Laboratory Exercises in

INVERTEBRATE PHYSIOLOGY

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by

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PREFACE

TO THE 1949 EDITION

While it would be amiss to dignify this revised manual as an edition, we may here point out that it has evolved to its present condition slowly, over a period of a dozen years. When the senior author instituted a course of the sort for which the present manual is designed, it was the practice merely to issue to the students separate sheets containing problems or suggestions and lists of pertinent references. As time went on it became apparent that each student in a class could benefit by having a complete set of problems and references in order better to integrate the diverse material being studied and reported upon in the laboratory. Accordingly, in 1946, a collection entitled "Laboratory Exercises for Experimental Invertebrate Zoology" was compiled and mimeographed at the Harvard Biological Laboratories for the use of the authors' classes at their respective schools. The manual was thus of special rather than of general utility and was intended to aid the students in becoming acquainted with scientific journals and other resources available in large universities and to develop their powers of ingenuity and original thinking. For this reason, step-by-step descriptions of procedure were largely omitted and the student was left free to develop his own problem as he thought best. Despite this limited approach, the manual has apparently proved useful in some other schools, either in class or as a sourcebook for instructors. Therefore, when it became necessary to reissue the manual it has seemed best to let it be printed commercially in order to make it available to those who might wish to make use of it. But in so doing, the authors have not departed from their original standard of having a manual primarily to meet their own needs. The result is a work whose general usefulness is probably limited to a relatively few courses in schools where comparative and invertebrate physiology is taught on a flexible and independent basis. It has many shortcomings; some we are aware of; others will become apparent. We publish it without apology, but would appreciate receiving criticisms from any source.

In the course of working out this manual, we have already received an untold amount of constructive criticism from many students, teaching assistants, and other teachers. While we would like to make a complete list of acknowledgements, it seems fairest to the group as a whole to leave most of these contributors nameless, since many of their suggestions, offered informally in conversation or laboratory reports, have become so blended with ours that the identity of the individual suggestions has been lost. We are none the less grateful to them. Some may feel that we have altered their contributions beyond recognition; this is a metamorphosis for which the authors alone must be responsible.

PREFACE

TO THE 1960 EDITION

Since the first appearance of this manual in printed form, many advances in the field of invertebrate physiology have been made. We have accordingly rewritten rather than simply revised most of the exercises, deleted some, and added a number of new ones. With the increased availability of reliable electrophysiological recording equipment, we have felt it well to expand considerably in that area of studies, and to add a new appendix (V) on methods. The general plan and spirit of the 1949 edition have been retained; as before, we are aware of inadequacies and will welcome corrections and criticisms.

The authors continue to owe a great deal to the suggestions and contributions of others. We are especially grateful to Dr. Betty Twarog, who has written Appendix V (Electrophysiological Methods) and several related exercises.

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INTRODUCTION

The exercises collected in this manual are an attempt to provide laboratory problems suitable for the inquisitive student who is entering a field of great diversity, full of unanswered questions. These exercises* are planned as individual problems, designed to acquaint the student with the activity of the living invertebrate. The presentation is that of small research problems, in the solution of which recourse must be had to original literature. The experiments suggested in these pages must be planned in more detail by the student, animals and apparatus assembled and readied, the experiment and controls carried out, and the results summarized. Since each individual in a laboratory section will ordinarily be carrying out in detail only one exercise in a given period, each must present his results in a clear and understandable fashion in order that the knowledge gained may be made available to the class as a whole. The preparation of demonstrations and oral reports is in itself valuable training.

To provide a schedule flexible enough to fit into the college half-year, we have divided the material into six sections, based on the functions of:

I. Feeding and Digestion.
II. Respiration and Circulation.
III. Osmotic Regulation and Excretion.
IV. Coordinating Systems.
V. Effector Systems.
VI. Receptor Systems and Response Mechanisms.

Each section normally occupies two weeks of the semester, leaving two or three weeks for field trips, term papers, special problems, or subjects not fully covered previously. There is obviously a considerable overlapping of material. For instance, cilia and nematocysts receive attention both under "Feeding and Digestion" and "Effectors"; the salt concentration of blood is related both to "Respiration and Circulation" and to "Osmotic Regulation and Excretion". It is not intended, nor would it be desirable, to focus the student's attention so closely on his problem that he fails to receive an adequate picture of the organization of the animal he works on and its place in nature.

It will be noted that certain areas of the broad field of physiological invertebrate zoology are scarcely touched. In some fields, such as genetics, material in abundance is available from other sources. In other cases, the special conditions attendant on the procurement and handling of material, and the delay involved in completion of experiments, have made it seem unwise to include exercises of particular kinds. Hence we have in general excluded work on experimental embryology, growth, and complex instinctive or learned behavior, despite the activity now prevailing in these fields. There is far less material than we should have liked to include on problems of an ecological nature, to be studied in the field. However, the alert student or teacher will repeatedly see opportunities where, by a change of emphasis, many of the essentially laboratory problems in this manual may be applied to studies made under field conditions. For this reason, and because of the limitless diversity of problems in behavior, ecology, and natural history, we have been forced to satisfy ourselves with laboratory types of problems. It may be desirable in some instances for the instructor to assign certain simpler

*Note that some exercises are too extensive to be covered by one student in a reasonable period of time. Such exercises are broken up into sections, designated by capital letters, which may be assigned to two or more students, or one student may confine his efforts to that section which seems to promise the best results according to the immediate availability of materials, etc. Where an exercise may be reasonably covered by one student, it may be divided into parts designated by numerals. A student may do all parts, or may make some selection as circumstances warrant.

types of work, such as the dissection of organ-systems, to certain students whose preparation in invertebrate zoology has been weak, or where this approach serves to render the subject matter more comprehensible.

Invertebrate physiology could be approached from several angles, the method of approach used by a given instructor being important chiefly to the extent that it makes the best use of existing facilities. Provided that a course possesses a coherent, comprehensive, and logical plan, it can be made successful.

If we were to use the phylogenetic approach, we could discuss in turn the total knowledge of the physiology of protozoa, coelenterates, molluscs, arthropods, etc. In so doing we might lose sight of fundamental physiological principles and face more complex detail than could be absorbed in a brief general course. We could go to the other extreme and restrict our attention to physiological processes as such, using a few easily kept laboratory animals: amoebae, snails, crayfish, or whatnot, to illustrate the major and similar processes of living animals. Here we tend to lose sight of the invertebrates. To adopt a middle course has seemed best. We have arbitrarily divided invertebrate physiology into several aspects and concentrated on each for a period of time, within which we have attempted to illustrate the principles with as wide a variety of invertebrate types as are available. The alert student should receive a broad view of invertebrates as diverse, living creatures, and at the same time should receive a basic knowledge of their comparative physiology. The experimental approach provides an introduction to the use of zoological literature and to some of the methods, difficulties, disappointments, and satisfactions of investigation.

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A survey of the feeding habits and nutrition of invertebrates shows clearly that we know a great deal more about the feeding mechanisms of invertebrates than we do about their actual nutritional needs. We know, for instance, that certain animals collect floating plant life by means of mucus and cilia, but in most cases we know little about the nutritional requirements in terms of actual chemical substances, except for certain Protozoa and insects.

We may classify feeding mechanisms of invertebrates according to the size and nature of the food. Thus we recognize as "microphagous" feeders those animals which strain microscopic organic material from the water by an array of cilia, mucus, legs, bristles, setae, and nets. Then there are the "macrophagous" feeders which subdivide larger masses of food by teeth or jaws, or by the action of enzymes after swallowing. Smaller groups would include sucking animals, modified to extract fluids from prey, and those parasitic forms which are bathed in nutrient absorbed through the body surface.

We may classify digestive processes according to whether the food is reduced to a completely soluble state before being taken into the cells of the body (extracellular digestion), or simply engulfed in amoeboid fashion while still in the particulate state (intracellular digestion).

We may consider the problem of digestive enzymes as such, demonstrate them in various animals, and attempt to relate them to the diet.

Finally, most difficult of all, we may try to determine the actual food requirements by selective feeding. Special problems arise in the case of animals sheltering symbiotic algae, fungi, or intestinal protozoa, which may or may not contribute to the nutrition of the host.

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Among the numerous references, three will be most useful in providing background, and should be read by all:

Trager, W., 1941. The nutrition of invertebrates. Physiol. Rev., 21:1-35.
Yonge, C. M., 1928. Feeding mechanisms in the invertebrates. Biol. Rev., 3:21-76.
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Additional references that contain material of wide applicability:

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Vonk, H. H., 1955. Comparative Physiology: Nutrition, feeding and digestion. Annual Rev. Physiol. 17:483-498.

EXERCISE 1

FEEDING METHODS OF PARAMECIUM

Paramecium is a microphagous, continuous feeder whose cilia constantly sweep minute particles, mostly bacteria, into the gullet where they collect in food vacuoles which circulate in a characteristic manner through the cell and within which digestion takes place. Since Paramecium seems to be capable of digesting the three major classes of foods, it presumably has the necessary enzymes for doing so. It is a remarkable fact that a single cell is capable of forming and using such a variety of digestive enzymes. Particles which a Paramecium is incapable of digesting are given off through the "cell anus" or cytopyge, a process seldom seen except after long and patient observation.

A. FEEDING REACTIONS:

Many of the phenomena of feeding and digestion in Paramecium may be observed through use of the procedure of Buck (1943), employing yeast stained with congo red:

> Take 3 grams of Fleischmann's compressed yeast, 30 mgm. of congo red and 10 ml. of distilled water. Mix thoroughly and boil gently for ten minutes. Make a thin ring of vaseline 15 mm. in diameter on a microscope slide. Into this ring place a drop of a thick suspension of Paramecium. Dip a dissecting needle into the congo red yeast and stir the drop of Paramecium. This adds an appropriate amount of "food". The color of the drop should be pink, not red. Add a cover-glass, press down gently, and start your observation immediately.

Observe with the microscope the feeding reaction, first under low power and then under high. Note the action of the cilia in setting up currents which direct food into the oral groove (see Mast and Lashley, 1916) and the formation of food vacuoles. How long does it take on the average for a food vacuole to form after feeding first begins? Does the rate of formation slow up as a Paramecium becomes filled with food? Do your timing with a stop-watch and record sufficient data to get an idea of maximum and minimum rates of vacuole formation. After the Paramecium has become packed with vacuoles filled with yeast, observe carefully to determine, if possible, whether the yeast is being rejected in favor of bacteria (probably preferable as food). If so, can you determine the mechanism of this selection? Is it entirely on the basis of particle size? Determine the course taken by food vacuoles as they circulate about the cell and record by means of diagrams. This is not as easy as it sounds.

As an alternative or follow-up to the above approach, look up and try the method of Kempton (1958) for observing the cyclosis of food vacuoles and the defecation of vacuolar material through the anal pore.

B. PH CHANGES DURING DIGESTION:

As you study the feeding of your animals, note changes in color of the congo-red-stained yeast. Congo red is an indicator, bright orange red above pH 5.0 and blue around pH 3.0. Be sure that you understand what is meant by pH. What can you deduce concerning changes in acidity within the food vacuoles? What is the significance of this? What pH changes occur in our own digestive tract as food passes through? You may wish to look up information on pH optima of enzymes in a textbook of biochemistry.

Further information about the change in reaction of food vacuoles may be obtained by staining Paramecia fed on their natural food with neutral red, which is red below pH 6, rose at pH 7, orange at pH 8, and yellow at pH 9. In staining, add a drop of 0.02% (or less) neutral red at the side of the cover-slip and draw the stain under.

REFERENCES

Papers by Phelps (1934) and Hetherington (1934, 1936) are concerned with the nutritional requirements of Paramecium and related ciliates and some of the material of these papers should be included in the report on this exercise. For methods of culturing ciliates and other protozoa see

Needham et al. (1937).
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Needham, J. F. et al. "Culture Methods for Invertebrate Animals." Comstock, 1937.
Phelps, A., 1934. Studies on the nutrition of Paramecium. Arch. f. Protistenkunde, 82:134-163.

EXERCISE 2

FEEDING AND DIGESTION IN AMOEBAE

Using Amoeba or its larger relative Pelomyxa (Chaos), observe the uptake of food; use of the ciliate Tetrahymena as prey provides the most striking example. It may be well to starve the amoebae for a day before the observations by placing them in culture fluid from which most of the food material has been removed by filtration or centrifuging. Note the formation of the "food-cup" as the prey is captured.

To observe the digestive process, place individual giant amoebae into drops of a crowded suspension of the reddish ciliate Blepharisma. It may be necessary to concentrate the latter by hand centrifuging. Observe carefully the killing of the prey. Does the vacuole show any changes in size as the ciliate is killed? Note the changes in the color of the pigment of Blepharisma, which is an indicator, being red in acid or neutral, colorless in an alkaline medium. If Blepharisma are unavailable, try other ciliates fed on Congo-red-stained yeast, as in Exercise 1.

Recent studies on the phenomenon of pinocytosis or "cellular drinking" in Amoeba and in tissue-culture cells suggest that this process may not be unrelated to the process of food-vacuole formation. Since it is probable that in the near future more information on these processes will be published, the student should read the reviews by Holter (1959) and Marshall, et al. (1959) in order to become acquainted with the interesting prospects in this field.

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Schaeffer, A. A., 1916. On the feeding habits of Amoeba. J. Exp. Zool., 20:529-548.

EXERCISE 3

FEEDING BEHAVIOR OF HYDRA

Although Hydra, our only common freshwater coelenterate, is probably extremely specialized in an evolutionary sense, its simplicity of structure and general availability make it a favorite experimental animal. It may be mass-cultured by the method of Loomis and Lenhoff (1956), using Artemia larvae as food.

A. STUDY OF FOOD-CAPTURE AND NEMATOCYSTS:

Coelenterates are characterized by the possession of nematocysts, used in the capture of food as well as for other purposes. Starve brown Hydra for a day or two, and allow them to become well relaxed; note the extreme length attained by the tentacles. Feed such Hydra by introducing among them small (pinhead sized) daphnids, strained from a thriving culture, or Artemia larvae that have been washed in fresh water. Record your observations on the capture and swallowing of the prey. By teasing apart a crustacean removed from the tentacles of its captor, and examining the pieces under the higher powers of the compound microscope, one can demonstrate the large stenoteles (penetrant nematocysts) embedded in the cuticle, and the small desmonemes (volvents) coiled tendril-fashion about the fine bristles. In tentacles of Hydra, observe the "batteries" of nematocysts in the ectodermal cells. Further information on nematocysts may be found in Exercise 4 of Section V.

B. THE CHEMICAL STIMULUS TO FEEDING BEHAVIOR:

Great interest attaches to the demonstration by Loomis (1955) of the role of reduced glutathione in eliciting the feeding response (although the use of the term "hormone" for this substance seems unfortunate). Attempt to feed Hydra on Daphnia or Artemia that have been dead for several hours. Compare the response with that obtained if such long-dead prey animals are presented after being moistened with the juice of freshly-crushed animals. Add reduced glutathione to a vessel containing relaxed Hydra so as to produce a final concentration of 1:1,000 or 1:10,000 (10^{-3} or 10^{-4}) glutathione. The solution should be fresh; otherwise the glutathione becomes oxidized and inactive. Observe the response to dead prey and to inert material presented to the Hydra.

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Loomis, W. F., 1955. Gluthathione control of the specific feeding reactions of Hydra. Ann. N. Y. Acad. Sci., 62(Art. 9):209-227.

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EXERCISE 4

FEEDING REACTIONS OF SEA ANEMONES

Anemones are excellent material for the study of simple feeding behavior. Young Metridium and Diadumene (=Sagartia) have proved most favorable, but other species may be used if they are properly attached. Metridium should be collected with a minimum of damage to the base, and set out, two or three to a fingerbowl, in cool clean seawater a day or two in advance to allow them to become well attached and relaxed.

A. THE RESPONSE TO FOOD SUBSTANCES:

Rub small fragments of clam or fresh liver in powdered carmine. Present a food object by placing it on the tentacles of the anemone, and observe the pattern of responses leading to ingestion of the meat. Repeat, but place the meat upon the oral disc. Test the coordination of response when the food is brought into contact with only one or two tentacles. Evaluate the roles of tentacles, disc musculature, and cilia in the ingestion of food.

After the ingestion of food by the anemone, observe through the transparent upper wall the activity of the acontia. Note the rapid movement and circulation of stained food material and the areas where food is actually taken up by the tissues (see Exercise 5, Intracellular Digestion).

B. THE REJECTION RESPONSE:

Having become familiar with the responses to food substances, test and compare the responses to such inert material as sand grains, bits of tinfoil, or filter paper dropped upon the disc. The reversal of cilia involved in the rejection response of Metridium is almost unique among metazoan animals, but do not overlook muscular responses. A

more searching examination of the rejection response may be made by using uniform bits of filter paper soaked in a diverse series of attractive or repellent substances (meat or clam juice, mucus, animal fat, acid, alkali, etc.).

Other problems may lead out from the above simple studies (which are, however, not brief if thoroughly worked out), such as those on nematocyst excitation (Exercise 4 of Section V). It frequently happens that anemones in the laboratory appear contracted and uncooperative in feeding experiments. The use of a small amount of mussel extract, as described by Batham and Pantin, may induce expansion. The importance of cool, adequately aerated seawater and of patience in dealing with such slowly-responding animals cannot be overstressed to the student starting work with anemones.

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EXERCISE 5

INTRACELLULAR DIGESTION

Many of the more primitive invertebrates, and some higher ones as well, utilize the process of intracellular digestion, whereby particulate food material is digested to the soluble end-products only after its ingestion by gastrodermal cells. The process is in contrast to that of extracellular digestion, in which foodstuffs are reduced to soluble digestion products by enzymes secreted into the gut, and only these soluble end-products are actually taken in by the absorptive cells. It should be clear that these processes are not mutually exclusive; for instance, extensive extracellular proteolytic digestion may precede particle uptake in coelenterates, and amylolytic enzymes are released into the lamellibranch stomach even though particles are ingested by cells of the digestive diverticula. This matter is well reviewed by Yonge (1937).

It would be best for the student to concentrate on but one of the following problems, utilizing additional information on the food-gathering process from related exercises.

A. HYDRA:

Feed brown Hydra (starved for a day or two) on small daphnids that have been allowed to swim and feed in a suspension of carmine or carbon particles. See Exercise 3 for references on feeding behavior. At intervals after the ingestion of the daphnids, fix specimens of Hydra in 10% formalin for hand sectioning, or in Helly's or Fleming's fluid for paraffin sectioning. Intervals of 1, 2, 8, 24 hours and 2, 4 and 6 days are suggested.

Hand sections may be mounted in glycerine for study. In addition, it may be revealing to follow the process of digestion in the living animal, using such methods as slight flattening under the cover-slip, or gentle teasing with fine needles. In this way the amoeboid activity of certain of the endodermal cells may sometimes be seen.

B. SEA ANEMONES:

This problem may be started as indicated in Exercise 4, in collaboration with the student studying feeding behavior of anemones. Feed an anemone on clam or liver ground up with powdered carmine. At intervals after feeding, relax the anemone thoroughly, using equal parts of sea water and isotonic magnesium chloride (73 grams of $MgCl_2.6H_2O$ per liter of fresh water). Full strength $MgCl_2$ solution may be used to complete the process. Dissect out the mesenteries and parts of the body wall, and pin them out for direct study of the sites of food ingestion as shown by the carmine. If desired, the tissues may be fixed in formalin and hand-sectioned for study in glycerine mounts, or sectioned in paraffin or Carbowax,* but immediate study of the fresh material is usually most effective.

C. PLANARIANS:

Planaria or other turbellarians, if starved for a few days, will readily consume fresh liver or hard-boiled egg yolk ground up with powdered carmine. Fix at intervals after feeding, and section by routine paraffin or freezing methods, or by embedding in Carbowax* and hand sectioning. Look for ingested particles in the cells of the gut. Fresh frozen sections may be stained with alcoholic Sudan III to show the presence of fat globules or fatty acid within the gut cells.

D. INGESTION BY AMOEBOCYTES IN MOLLUSCS:

Ingestion of particulate matter and oil droplets by amoebocytes in the gut and mantle cavity of bivalves has been reported, but the matter is one about which there are differences of opinion. The student who is interested should consult the papers of Yonge (1928) and George (1952).

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* See Exercise 6 in Section V.