. Is the evidence sufficient to say that snails follow trails? _____

_____

7. What do you recommend as the next step in this investigation? _____

_____

_____

Further trials by the experimenters provided enough data to demonstrate that the snails did indeed show a tendency to follow trails left by their predecessors.

Today's exercise will allow you to use the chi-square test to help you decide whether or not hermit crabs exhibit certain behavior patterns.

Preliminary Information

It is part of life to be able to respond to one's environment. As animals evolved the means of movement, it became necessary for them to detect events at a distance. The range and direction of objects had to be determined in order for appropriate action to be taken. A "decision" was required to flee from a potential predator or to attack potential prey. All these actions required the animal to make predictions about what was going to happen next. Can animals as primitive as cockroaches, spiders, and worms "decide" on appropriate actions or do they simply have a small repertoire of automatic responses which they have inherited?

> One could, in principle, conceive of an animal in which all possible events were foreseen, with appropriate reactions built in as a fixed property of the neuro-muscular machinery. The nerve arrangement could, in principle, be determined genetically, just like the overall shape of the animal or the color of its eyes, as a result of natural selection.*

This means that an animal would have built-in, automatic responses to cover the *full range of its behavior* and would never vary from these responses.

8. Consider an individual organism crawling on the bottom of the sea. Suppose it has inherited a pattern of automatic responses which have evolved over millions of years. If a predator approaches, which would be more rapid, an automatic inborn response or an on-the-spot decision requiring "thinking"? _____

9. Compare the potential for error in an emergency situation between an animal having an automatic response which it has evolved over thousands of years and one which must figure out a new response. Which would have a greater chance to make a disastrous mistake? _____

_____

10. So far, logic seems to point to the advantages of inherited, automatic behavior. Is this true in terms of the evolution of behavior over vast periods of time? Explain which is more advantageous, automatic behavior or the ability to modify behavior according to environmental conditions,

*M. Wells, *Lower Animals*, McGraw-Hill, New York, 1968, p. 249.

over the millions of years of the evolution of the organism. _____

_____

_____

_____

In practice, as always when dealing with living things, one discovers a series of compromises. Some actions are learned, others follow automatically from the construction of the animal's nervous system. Since the broad outlines of environmental conditions tend to remain stable for long periods, many behavioral responses can be built in with advantage and little potential danger. It is wise for a crab or a cockroach to remain still or scuttle away when confronted with anything large and moving, just as it is biologically good sense for a tick or a tsetse fly to do precisely the opposite. It is very unlikely that these will suddenly cease to be appropriate responses.*

But consider the above-mentioned crab going about its daily life. Is it logical that it would respond to every moving shadow by running or hiding? _____ If you answered "yes" to this question, reconsider in the light of the fact that crabs live in areas where large masses of seaweed are constantly moving to and fro with the waves. The crab must learn not to go on responding when some event repeatedly proves to be harmless. This learned behavior is called **habituation.** When animals cease to respond to stimuli that have proved to be irrelevant to them, they are exhibiting habituation.

In general, most animals have the broad outlines of their behavior laid down genetically, with the details filled in as the result of individual experience. The inborn inherited aspect makes sure that members of a species collectively make relatively few fatal mistakes. The learned component ensures that each individual behaves economically with respect to its own local environment.

Today's exercise deals with habituation in hermit crabs (see Figure 12.2). We will expose the crabs to a stimulus they have never faced before in their natural habitat, striking the top of their "world" (a 250 ml beaker) with a book.

11. What do you suppose the normal response of the crab will be when the book's shadow and impact on the beaker reach it? _____
(If you don't know the answer to this question, look again at Figure 12.2.)

12. Is it likely that the crab has inherited a mechanism capable of responding to the shadow of and vibrations caused by a book hitting the top of a beaker? _____

Ibid.

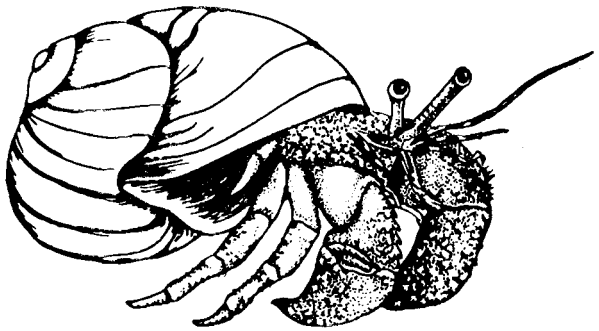


Figure 12.2. The hermit crab, *Pagurus longicarpus.*

Is it likely that the crab will become habituated to this unnatural stimulus? _____
(This answer is today's hypothesis.)

THE PROBLEM: ***Will the hermit crab, Pagurus longicarpus, become habituated to the shadow and impact of a book striking its habitat?***

Work in pairs; the data will be pooled on the chalkboard by the instructor. Obtain three 250 ml beakers. Fill two with 200 ml of seawater. Use the third beaker to pour the water back and forth between two beakers to ensure that it is maximally oxygenated. Do this five times for each of two beakers. Put a hermit crab in each of the two beakers. Place one beaker in front of you and allow the crab to adjust to its new home for five minutes. Place the other beaker, with another crab in it, far enough away so that it cannot see or feel the stimuli applied to the first crab. (It may be necessary to place this crab at the back of the room.)

Make sure all environmental factors which might influence the behavior of the crabs are as identical as possible for each beaker. Then take a thin book and bring it down sharply on the top of the beaker so as to cast a shadow over the crab and create vibrations it will feel. This is your experimental crab. Record a plus (+) if the crab withdraws into its shell, a minus (−) if it does not, next to number 1 on Table 12.4. Allow 30 seconds to pass and repeat. Do this for 30 trials, each after a 30 second interval. While you are waiting between trials, set up the graph on page 227. The abscissa (horizontal axis) should be "Trial number" (0–30). The ordinate (vertical axis) should be "Number of positive responses" (withdrawals into shell) (0–12). Use intervals of four spaces on each axis.

After the 30 trials are completed, enter your data on the chart your instructor has constructed on the chalkboard. When all teams in the class

TABLE 12.4. Number of Positive Responses (Withdrawals) of Hermit Crabs

Your Data		*The Class Data*	
Trial Number	*Response* (Record a plus if the crab withdrew, a minus if it did not.)	*Trial Number*	*Total number of pluses for each trial* (Add up the number of + signs next to each trial number after all teams have recorded their data on the chalkboard.)
1		1	
2		2	
3		3	
4		4	
5		5	
6		6	
7		7	
8		8	
9		9	
10		10	
11		11	
12		12	
13		13	
14		14	
15		15	
16		16	
17		17	
18		18	
19		19	
20		20	
21		21	
22		22	
23		23	
24		24	
25		25	
26		26	
27		27	
28		28	
29		29	
30		30	

have recorded their data on the same chart, add up the positive responses for each trial and transfer the data to Table 12.4. Use them to plot your graph later.

Immediately after you have recorded your data in Table 12.4 and on the chalkboard—but before completing Table 12.4—perform five more trials at 30 second intervals on both the experimental crab and the control crab which has been sitting in the other beaker. *Do this as soon after you have finished testing your experimental crab as possible.*

These trials will compare the behavior of a crab which has been exposed to the shadow and vibrations of the book striking its beaker for 15 minutes with the control crab which has had no such exposure and which will respond to a new stimulus. Make sure to test the crabs together, each partner administering the stimulus to one of the crabs at the same time as the other, or place the beakers next to each other and apply the stimulus to both at the same time.

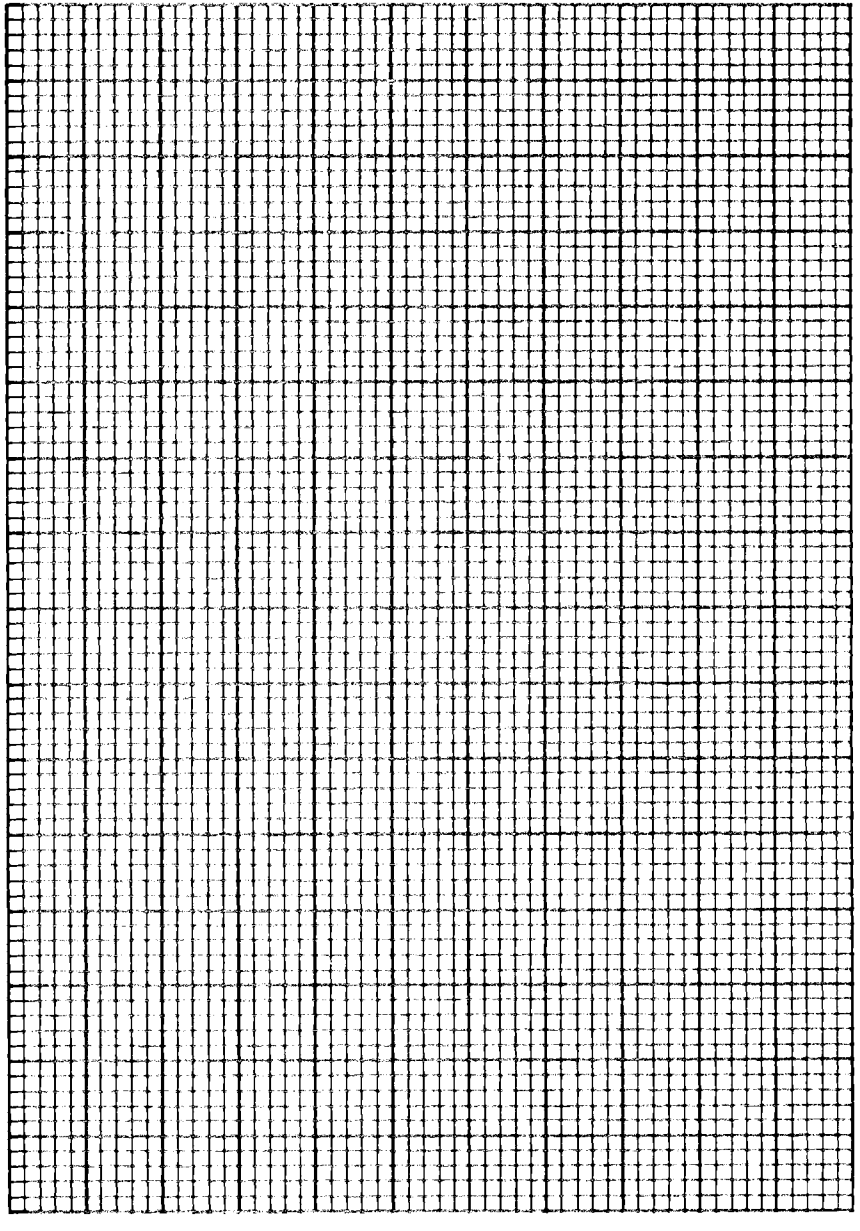
Record your data on Table 12.5. Obtain the data from three of your neighbors so that you have 20 trials of experimental crabs and 20 trials of control crabs.

TABLE 12.5. Comparison of Responses of Crabs with 30 Previous Exposures and Responses of Unexposed Crabs

(A plus signifies withdrawal.)

Crabs with Previous Exposure		*Crabs with No Previous Exposure*		*Crabs with Previous Exposure*		*Crabs with No Previous Exposure*	
Trial	*Response*	*Trial*	*Response*	*Trial*	*Response*	*Trial*	*Response*
				Data from neighbor 2			
1		1		1		1	
2		2		2		2	
3		3		3		3	
4		4		4		4	
5		5		5		5	
Data from neighbor 1				*Data from neighbor 3*			
1		1		1		1	
2		2		2		2	
3		3		3		3	
4		4		4		4	
5		5		5		5	

Now complete your graph using only the data of Table 12.4 (*not* Table 12.5).



13. Can you use your graph to make any statement about the solution of the problem? _____ The problem was:

Will the hermit crab, Pagurus longicarpus, become habituated to the shadow and impact of a book striking its habitat?

14. What is your interpretation of your graph? _____

_____

_____

The graph is useful in delineating trends. It is not, however, a sensitive instrument for determining whether or not the observed phenomenon shows a real change in the behavior of the subjects.

A more precise method is the chi-square test. Analyze the data of Table 12.5 to determine if the behavior of "exposed" crabs differs from that of "unexposed" crabs when both are compared with chance.

15. According to chance, what would be the expected frequency of positive responses out of 20 trials? _____ Of negative responses?

_____

16. What is the total of positive responses of "exposed" crabs from Table 12.5? _____

Of negative responses? _____

Compare these responses to the chance frequencies.

$$X^2 = \frac{\left(\begin{array}{c}\text{Positive responses of "exposed" crabs}\end{array} - \begin{array}{c}\text{Expected positive responses}\end{array}\right)^2}{\text{Expected positive responses}} + \frac{\left(\begin{array}{c}\text{Negative responses of "exposed" crabs}\end{array} - \begin{array}{c}\text{Expected negative responses}\end{array}\right)^2}{\text{Expected negative responses}}$$

X^2 = _____

Look up your chi-square number in Table 12.6.

TABLE 12.6. Chi-square Values at One Degree of Freedom

Level of Confidence	.50	.30	.20	.10	.05	.02	.01
Minimum Value of Chi-square at DF1	.455	1.074	1.642	2.706	3.841	5.412	6.635

17. Did the chi-square exceed the .05 level? _____

18. Do you accept or reject the null hypothesis? _____

Now use the total positive _____ and negative _____ responses of "unexposed" crabs to calculate the following chi-square.

$$X^2 = \frac{\left(\begin{array}{c}\text{Positive responses of} \\ \text{"unexposed" crabs}\end{array} - \begin{array}{c}\text{Expected positive} \\ \text{responses}\end{array}\right)^2}{\text{Expected positive response}} + \frac{\left(\begin{array}{c}\text{Negative responses of} \\ \text{"unexposed" crabs}\end{array} - \begin{array}{c}\text{Expected negative} \\ \text{responses}\end{array}\right)^2}{\text{Expected negative response}}$$

X^2 = _____

Look up this chi-square number in Table 12.6.

19. Does it exceed the .05 level? _____

20. Do you accept or reject this null hypothesis? _____

21. Clearly state your solution to the problem below. Include reasons for your conclusions.

Exercise II. Anthropomorphism
Animal Behavior 2: Kineses and Taxes

Anthropomorphism, the subject of the first portion of this exercise, is a common source of disquietude among young people who are being asked to experiment with living organisms. It is important that they come to terms with the problem of the relationship between human behavior and that of other animals. So often students will ask, "Can it feel pain?" And when two isopods or snails are in close proximity someone always says, "They like each other." There is a lot of emotion invested in animal-human relationships. Irrational hatred of cockroaches and earthworms and unwarranted affection

for certain rodents can impair the functioning of graduate students and undergraduates alike. They may avoid choosing a topic for research involving the use of experimental animals because of a culturally induced tendency to give them human characteristics. Students dropped my course in parasitology because they refused to use live cockroaches or to inject mice with malaria. On the other hand, it is conceivable that forcing students to remove the beating heart of a terminally anesthetized frog will so traumatize them that they will fall prey to anti-vivisectionist propaganda, unless they have the opportunity to think out their own attitudes towards animals.

Part II of the exercise, the determination of the behavioral pattern of snail or isopod as either taxis or kinesis, requires the student to set up his own experiment. He tests his own hypothesis and analyzes the data by means of chi-square. This aspect of the chapter deliberately offers little guidance and requires independent thinking.

Biological concepts covered include: ecological niche, taxis, kinesis, carnivore, herbivore, "rotting log community."

Take 45 minutes for class discussion. The first few minutes may be devoted to doing the anthropomorphism survey *before* they read what has been written below it. Accumulate the responses on the chalkboard and let the students interpret them. Almost invariably, eight or ten will characterize parakeets, cats and dogs as capable of dreaming, while other, less familiar, members of the same taxa, such as chickens, lions, and wolves are not described as dreamers. Make it clear that we are using the concept of dreaming as an indicator of the tendency to give animals human characteristics.

After this discussion, do an example of chi-square analysis. Emphasize the null hypothesis and levels of confidence.

Finally, demonstrate the use of the trays and T-maze. A waterproof magic marker should be used to mark off four quadrats in the enamel tray, marked I, II, III, and IV. The assistants should place 10 isopods and 10 snails in each of two petri dishes or covered finger bowls before class for each pair of students. Both dishes must have a floor of wet filter paper or paper toweling to keep the humidity high. The students should be instructed to place a disc of wet filter paper in the center of one of the quadrats. They should drop the isopods into the center of the tray, where the lines cross, by inverting the dish and tapping the bottom. Eventually the isopods will accumulate on the wet disc. After the observations are recorded, the isopods are placed back into their petri dish and the procedure is repeated with the snails. They, however, will not necessarily be distributed in any pattern on the tray.

The T-maze is a simple instrument to use. Unscrew the bottle and place the isopods or snails inside. *Keeping the bottle vertical,* screw on the plastic tube containing the two rubber stoppers. A wad of wet cotton should have been placed against one of the stoppers. The maze should be placed horizon-

tally or vertically on the table and *the initial direction each organism takes* as it leaves the bottle and the number of organisms in each side of the maze should be recorded at specific time intervals.

Obtaining and culturing snails and isopods:

Isopods (sowbugs or pillbugs) can be obtained everywhere, from desert to mountainside. They have every virtue, being easy to culture, relatively active, harmless, and not easily damaged by handling. There is little cultural antagonism towards them, and most people are willing to pick them up.

A good technique for providing a constant supply of isopods is to take a squashed carton or a piece of plywood and place it on the ground in a deserted area. After a few weeks you can return to find dozens of isopods underneath. Rotting logs or lumber always have dozens or hundreds of isopods beneath them. Collect isopods before the frost.

To culture, place a handful of isopods in a plastic container, such as are used to store food in refrigerators, add moist filter paper, and cover. Keep in a dark place. The isopods will live out their life cycle in the container. They need no food, since they will eat the paper, and no attention, since adequate air diffuses into the container. Do not make holes in the top, as this will reduce the humidity level. They can be kept in this manner for weeks. For long-term storage, cut a potato in half and place it, cut side down, on crumpled wet paper. When you need the isopods, simply turn over the potato and you will find them clinging to the bottom. Check occasionally as the potato often grows shoots which force the lid up, allowing the contents of the container to desiccate.

Snails can be collected and kept in the same manner, but they are not as ubiquitous. You may have to order them. While they, too, will eat moist paper, they are best kept in a terrarium and fed lettuce. Land snails will often attach themselves to the side of the tank, closing off their shells with a barrier of mucus. To activate them before use, place them in a beaker of lukewarm water for a few minutes. They will rapidly crawl to the surface of the water.

Other organisms which can be used, instead of isopods and/or land snails, are guppies and pond snails, *Daphnia,* beetles, or whatever is being cultured by someone in your building, as long as they are relatively active, harmless, and not repugnant to the students.

Note: Although both isopods and snails are, to me, animals of great perfection, this chapter can be even more instructive if the hermit crab, *Pagurus longicarpus* (used in the previous exercise), and the mud snail, *Nassarius obsoleta,* are used. These organisms require salt water, but the extra effort is worth it. There is no need to use a target substance (stimulus) like a wad of wet cotton; the light variation in the room will do. When the ten hermit crabs are placed in the tray, each immediately establishes a territory and defends it, to the great fascination of the students. The snails, if properly

acclimated, will rapidly glide along the sides of the tray in what looks like a parade, as they follow the trails of their predecessors.

The propensity of crabs to avoid close proximity to other crabs makes them good subjects for use with the T-maze, as they rapidly move out of the bottle and into the arms of the maze. The snails, too, can be induced to do this by placing the bottle in a vertical position.

Use the sources previously described to order the snails and hermit crabs. If you find these organizations undependable, write to me and I will send the organisms to you.

Time: One three-hour period or two two-hour periods. This exercise is crammed into the time period, so don't make introductory remarks overlong.

Student Materials

Per pair of students:

1 white enamel tray, 12″ × 8″ × 2″, Turtox #73–473, or, if aquatic forms are used and there is little tendency for them to crawl over the sides of the tray, Ward's #14W7030, 23″ × 12″ × ¾,″ is excellent because of its large size.
10 isopods, Carolina #L624 or L630d, Ward's #87W5520.
10 snails, land, *Polygyra* sp. Ward's #87W4300, or small *Helix pomacea.* (These are called "escargot" by the French. They are edible and can sometimes be bought by the pound in fish stores.)
2 petri dishes or finger bowls with covers, one containing the 10 isopods, the other, the 10 snails.
2 discs of filter paper, 10 cm in diameter.
1 small metric ruler.
1 small animal T-maze (Micro-Biome Behavior Chamber). Available from:

Scientific Manufacturing Industries
1399 64th St.
Emeryville, CA 94608

Instructor's Materials

Per class:

1 package of cotton wool.
1 glass tank containing a wet paper toweling floor and approximately 30 isopods. Cover with glass lid or Saran Wrap.

1 glass tank containing a wet paper toweling floor and approximately 130 snails. Cover with glass lid or Saran Wrap.

Note: Isopods and snails can be re-used by successive classes.

Anthropomorphism*
Animal Behavior 2: Kinesis and Taxis

Before reading on, fill in Table 12.7. *Place a check next to each kind of animal on the list which you feel is capable of dreaming while asleep.*

TABLE 12.7.

Pigeons _____	Parakeets _____	Goldfish _____	Wolves _____
Earthworms _____	Snakes _____	Snails _____	Chickens _____
Guppies _____	Lions _____	Dogs _____	Clams _____
Cats _____	Giraffes _____	Alligators _____	Monkeys _____
Cockroaches _____	Grasshoppers _____	Crabs _____	Humans _____

1. Which organisms are you sure are capable of dreaming? _____

2. What evidence do you have? _____

Your instructor will tabulate the responses of the class on the chalkboard.

3. Has a pattern appeared? _____ Summarize the attitudes of your class toward the ability of animals to dream. _____

*PROBLEM SOLVING IN BIOLOGY, Second Edition, by Eugene H. Kaplan. Copyright © 1976 by Eugene H. Kaplan. Reprinted by permission of Macmillan Publishing Co., Inc. and the author.

Dreaming, as far as we can tell, is a human trait, although there is some evidence that dogs exhibit the rapid eye movements (REMs) which characterize human dream periods.

The attribution of human characteristics to animals is called **anthropomorphism.** Usually the closer an organism is to man, either phylogenetically (for example, vertebrates like dogs and cats are closer on the evolutionary scale than insects or clams) or in familiarity (for example, goldfish and parakeets are more familiar to us than carp and orioles), the more likely people are to ascribe human characteristics to it.

Living with animals has always been a part of human experience. Sharing one's home with a flock of goats is a dimly remembered ancestral memory to some of us, although it is still practiced in many parts of the world today. But the luxury eighteenth-story apartment in midtown is not free of its complement of animals. The cockroach is found wherever man lives. The ancient silverfish, a primitive insect which has existed unchanged for millions of years, can live as comfortably on the glue of bookbindings as it did on scraps from the caveman's table or on rotting plants in the moist fern forests which existed before vertebrates evolved.

Attitudes toward animals vary with human experience. Some people are offended at the idea of placing stones in the uterus of female camels being prepared for a long caravan trip across the desert, a form of birth control practiced by Bedouin tribesmen. The same people may be less offended when an offshoot of this practice, the IUD (intrauterine device) or "coil," is placed in a woman's uterus for the same purpose—to prevent conception.

What are the behavioral limitations of nonhuman animals? The cat, the crocodile, and the scarab beetle were worshipped by the ancient Egyptians as gods capable of superhuman feats. The same beetles in our culture are often squashed on sight. Do insects have "feelings"? Do they, for example, feel pain? The child brought up on a farm is accustomed to the sight of animals being slaughtered for food, and he finds it relatively easy to participate in preparing a hog for market. His city cousin is horrified at the idea, yet he squashes every spider and insect in his apartment, and the recognized method of removal of mice is to flush them down the toilet.

The emotional response to animals (genuine love for dogs and cats; abhorrence of snakes and spiders) can cloud rational behavior. Antivivisectionists describe bloodcurdling experiments supposedly performed in hospitals and laboratories on dogs and cats. To attribute excessive cruelty to animals to scientists is to deny the scientists the humanity they are entitled to, just as to destroy an animal because it offends one's esthetic sense deprives that animal of its inherent dignity as a living thing.

4. Does man have the right to use animals in place of humans for legitimate experiments which might help improve human health or well-being? ___ Explain.

How "human" are the lower animals? Do they think? Is their behavior governed by emotions? The octopus, a close relative of the clam (both are molluscs), clearly exhibits emotions. We can tell, because it "blushes." It changes color rapidly with its "mood," and since the colors are associated with different "states of mind" (a dull color when at rest, rapid color changes to purple and pink when disturbed), we can relate these color changes to its behavior.

Today's exercise will give you a chance to test the level of behavior of another relative of the octopus, the snail, and a species which has survived for millions of years before man appeared, the isopod.

Preliminary Information

An **ecological niche** is that part of the environment which includes food, shelter, and other factors necessary to sustain an organism. An unusually complex and well-inhabited ecological niche is found under the surface of a rotting log in the forest. Animals and plants living there have become adapted to a narrow set of conditions. Vary the delicate balance even slightly and the organisms will die. The major *limiting factor* is the availability of moisture. Animals which seek dark, moist environments by tunneling through the deteriorating wood live in an atmosphere of 100% humidity. Isopods, important members of the rotting-log community, have evolved from sea-dwelling forms and have never really achieved a satisfactory substitute for gills. They will die if kept in the very low humidity of a heated house or classroom for more than an hour or so. (The relative humidity of your house in the dead of winter with the heating system operating maximally is lower than that normally found in the Sahara Desert.)

The rotting log has its **herbivores** (termites which eat the wood, millipedes which graze on molds which, in turn, obtain their sustenance from the decaying organic matter in the log). Also living in the tunnels and spaces which honeycomb the log are the **carnivores,** especially the centipedes which have a pair of poisonous claws with which to paralyze the small worms, insects, and isopods upon which they prey. Snails, moving along on trails of mucous, are relatively impervious to the centipedes because they can withdraw into their shells. A snail breathes through a small hole in its side, called a pneumostome, which opens into a moist chamber surrounded by blood. This primitive lung is adequate in the moist air of the rotting-log community.

Two highly successful inhabitants of rotting logs are the isopod *Armidillidium vulgare,* often called the sowbug, and land snails of the genus *Polygyra* (Figure 12.3).

Part I. Observations

At your table will be found two closed containers, one labeled "Isopods" and one labeled "Snails," and a white enamel tray marked off into four quadrats. Work in pairs, one partner obtaining ten isopods from the container and placing them into a petri dish. The other partner will obtain ten snails in another petri dish, an enamel tray, two discs of filter paper, and a ruler.

Observation 1

Isopods are particularly susceptible to a lack of moisture. To see if their behavior is affected by availability of water, wet the disc of filter paper and place it in the middle of one quadrat. Pour all ten isopods into the center of the tray and record in Table 12.8 what happens after 30 seconds, 1 minute, 3 minutes, and 5 minutes. Repeat the procedure three times, or until you are satisfied that you have accumulated enough data in Table 12.8 to make a generalization.

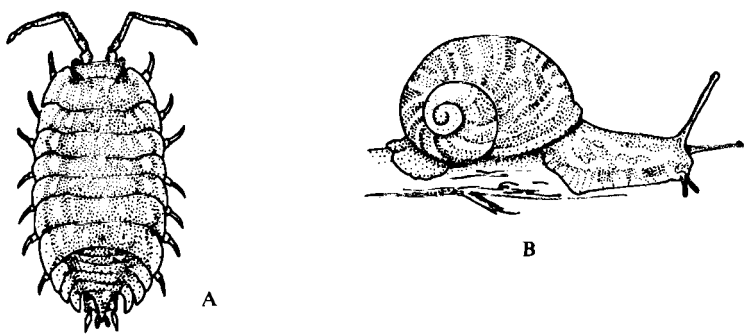


Figure 12.3. A. *Armidillidium,* an isopod. B. *Polygyra,* a land snail.

TABLE 12.8. Isopod Distribution

Time	*Number of Isopods in Quadrat I*	*Number of Isopods in Quadrat II*	*Number of Isopods in Quadrat III*	*Number of Isopods in Quadrat IV*
30 sec.				
1 min.				
3 min.				
5 min.				

5. Make a generalization about the distribution of isopods in the space below.

6. Are you reasonably sure of this generalization? _____

   Explain why or why not. _____

   _____

   _____

7. Is there a stimulus for this behavior? That is, if the isopods seem to move in a particular direction, hypothesize as to what it is they are moving toward or away from.

☐ **Hypothesis:** _____

_____

Test your hypothesis. Explain what you have done and the results in the space below. Keep testing alternative hypotheses, if necessary, until you feel that you have adequately explained the cause of the behavior of the isopods as far as their distribution is concerned. Record what you did below.

8. What do you believe to be the cause of the isopods' behavior? _____

_____

_____

_____

Measure the distances between adjacent isopods in the quadrat which has the most isopods in it. For example, if quadrat II has six isopods in it and the other quadrats have fewer than six, you would measure the distance of each isopod from its nearest neighbor in quadrat II. Calculate the average distance:

$$\text{Average} = \frac{\Sigma X}{N} \text{ or } \frac{\text{sum of all measurements}}{\text{number of measurements}}$$

Average distance between isopods: _____ mm.

Put the isopods back into the container. Close the lid.

Observation 2

Repeat for the snails the procedure for the isopods, using ten snails. Fill in Table 12.9.

TABLE 12.9. Snail Distribution

Time	*Number of Snails in Quadrat I*	*Number of Snails in Quadrat II*	*Number of Snails in Quadrat III*	*Number of Snails in Quadrat IV*
30 sec.				
1 min.				
3 min.				
5 min.				

Repeat the procedure three times, or more if necessary.

9. Is the snail behavior the same as that of the isopods?

_____

10. Make a generalization about snail behavior in the space below.

Measure the distances between neighboring snails in the most highly populated quadrat. Calculate the average distance between snails:

_____mm.

Summary

Examine all the data you have accumulated and the explanations of behavior you have tested. In the space below, discuss the behavior of isopods and snails, including *factors affecting their distribution.*

Part II. Experiments

The isopod *Armidillidium vulgare* exhibits a clear-cut behavior pattern which governs its distribution.

11. Does this pattern have survival value? _____ Describe an instance in which this behavior might help the isopods survive. _____

_____

_____

_____

Two problems have arisen from the observations we have made. These concern the degree to which the behavior of these animals is consciously controlled. In other words, do the organisms "decide" on the type of response they will make to a stimulus?

There are several categories of behavior exhibited by lower animals. The simplest type is a **kinesis.** This type of behavior is characterized by random movements which become more and more vigorous as the stress from an unfulfilled physiological requirement becomes greater and greater. For example, if an animal is hungry, it will move about rapidly until it finds food (**appetitive phase**). When it finds the food, it engages in a **consummatory act** (eating), triggered by the stimulus of the food itself. The final phase is **quiescence.** The animal slows down its activity related to satisfying the original physiological requirement. It might even go to sleep after its meal, for example.

A kinesis, then, is characterized by **random movement** which continues until a particular physiological condition is reached. Then it stops. The physiological condition may be hunger, thirst, sexual fulfillment, or habitat selection.

A **taxis,** on the other hand, is not random. The organism points itself at (or away from) the stimulus and approaches (or moves away from) it *directly.* When it reaches the origin of the stimulus, it engages in the consummatory act and becomes quiescent. (Or when it gets far enough away so that the stimulus is no longer effective, it becomes quiescent.)

The response may be either positive or negative. The name of the taxis usually has the appropriate Greek prefix attached to it. Thus an attraction toward the light, such as is exhibited by a moth, would be *positive* **phototaxis** (*photos,* the light), movement *toward* light. An organism crawling *upward* in a dark place (that is, uninfluenced by light) would be exhibiting *negative* **geotaxis** (*geo,* earth), movement *away from* the center of the Earth's gravitational field.

PROBLEM I: ***Is the behavior affecting the distribution of isopods an example of kinesis or of taxis?***

PROBLEM II: ***Is the behavior affecting the distribution of snails an example of kinesis or taxis?***

At the front table you will find a T-maze. Obtain 10 snails and 10 isopods and use the T-maze to solve each problem. On the Report Sheet (page 248) be sure to record the following after you have completed your experiments and analyzed your data.

(1) Your hypothesis.
(2) The observations which led to your hypothesis.
(3) All the information you obtain from testing your hypothesis.
(4) Your statistical analysis (see pages 216–222 and pages 243–245 for explanations of the chi-square test).
(5) Your experimental and control data.
(6) The solution to the problem.

Use of the T-Maze (Figure 12.4)

(1) Obtain a wad of cotton wool, wet it, squeeze out excess water, and place it at one end of the T-maze, against the stopper. Make sure both stoppers are in tightly.
(2) Unscrew the bottle and place organisms inside. Screw bottle back in tight. Keep bottle vertical so that organisms do not fall out.
(3) Place the maze flat or vertical, whichever seems more appropriate. If the flat placement is selected, the bottle should be supported by a pencil on the table, at right angles to the bottle, to keep it level.

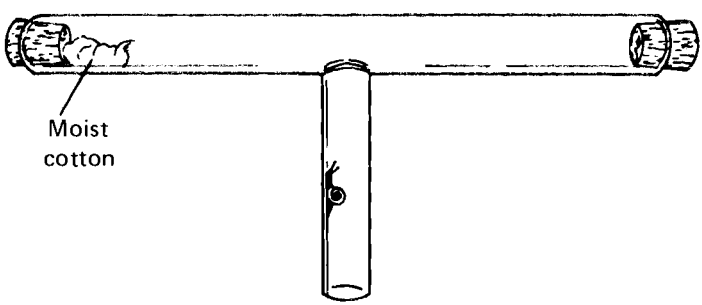


Figure 12.4. T-maze setup.

Begin your experiments now. Record your data below. Repeat each experiment at least five times and organize your data in a table.

Analysis of the Data

One of the most discomforting problems facing the scientist is that of determining whether or not his data are significant. In short, if you used 10 snails and 6 went left and 4 went right, could you make any generalization about the response of snails to a possible stimulus on the left? What if 7 went left and 3 went right? If all 10 went left, would you then be sure that the proposed stimulus was the cause of their behavior?

There are just a few basic tests of significance that are usually applied to find out the significance of differences between populations above 30, where chance can operate. One of these is the chi-square test. It has additional usefulness because it is not as dependent as some of the other techniques on sample size and can be used with relatively small samples, such as 10. Unfortunately, the smaller the sample size, the greater must be the difference between groups in order to show significance. In other words, if 7 out of 10 snails were found on the left, it is more likely that this may have been an accident (chance) than if 700 out of 1000 snails turned left. With this understanding, apply the following chi-square test to each of your sets of data to find out whether or not it was significant.

The Chi-square Test

The purpose of this statistical test is to determine whether the frequency of a particular response in your samples is accidental (that is, by chance) or the result of some systematic influence. In order to find this out, you compare the frequency of responses exhibited by your isopods or snails to the expected frequency (chance frequency).

12. In a situation where the animals could go either left or right, what would you expect the chance frequency to be (that is, what percent would go left by chance)?— _____ % (If you are in doubt, read the discussion on pages 216–222.)

In order to compare the obtained frequency (your data) with the expected frequency, (chance data) follow this procedure:

(1) Compute the number of snails you would expect to turn left if chance were operating. Your sample size was probably 10. Of these 10, how many would normally turn left if there was no particular influence acting on them? _____. How many would turn right? _____. These numbers are your ***expected*** frequencies.

(2) Take a set of data from your table on page 242. Record the number of snails which turned left _____ and right

(3) Fill in the spaces in the formula below.

$$X^2 = \frac{(\text{Number of snails turning left} - \text{Expected frequency})^2}{\text{Expected frequency}} + \frac{(\text{Number of snails turning right} - \text{Expected frequency})^2}{\text{Expected frequency}}$$

(4) Subtract the expected frequency from the actual frequency of left turns and of right turns. If the expected frequency is greater, your answer will have a minus sign in front of it. This will disappear when you square each difference, but you must keep it in mind in order to determine whether or not the difference in frequencies is positive or negative.

(5) Square each difference and divide the result by the appropriate expected frequency. Add these numbers together. Record your answer: $X^2 =$ _____

(6) To find out what your chi-square number means, find the number *just lower* than yours in Table 12.10.

The upper row is the level of your confidence that your results *did not* occur by chance. For example, let us suppose that your chi-square number was 1.73. The number on your chi-square table closest to it (and just below it) would be 1.642. The heading of the column above 1.642 is its level of confidence, .20. This means that you could expect results like this to occur less than 20% of the time by chance. Seems like a good bet? Not good enough.

TABLE 12.10. Chi-square Values at One Degree of Freedom

Level of Confidence	.50	.30	.20	.10	.05	.02	.01
Minimum Value of Chi-square at DF 1	.455	1.074	1.642	2.706	3.841	5.412	6.635

Depending on the nature of the data, scientists set levels of confidence at .05 or .01 *before they begin their experiments.* (.05 means you would expect results like this to occur rarely by chance– less than 5% of the time.)

13. Why is it necessary to set up levels of confidence before starting an experiment?

_____

_____

_____

In any field of investigation, including psychology, education, physics, etc., a level of confidence is always indicated, the .05 level usually being the lowest acceptable. It means that the results of the experiment (obtained frequency) differ from the expected frequency (chance frequency) so that only 5 times out of 100 would the event be expected to occur by accident (chance).

We will use the .05 level of confidence for our investigation.

Calculate the chi-square of each experimental trial you have performed.

Record the chi-square number you calculated in column 1 of Table 12.11. Record the .05 level of confidence number found on the chi-square table in column II. Is your chi-square number higher? If so, write "reject" in column III. This means you *reject the null hypothesis.* The null hypothesis is "There is no significant difference between the experimental and control variables."

TABLE 12.11. Comparison of Obtained Chi-squares with .05 Level of Confidence

	I *Chi-square Number*	*II* *.05 Level of Confidence*	*III* *Accept or Reject Null Hypothesis*
Isopods			
Trial 1			
Trial 2			
Trial 3			
Trial 4			
Trial 5			
Snails			
Trial 1			
Trial 2			
Trial 3			
Trial 4			
Trial 5			

Or, in our investigation, "There is no significant difference between the expected frequency and the obtained frequency." To reject the null hypothesis means that you *do not* believe that the results are caused by chance so they *are* the result of some systematic factor in your experiment.

Now it is finally possible to decide whether or not the snails and isopods we studied exhibited kinesis or taxis in their distribution patterns.

14. Do we have any information regarding the presence of nonrandom factors affecting the distribution of snails and isopods? Indicate why you do or do not believe in the existence of a factor or factors which affect the distribution of:

a. Isopods _____

b. Land snails _____

_____

_____

15. If there was any systematic behavior on the part of the snails or isopods, it may have been a kinesis or a taxis. How could you tell which it was? _____

_____

_____

_____

(If you could not answer that question, go back to page 241 and reread the definitions of kinesis and taxis.)

Conclusion (Also include the answers to these questions on your report sheet)

16. Is the behavior affecting the distribution of isopods an example of kinesis or taxis? _____ Explain. _____

_____

_____

17. How sure are you? (Were most of your chi-squares above the .05 level of confidence?) _____

18. Is the behavior affecting the distribution of snails an example of kinesis or taxis? _____ Explain. _____

_____

_____

19. How sure are you? (Were most of your chi-squares above the .05 level of confidence?) _____

20. Someone said that if you reject the null hypothesis the behavior is a taxis, and if you accept the null hypothesis, it is a kinesis. Is such a statement reasonable? _____

Explain. _____

_____

_____

REPORT SHEET Name _____

Instructor's name _____

State problems, hypotheses, results, and conclusions. Include evidence and results of statistical analysis.

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The classical study which inspired the development of the laboratory exercise described in this chapter. Includes data.

Chapter 13

An Investigative Laboratory: The Study of a Freshwater Stream

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Introduction

The primary objective of this exercise is to allow students to complete a comprehensive and efficient study of a freshwater stream. The authors use this laboratory exercise as the culminating activity to a rather extensive study of ecology in their non-major general biology course.

The laboratory was designed to allow a large number of students (total of 100 or more) to enter the field with a minimum of confusion and destruction to the environment.

Organization is a must. The authors have found that student groups of four to six individuals seem to be best. This allows for a rather even distribution of the workload within the group.

During the regular spring semester, when students meet for laboratory activities once a week, the freshwater stream study extends over three to four weeks. Summer sessions allow for completion in about one week.

Student Materials

Introduction

The concept of a biotic community is difficult to grasp from verbal descriptions alone. This difficulty may be overcome by gaining direct experience with a community, that is, by examining the physical, chemical, and biological characteristics of a selected community in our area.

Our purpose in this laboratory exercise is to have you undertake a brief, but comprehensive study of a small, aquatic community. In doing so it is hoped that you will learn something about the complexity of such a community and appreciate the numerous difficulties ecologists experience when they study complex ecosystems.

Behavioral Objectives

At the end of this laboratory you should be able to write a laboratory report which

1. describes the stream site used in your study geographically, historically, and biologically.
2. summarizes the data on the physical, chemical, and biological characteristics and states the importance of each to the organisms in the stream.
3. based on the data collected, offers a conclusion concerning the quality of your stream.

Procedure

I. Preparatory Activities

Our first laboratory period will be a tool session: a time for us to learn to use the pieces of equipment required to plan our work and complete

this study. Our activities will include the following discussions and demonstrations:

A. Locating stream site and watershed
B. How to collect microscopic forms
   1. Submerged slide technique
   2. Plant and rock scrapings
   3. Stream sediment
C. How to collect macroscopic forms
D. Obtaining most probable numbers for coliform bacteria
E. Demonstration of Hach Kit used in chemical analysis
F. Student division of duties and approval by the instructor

II. Field Activities
   A. General Characteristics
      1. Geography
         On the way to the stream copy down route numbers, directions, and the general lay of the land that will allow you to accomplish the following back in the laboratory:
         a. Find your location on the map in the laboratory and then identify the stream. _____
         b. Trace the stream to its source _____ and to its juncture with a larger body of water. _____
         c. In which watershed is your stream found? _____
      2. History and Aesthetics
         Upon arriving at the stream site, sit down for a moment and look the area over carefully; become a true part of the environment for awhile. Talk to any people who might be living close by and then answer the following:
         a. Look at the stream from the standpoint of the changes it has undergone. Record your observations, predictions, and feelings below.

Evidence of Changes in Environment	What Might Have Caused Them	Effect on Environment

b. How do you think this area looked about 25–50 years ago?

c. How do you think this area will look 25–50 years in the future?

d. Do you have any feelings about these changes? Describe them.

3. Streamflow (Velocity)
Probably the one most important characteristic of a stream, in terms of the organisms in it, is its current. Follow the below procedure to determine this important characteristic.

a. Measure and mark a 100-foot distance along a straight section of your stream. If you can't find a 100-foot section, use 25 or 30 feet. Throw a stick (2 or 3 inches long) in the water above the upstream marker. Record the number of seconds it takes to float downstream between the markers. Record below. Now divide the 100-foot distance by the total seconds it took the stick to float between the stakes.

$$\frac{100 \text{ ft.}}{\text{Total Seconds}} = \_\_\_\_\_ \text{ ft. per second}$$

b. Find the average width of your section of the stream. Measure the width of the stream at 3 places within the 100 foot area. Divide the total by 3 to get the average width of the stream.

First measurement _____ feet

Second measurement _____ feet

Third measurement _____ feet

Total _____ feet ÷ 3 = _____ feet (average width)

c. Find the average depth of your section of the stream. Measure the depth of the stream in at least 3 places across the stream in a straight line. Divide the total by 3 to get the average depth of the stream.

First measurement _____ feet

Second measurement _____ feet

Third measurement _____ feet

Total _____ feet ÷ 3 = _____ feet (average depth)

4. Temperature
   Hold a thermometer several inches below the surface of the water for several minutes and then record the temperature of the water in degrees Celsius: _____

B. Biological Characteristics

1. Gross Biological Characteristics
   Although you will be spending considerable time in the laboratory examining the living forms from the stream, you should note the gross biological characteristics of the area while you are in the field (forest, meadow, swamp, etc.). Photographs of the area are ideal, but good observation notes are necessary.
2. Collection of Organisms and Water for Study
   Many microscopic forms may be collected by placing microscope slides in the water tied to a piece of string. Secure the other end to a rock or branch. Microscopic forms will collect on the slides during the next several days. Several days later place the slides carefully into a small jar of stream water for transport to the laboratory. Be certain to place the slides on end and not touching each other.

   To collect macroscopic invertebrate animals it is best to pick up rocks carefully and remove the worms, crustaceans, insect larvae, etc., from the bottom. You may also overturn rocks with your foot and generally stir the water while a partner collects the organisms by holding a net or cloth downstream.

   You should also count forms like fish and crayfish that you need not bring back to the laboratory.

   You will also need to collect at least 500–1000 ml of water for your chemical tests. Water for the coliform test should be collected in a sterile bottle. This will be provided in class.
3. Dissolved oxygen (DO) and carbon dioxide should be measured at the stream site since the solubility of both vary with temperature and agitation of the water. Your group will sign out a Hach Kit from the laboratory for measuring these dissolved gases. Since these tests take considerable time to do, you should start soon after your arrival at the site.

III. Laboratory Activities

A. Diversity Indices of Organisms

One could and some do devote a lifetime of study to the organisms of a freshwater community. We do not have the time nor is it essential for you to identify by name the organisms you have collected.

It is our goal, however, to determine the diversity of organisms within this freshwater community. High diversity indices indicate a healthy, stable community.

In examining the organisms you have collected you need not identify each form. You need only two pieces of data to calculate a diversity index, the total number of organisms counted and the number of each look-alike group within the total. A look-alike group means just that; from your observations all organisms in the group seem identical in structure. We will *assume* for convenience that these look-alike groups are species groups.

For example, let's assume that when you placed your collected macroscopic animals in a white pan and separated them into look-alike groups (species) these are the data you collected:

A total of 30 organisms

Species A— 5 individuals
Species B— 20 individuals
Species C— 5 individuals

From these data, you can calculate a diversity index. This method was developed by Simpson ("MEASUREMENT OF DIVERSITY," *Nature,* 163:688, 1949).

$$\text{Diversity Index} = \frac{(\text{Total Number of Organisms})^2}{(\text{Num. of Sp. A})^2 + (\text{Num. of Sp. B})^2 \text{ etc.}}$$

$$\text{DI} = \frac{(30)^2}{(5)^2 + (20)^2 + (5)^2} = \frac{900}{25 + 400 + 25} = \frac{900}{450} = 2$$

Now, calculate the DI of your macroscopic animals in this fashion.

Various techniques are available for collecting aquatic vascular plants; however, for this study you should simply collect sample specimens of each type of plant growing in the water and rooted to the bottom. Estimate the density of each plant species by using such terms as light, moderate, or heavy growth.

The calculation of the diversity index for the microscopic forms requires some special consideration. Wipe off one side (bottom) of the

slide, add a cover slip to the top and place it on the stage of the microscope. Observe 5 fields of view and count the number of organisms in each look-alike group. You may use either low (10x) or high (43x) power; just be consistent. Do this for both microscopic plants and animals. You will need to make some simple line drawings of the organisms to keep your collected data accurate. Now, calculate a DI for these two groups.

B. Coliform Count (most probable numbers)

Coliform bacteria are normal nonpathogenic bacteria that live in the intestinal tract of man and other warm-blooded mammals. Their presence is an indication of sewage contamination and possible presence of the bacteria causing typhoid fever and dysentery which also live in the intestinal tract.

Coliforms are among the few bacteria that can ferment the sugar lactose. In this test you will use a special lactose medium.

1. Preparation of Phenol Red Lactose Broth
   Secure 3.0 grams Phenol Red Lactose Broth from the stock bottle and dissolve in 150 ml distilled water.
2. Preparation of Durham Fermentation Tubes (7 per test)
   Obtain seven large and seven small tubes. Place the seven small tubes inverted in the larger tubes. Distribute the medium equally in the seven tubes, stopper with a cotton plug and autoclave. After the tubes have been autoclaved and have cooled, the smaller tubes will be filled with broth; i.e., there should be *no* air space in the smaller tube (Figure 13.1).
3. Addition of Water Sample to Fermentation Tubes
   To five of your large fermentation tubes add 10 ml of the water to be tested. To the sixth tube add 1 ml and to the seventh tube add 0.1 ml of the water sample. Restopper and place the seven tubes, after they have been properly labeled, in the incubator for 48 hours.
4. Reading the Tubes
   Tubes must be read within 48 hours (not before 24 hours). You should look for gas bubbles in the smaller tube and a yellow color. If both of these conditions are present, you have a positive reading for that tube (+ sign on your chart). The chart below should be used to determine the most probable numbers (MPN) of coliform bacteria present in your water sample.

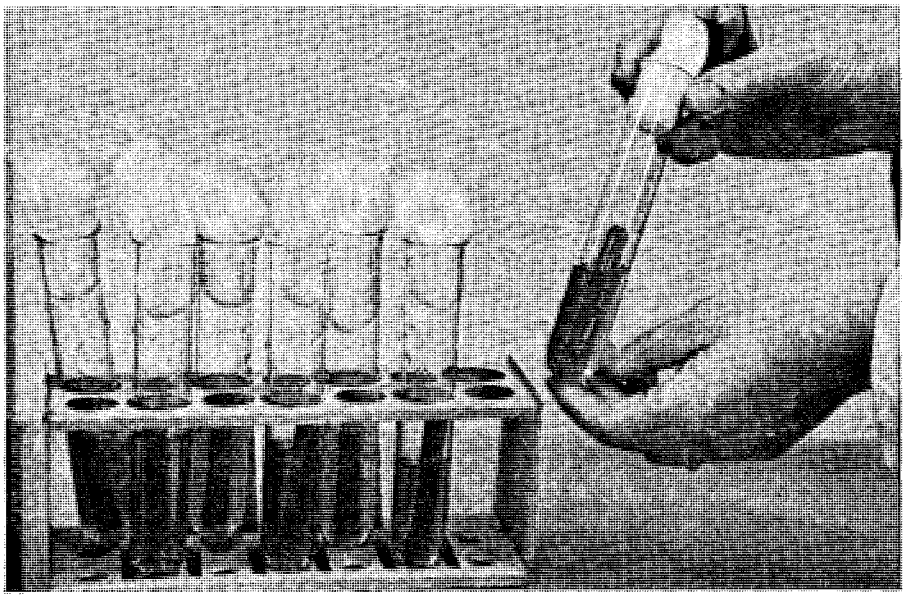

Figure 13.1. Setup of Durham fermentation tubes.

10 ml portions	1 ml portion	1/10 ml portion	MPN
− − − − −	−	−	less than 2/ 100 ml
+ − − − −	−	−	2
+ + − − −	−	−	5
+ + + − −	−	−	8
+ + + + −	−	−	15
+ + + + +	−	−	38
+ + + + +	+	−	240

Most probable numbers table adapted from:

Standard Methods for the Examination of Water and Wastewater

Fourteenth Edition, 1976

Available from:

American Public Health Association, Washington, D.C.

The test you have completed is called the presumptive test. To confirm that these are fecal coliforms you must complete the following confirmation test:

Obtain a prepared *E*osin *M*ethylene *B*lue agar plate. Streak the plate with a loop of broth from a positive (+) fermentation tube. Incubate in an inverted position for 24 hours. A positive confirmation test for coliforms will show colonies with dark centers that may have a greenish metallic sheen.

C. Chemical and Physical Tests
   1. Use the Hach Kit to accomplish the following tests:
      a. dissolved oxygen (ppm)
      b. carbon dioxide (ppm)
      c. nitrate (ppm)
      d. phosphate (ppm)
      e. total hardness (ppm)
      f. pH (hydrogen ion concentration)
      g. turbidity (% transmittance)
   2. Biochemical Oxygen Demand (BOD)
      This test (biochemical oxygen demand) allows a general estimate of the predisposition of your stream to develop an oxygen deficiency. It gives an indication of the amount of organic material present in the water that will decompose (oxidize) and demand oxygen.

      The BOD is determined in the following manner:
      a. Determine the parts per million (ppm) of dissolved oxygen (DO) in your water sample. If the dissolved oxygen is not at least 7 ppm, bubble air through your sample until this concentration is reached. Incubate a sample of this water in a BOD bottle for 5 days at 20°C in a darkened environmental chamber.
      b. Determine the dissolved oxygen content of this incubated sample. Subtract this second reading from the initial reading taken in the laboratory. The difference expressed in ppm of oxygen is your BOD reading.

Evaluation

Although you have worked in a group to carry out this research, it is the responsibility of each member to write their own paper. The following outline should be used as a guide:

1. Prepare an assignment sheet showing the duties and responsibilities of each member of the group.

2. Geographic Information—A description of the stream location including a map of the area (a sketch will do).
3. History—Aesthetics.
4. Test Results—For each of the following tests indicate the results for your stream. In addition, answer each question by searching the appropriate literature and using your class notes.
   a. Phosphate—Why are phosphates needed in aquatic ecosystems? What do high phosphate concentrations tend to cause?
   b. Nitrate—Same as "a."
   c. pH—Acidity/Alkalinity—What is the normal pH in a freshwater stream? Are extremes of pH dangerous?
   d. Carbon Dioxide—Why is carbon dioxide needed in aquatic ecosystems?
   e. Dissolved Oxygen—Why is oxygen needed in aquatic ecosystems? What are minimum levels for a healthy stream?
   f. Biological Oxygen Demand—What are the detrimental effects of a high BOD level?
   g. Total Hardness—Attempt to relate the hardness of your stream to its location.
   h. Turbidity—How does water with a high turbidity level influence life in a stream?
   i. Velocity—How does the velocity of a stream affect the abiotic characteristics and the type of organisms that live there?
   j. Coliform Count—What is the significance of a high coliform count?
   k. Diversity Indices—What is the meaning of a high diversity index?
5. Environmental Profile Chart.
6. Conclusions—Attempt to interrelate all of the data collected and offer your interpretation of the quality of your stream.
7. Bibliography.

Instructor's Materials

Procedure

I. Preparatory Activities

It is important that the instructor organize the procedures and materials carefully since many students will be working on their own in the field.

The first laboratory is devoted to explaining the tools, techniques, and field activities. Sites are also selected and groups organized. The instructor may choose several sites beforehand or allow the students to choose their own sites.

ENVIRONMENTAL PROFILE CHART

Velocity ft/sec	0		0.5		1.0		1.5		2.0		2.5		3.0
Transmittance %	40		50		60		78		80		90		100
Temperature (Celsius)	25		20		15		10		5		0		
Diversity Indices													
Macroscopic Animals	10		8		6		5		4		3		2		1
Microscopic Animals	10		8		6		5		4		3		2		1
Microscopic Plants	10		8		6		5		4		3		2		1
Coliform Count (MPN)	240		38		15		8		5		2		
Oxygen (ppm)	0		2		4		6		8		10		12		14
BOD (ppm)	10	9	8	7	6	5	4	3	2		1		
CO_2 (ppm)	24	22	20	18	16	14	12	10	8	6	4	2	1	0	
Nitrates (ppm)	18	16	14	12	10	8	6	4	2	1	0				
Phosphates (ppm)	5		4		3		2		1		.5		0		
pH	10		8		6		5		4		3		
Hardness (ppm)	300		250		200		150		100		50		

II. Field Activities

A. Geography

Students may draw their own maps of the area or the instructor may secure maps of the area from the Geological Survey for copying. Order forms for maps may be obtained from:

U.S. Geological Survey
1200 South EADS Street
Arlington, Virginia 22202

B. History and Aesthetics

Students interested in history may pursue this aspect of the study. We have found that much information can be obtained from local agencies (e.g., courthouse records) and from residents in the area where the site is located.

C. Stream Dimensions and Velocity

Each group should have a tape measure and a yard stick.

Although a stick is suggested for measuring velocity, an orange may be better. Its density, color, and shape enable it to float almost submerged yet still remain visible because of the color, and the round shape helps to keep it from becoming tangled in logs and floating plants.

D. Biological Characteristics

Microscopic forms may be easily collected by using the suspended slide technique. Strings may be either tied or glued to the slides and then anchored to a rock, tree limb, or stake. It takes about 48 hours to get a good collection of organisms. If plankton nets are available, these may also be used.

Macroscopic forms may be removed from rocks and plants with thin metal spatulas, medicine droppers, or forceps. We discourage the collection of large animals since they are often few in number and difficult to keep in the laboratory for any length of time. Such forms may be counted at the site.

We assume that the collection of organisms is somewhat random. If the instructor wishes to improve on collection techniques he should consult one of the limnological references listed in the bibliography.

We urge that the determination of dissolved oxygen and carbon dioxide be completed at the site for the reasons mentioned in the laboratory write-up. Several kits are available; the authors use the following:

Hach Chemical Test Kit for
Carbon Dioxide and Dissolved Oxygen
(Model CA–10)

Hach Chemical Company
Ames, Iowa 50010

III. Laboratory Activities

A. Diversity Indices

For non-major students the "look-alike" groups concept works well in that it removes the frustration of trying to identify individual organisms. However, any classification of organisms is possible from phylum to species depending on the sophistication of the students and the time available. More precise identification raises the possibility of determining food chains or webs.

B. Coliform Count

The collection of water for this test should be done using a sterile bottle with a screw cap and a capacity of at least 250 ml. Be sure to loosen the cap before sterilizing at 15 pounds pressure for 20 minutes. Students should be cautioned not to loosen or remove the cap until they are ready to collect their sample at the stream site. Testing should be done as promptly as possible after collection.

Students seem to appreciate to a greater extent the significance of this test if they prepare some of the materials themselves. If they begin early in a three-hour laboratory, they should be able to complete the entire procedure including autoclaving and inoculation with stream water.

The media used for the presumptive test is phenol red lactose broth which is available from biological supply houses. The large tubes we use measure 25 × 150 mm and the small tubes 14 × 100 mm. Obviously one may use tubes that are not this exact size.

The second or confirmed test is done using Levine's Eosin Methylene Blue Agar. This is also a standard media available from biological supply houses. Pour plates should be prepared using the procedure described in any microbiological laboratory manual. Directions for making these prepared media appear on the container. If students do not know how to streak a pour plate, instructions should be given during the tool session. These procedures are also found in any microbiology laboratory manual.

C. Chemical and Physical Tests

As was mentioned before, the dissolved oxygen and carbon dioxide tests are best done in the field. The remaining chemical and physical

tests may be done in the laboratory or the field depending on the availability of the chemical test kits. Details concerning techniques for collection and quantity of water needed appear in Student Materials.

A wide variety of kits are available for purchase from several vendors for water testing. The authors use model DR-EL from the Hach Chemical Company. This particular model contains reagents for more than a dozen chemical tests. Many students become confused with this large collection and we have found it best to remove all reagents except those used in the tests that apply to this study. These kits are very accurate but certain precautions should be taken. Water from the demineralizer bottle should be used in preference to distilled water. Care should be taken to see that all glassware is cleaned thoroughly between each test. Be sure to check batteries before each use. Worn batteries will result in erroneous readings. Directions for doing this are in the Hach manual. It is recommended that each test be done twice by different team members to check results. All chemical tests can usually be done in one three-hour laboratory session. There is a wide diversity of kits available from the Hach Company. For information, instructors should write to the:

Hach Chemical Company
Ames, Iowa 50010

The Hach Chemical Company will also supply information concerning the chemical basis of the mineral tests if the instructor wishes to pursue this topic in depth.

D. Biochemical Oxygen Demand
Standard BOD bottles should be used for this test. They are available from supply houses such as Fisher Scientific Company, Central Scientific, and Sargent-Welch. A BOD cabinet or an environmental chamber is needed to maintain the temperature at 20 degrees centigrade in a darkened condition.

Evaluation

1. Environmental Profile Sheet
The profile sheet can be used in different ways according to the type of study being conducted. A comparison could be made between profiles of fast-moving mountain streams and slow meandering valley rivers. Another study could compare different sites along a single stream for a considerable distance. If the same sites are used these data can be compared year to year.

2. Final Report

   The authors allow each student in a team to assume responsibility for writing a section of the final report. These sections are then duplicated and shared with other team members. Each student is then expected to write his/her own conclusions.

References

Amos, W.H. Limnology, an introduction to the freshwater environment. Chestertown, MD: LaMotte Chemical Products Co., Education Division; 1969.

An extremely simple but good introduction to freshwater streams.

Colinuaux, P.A. Introduction to ecology. New York: John Wiley and Sons; 1973.

A good technical reference.

Mongan, A.H. Field book of ponds and streams. New York: G.P. Putman's Sons; 1930.

Out of print but may be available in some bookstores or libraries. Excellent field guide.

Needham, J.G.; Needham, P.R. A guide to the study of freshwater biology. San Francisco, CA: Holden-Day; 1962.

One of the best books for identification of organisms.

Needham, P.R. Trout streams. San Francisco, CA: Holden-Day; 1969.

Certain chapters contain excellent information on freshwater streams.

Smith, R.L. Ecology and field biology. New York: Harper & Row; 1966.

A good general reference.

Southwick, C.H. Ecology and the quality of our environment. New York: VanNostrand Reinhold Co.; 1972.

Contains some information on water pollution.

Water quality criteria. A report of the committee on water quality criteria. Environmental Studies Board, National Academy of Sciences, National Academy of Engineering, 1972. Available from: U.S. Government Printing Office, Washington, D.C.

An exhaustive compilation of data concerning the quality of freshwater streams. One of the few sources that provide standards for comparison.

Appendix I

Documents Relating to the Origins of A.B.L.E.

The University of Calgary
2920 24 AVE. N.W.
CALGARY, CANADA
T2N 1N4

FACULTY OF SCIENCE
DEPARTMENT OF BIOLOGY
(403) 284-5261

PLEASE BRING TO THE ATTENTION OF APPROPRIATE DEPARTMENTAL FACULTY

Committee to Establish a Laboratory Biology Teaching Organization and Library

Jon Glase
Cornell University

Don Igelsrud
University of Calgary

Patricia Paulus
Texas Christian University

Ruth Von Blum
University of California, Berkeley

Those of us who have been developing, teaching, and coordinating undergraduate biology laboratory courses for several years have been working on common problems that could be approached more effectively if better communication were possible between us. Laboratory instructors everywhere are confronted with the same problems: finding and maintaining *reliable* living materials, producing modern laboratory exercises involving living materials and investigative inquiry, training others involved in the teaching and preparation for laboratory classes, developing fair and relevant methods of examination, and so on. Because there is so much to be done, most of what is attempted is based on traditional procedures and materials that are themselves frequently unreliable. By establishing communication with other biology laboratory instructors we hope to begin to solve these problems. One of us, Don Igelsrud, has suggested that a library be developed so that other persons actively working in laboratory biology teaching can be identified and communication facilitated. The main function of this library will be to collect in one place descriptions of biological systems (and supportive materials) that the contributor believes are reliable and effective in undergraduate biology laboratory instruction.

As presently conceived, the library of laboratory teaching will collect written materials contributed by individuals within your department who are actively involved with teaching biology in the laboratory. We are most interested in acquiring information about reliable, possibly unique, biological systems for teaching undergraduates in a laboratory setting. Our goal is to produce an index which will allow persons with specific interests to communicate with each other. With good participation we should be able to acquire sufficient outside funding to produce a detailed and useful index which would be sent to each contributor.

The biology departments of The University of Calgary and Cornell University will fund the initial costs of the library. It will be located in The University of Calgary Biology Department which has a strong commitment to laboratory teaching, offers laboratories with appropriate courses, and has eight full time laboratory instructors, marine and freshwater aquarium facilities, greenhouses and a large support staff. The facilities and faculty are available during the summer months (May through August) and Calgary's close proximity to Banff and the Canadian Rockies make it ideal for winter as well as summer visits.

The establishment of the library will serve an immediate and important funtion: to identify a group of individuals actively concerned with the problems of teaching biology in the laboratory. We propose to establish an organization of these individuals that would meet regularly, communicate via a newsletter, and in some fashion encourage the publication of its members' efforts. We hope to establish the library as quickly as possible so that a conference on laboratory instruction can be held at one of the national meetings during 1978.

Contributors should send copies of their current laboratory manuals and preparatory information for undergraduate laboratory courses indicating the authors of each exercise and annotating those materials which are particularly reliable and effective. Authors may send only their best materials if they wish. *The materials will be used in the following ways depending on the author's preference*: 1. For identification to help establish an organization and then held in confidence. 2. Available for study by visitors to the library. 3. Released for copying with the author's written permission. 4. Available for copying without permission of the author. Unless otherwise stated the library will assume the contributor wishes the materials be used only to the first extent. If you are revising materials send a copy of what you are using now and the revision, when available, to:

Laboratory Biology Teaching Library
Department of Biology
The University of Calgary
Calgary, Alberta, Canada T2N 1N4
(403) 284-6127

NEWS RELEASE: September 26, 1977

A LABORATORY BIOLOGY TEACHING LIBRARY

A laboratory biology teaching library is being established at The University of Calgary in order to improve communication among persons actively involved in undergraduate biology laboratory instruction.

Jon Glase (Cornell University), Don Igelsrud (University of Calgary), Patricia Paulus (Texas Christian University), and Ruth Von Blum (University of California, Berkeley) have formed a committee to establish the library and are sending a letter requesting written materials to 3500 institutions in the U.S. and Canada. The committee will identify a group of individuals actively concerned with problems of teaching biology in the laboratory so an organization can be established.

The main function of the library will be to collect in one place descriptions of biological systems and supporting materials that the contributor believes are reliable and effective in biology laboratory instruction. The committee is most interested in acquiring information about reliable, possibly unique, biological systems for teaching undergraduates in a laboratory setting.

Contributors are being asked to send their current laboratory manuals and preparatory information for undergraduate laboratory courses indicating the authors of each exercise and annotating those materials which are particularly reliable and effective. Authors may send only their best materials if they wish. The materials will be used in the following ways depending on the contributor's preference:

1. For identification to help establish an organization and then held in confidence.
2. Available for study by visitors to the library.
3. Released for copying with the author's written permission.
4. Available for copying without permission of the author.

Unless otherwise stated the library will assume the contributor wishes the materials be used only to the first extent. With good participation, the library expects to obtain sufficient outside funding to produce a detailed index so persons with specific interests can communicate with each other. An index would be sent to each contributor.

A conference on laboratory instruction is planned for one of the national meetings in 1978 and will be organized in conjunction with the formation of the new organization. By establishing communication among laboratory instructors the group hopes to begin to solve some of the common problems confronted in the laboratory: finding and maintaining *reliable* living materials, producing modern laboratory exercises involving living materials and investigative inquiry, training others involved in the teaching of and preparation for laboratory classes, developing fair and relevant methods of examination, and so on.

The library was suggested by Don Igelsrud to identify persons actively working in biology laboratory instruction so that communication could be facilitated. The biology departments of The University of Calgary and Cornell University will fund the initial costs of the library. It will be located in The University of Calgary Biology Department which has a strong commitment to laboratory teaching, offers laboratories with appropriate courses, and has eight full time laboratory instructors, marine and freshwater aquarium facilities, greenhouses, and a large support staff. The facilities and faculty are available during the summer months (May through August) and Calgary's close proximity to Banff and the Canadian Rockies make it ideal for winter as well as summer visits.

The group recognizes a need for an organization that addresses itself to the problems of biology laboratory teachers. Most papers on biological education are philosophical rather than practical in nature. Commercial laboratory manuals are often based on traditional procedures and materials that are unreliable. The methods, materials and experience required to greatly improve laboratory instruction do exist, however. For example, many reliable living organisms are available but have not been widely used in teaching because of poor communication. An organization of biology laboratory teachers would encourage the discussion of common problems and facilitate the improvement of laboratory programs.

Biologists who do not see a copy of the letter but are actively involved in laboratory instruction and are interested in participating should write to: Laboratory Biology Teaching Library, Department of Biology, The University of Calgary, Calgary, Alberta, Canada, T2N 1N4 (403) 284-6127.

BIOLOGY LABORATORY TEACHING WORKSHOP

June 4 through 8, 1979

The University of Calgary

Sources of Living and Supplementary Materials for Laboratory Instruction Don Igelsrud (Calgary)

Preparing Karyotypes of *Rattus norvegicus*, *Mus musculus*, and *Rana pipiens* Joseph Larsen (Illinois)

An Investigative Study of Sick Potatoes
Daniel Burke (Mercer)

The Elucidation of the Biochemical Pathway for Pigment Production in *Serratia marcescens* Marcia Allen (Stanford)

An Investigative Laboratory in Cell Permeability Ruth Von Blum (Berkeley)

Plant and Animal Tissue Culture
Lester Eddington (Biola)

Animal Behavior, and Introduction to Statistical Analysis Eugene Kaplan (Hosstra)

The Structural Organization of Living Cells and Cell Organelles Osris and Susan Boutros (Pittsburgh)

The Honey Bee, *Apis mellifera*, an Ideal Laboratory Animal Jon Glase (Cornell)

Digestive Enzymes of the Cockroach
C. Leon Harris (SUNY Plattsburgh)

Brassica campestris, an Ideal Higher Plant for Teaching Loy Crowder (Cornell)

Liver and Muscle Glycogen—Effect of Fasting and Exercise Bruce Virgo (Windsor)

The Study of a Freshwater Stream—an Investigative Laboratory Richard Montgomery and William Elliott (Hagerstown Junior College)

The workshop will be limited to 50 participants and is intended for persons with permanent positions who are responsible for undergraduate laboratory teaching. Each presentation will last about four hours and will be limited to 25 participants. Participants will be selected by the Committee to Establish a Laboratory Biology Teaching Organization and Library (Jon Glase-Cornell, Don Igelsrud-Calgary, Patricia Paulus-Texas Christian, and Ruth Von Blum-Berkeley). The participants will form the nucleus of a new biology laboratory teaching organization. The group hopes to increase communication between the research and laboratory teaching communities to produce better living materials for instruction. Interested persons should write to the committee indicating their position and experience. They should also submit examples of their teaching materials. The materials submitted for selection of participants will be placed in the laboratory Biology Teaching Library if applicants wish. Write to: Laboratory Biology Teaching Library, Department of Biology, The University of Calgary, Calgary, Alberta, Canada T2N 1N4
Phone (403) 284-6127

NEWS RELEASE:

THE ASSOCIATION FOR BIOLOGY LABORATORY EDUCATION (A.B.L.E.)

An international group of biologists involved in teaching undergraduate biology laboratories met recently in Calgary, Canada and formed the Association for Biology Laboratory Education (A.B.L.E.). The organization was inaugurated with a series of workshops presenting specific creative laboratory exercises. One function of the Association will be to publish and disseminate material presented at the annual workshop sessions. The Association plans to publish a newsletter called *Labstracts* and is establishing a clearing house for laboratory teaching materials, exercises, organisms and techniques.

The second annual meeting will be held at the Urbana campus of the University of Illinois, June 2 to 6, 1980. Future meetings are planned at the State University of New York at Stony Brook in 1981 and at Stanford University in 1982. Membership is open to anyone involved or interested in laboratory instruction at the post-secondary level at a cost of $10 U.S. per annum. Persons interested should contact Rosalie Talbert, Treasurer—A.B.L.E., Department of Biology, Nassau Community College, Garden City, New York 11530.

Officers elected at the first meeting were: President—Don Igelsrud, The University of Calgary; Vice President—Joseph Larsen, University of Illinois; Secretary—Anna Wilson, Purdue University; Treasurer—Rosalie Talbert, Nassau Community College; Directors at Large—Marcia Allen, Stanford University, William Elliott, Hagerstown Junior College, Eugene Kaplan, Hofstra University, and Jenny Xanthos, McGill University.

The organization has four main committees and persons interested in specific problems should contact the appropriate committee chairman. The Workshop Committee is chaired by Don Fritsch, Department of Biology, Virginia Commonwealth University, Richmond, Virginia 23284. The organization will publish the proceedings of the annual workshop under the editorship of Jon Glase, Section of Neurobiology and Behavior, Division of Biological Sciences, Cornell University, Ithaca, New York 14853. The Laboratory Biology Teaching Library is now under the guidance of Daniel Burke, Department of Biology, Mercer University, Macon, Georgia 31207. Labstracts will be edited by James Waddell, Department of Zoology, University of Maine, Orono, Maine 04473. The publication will attempt to facilitate communication among persons trying to solve similar problems by running ads to exchange ideas, organisms, equipment, etc. To help improve local communication *Labstracts* has five regional editors based on time zones: Eastern—Janet Emerson, Department of Biology, Emory University, Atlanta, Georgia 30322; Central—Dennis Brown, Department of Biology, The University of Winnipeg, Winnipeg, Canada R3B 2E9; Mountain—John Gapter, Department of Biological Sciences, University of Northern Colorado, Greeley, Colorado 80639; Pacific—Don Mansfield, 2509 Whittier Drive, Davis, California 95616; European—Jaume Josa, Laboratorio Biologia General, Facultad de Biologia, Universidad de Barcelona, Avda. Jose Antonio. 585, Barcelona 7, España.

TESTED STUDIES FOR LABORATORY TEACHING

The proceedings from the annual conference/workshops of the Association for Biology Laboratory Education (ABLE) have been assimilated into volumes of innovative activities for the teaching laboratory. ABLE proceedings contain a wealth of useful information covering a great variety of topics.

- All volumes contain complete comprehensive studies that can be used in the teaching laboratory.

  Studies include: Background information covering each topic.
  Lists of materials and equipment.
  Specific instructions.
  Sources of supplies.
  References.
  The name and address of each author.

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