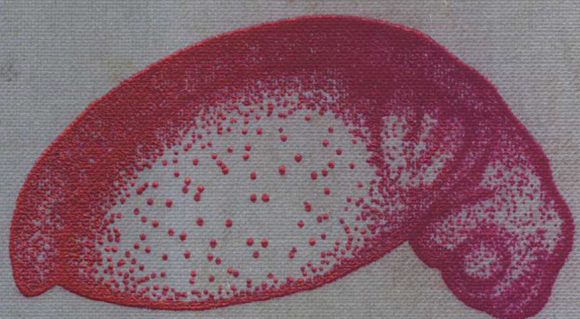
Roberts Rugh

Experimental Embryology



EXPERIMENTAL EMBRYOLOGY

Techniques and Procedures

by

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INTRODUCTION

The material of this "Experimental Embryology" represents many years of the most intense research on the part of innumerable embryologists, from all parts of the world. The author disclaims any originality except in those sections relating specifically to his particular investigations. The book is a compendium of data, directions, and references not generally found in textbooks, but information which is necessary in training the prospective experimental embryologist in the fundamentals of this relatively new and dynamic field of research.

There are contained herein some 50 separate experimental procedures, from Androgenesis to Xenoplastic Grafts, all of which have been tested in the course "Mechanics of Development" developed by the author while at New York University. The present, completely rewritten book incorporates all the improvements in the various techniques that have come to the author's attention. Each procedure is presented as foundational to some basic concept so that the qualified graduate student may be stimulated to pursue further research in the field. The approach is entirely experimental; the subject matter is exclusively the embryo.

The organization of each exercise is based upon the general plan of a publishable scientific manuscript. The usual historical background is omitted, and the discussion (if any) is limited because this is the function of related textbooks. The reference list contains only the most recent and pertinent papers, and certain review articles. Only occasionally there are included papers more than 15 years old, and these because they have been established as classics within the field. It is felt that interested investigators can acquire a complete bibliography through the references given.

It is Recommended that the Student's Report Include the Following:

1. EXPERIMENTAL PROCEDURE: Any modifications of the procedure as outlined.
2. EXPERIMENTAL DATA: This section must be complete in every detail.
3. DISCUSSION: This should be based upon "2" above.
4. CONCLUSIONS: These should be based entirely upon the findings of the student.
5. REFERENCES: Only new references which are not included in this exercise.

It would be impossible for any student, under any conditions, to complete the work outlined in this book during a single academic year. There are three solutions to this matter, all of which have been tried in our laboratory and any of which is satisfactory:

1. Assign a new procedure for each of the regular weekly laboratory sessions. This is a very heavy assignment and the student would necessarily spend more than the usual four hours per week in the laboratory. The plan has the advantage of making it simpler for the instructor to anticipate the needs of the entire class, from week to week. He can often schedule the procedures in such a way that they follow in a natural sequence and often conveniently overlap. The major disadvantage is that the student acquires only a passing acquaintance with the various techniques and is apt to assume that he is master of all of them.
2. Select a logical series of experimental procedures designed to be completed during the first semester, and progressing from the gross to the microscopic, the crude to the refined, the simple to the complex. There is no attempt to cover the entire gamut of techniques. The responsibility of representative selection falls on the instructor, but the student will be quite thoroughly grounded in the basic procedures, and will thereby be qualified later to pursue independent investigation. This has been

the most frequently followed program at New York Univeristy during the first semester. During the second semester the students have been assigned individual and original problems for investigation.

3. Assign some of the introductory procedures to the entire class, such procedures as "Induction of Ovulation", "Breeding and Care of Embryos", and "Temperature and Rate of Development". Then delegate each student to carry out two or three integrated procedures, with the responsibility of completed and thorough work later to be reported in full to the class. This plan deprives the student of experience in many of the techniques in experimental embryology, but it places upon him a responsibility to the entire class which often kindles the research attitude. By such a plan most of the exercises can be attempted by an average class of about 15 students.

A suggested sequence of exercises, which has been used at New York University, is given below. The assignment is based upon a weekly class session of about 4 hours, and supplemental time as may be required by the individual student.

1. INDUCTION OF OVULATION AND ARTIFICIAL FERTILIZATION

2. NORMAL DEVELOPMENT
   a. Relation of temperature to early development.
   b. Relation of osmotic pressure to early development.
   c. The appearance of behavior patterns.

3. EXPERIMENTS WITH THE EGG
   a. Germinal vesicle studies.
   b. Artificial parthenogenesis.
   c. Androgenesis.

4. EXPERIMENTS WITH THE CLEAVAGE STAGES
   a. The effect of unequal pressure on cleavage.
   b. The production of double embryos.
   c. The behavior of isolated embryonic cells.

5. EXPERIMENTS ON THE BLASTULA AND GASTRULA
   a. Vital staining and morphogenetic movements.
   b. The organizer.

6. EXPERIMENTS WITH THE NEURULA
   a. Parabiosis.
   b. Regeneration.
   c. Embryonic inductions in the blastema tissue.

7. EXPERIMENTS WITH LATER STAGES
   a. Wound healing.
   b. Hypophysectomy.
   c. Limb or eye transplantations.

This program would carry the student through about $1\frac{1}{2}$ semesters. There would remain about 2 months during which the instructor could direct the students in some of the more difficult techniques with either the fish or the chick embryos.

Through the very generous help of Dr. Jane Oppenheimer and Dr. Nelson T. Spratt, Jr. the sections on fish and on chick embryos have been expanded very considerably. It is believed that the traditional reluctance to use these forms is being broken down by the brilliant work of investigators such as these, and the laboratory of experimental

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embryology can no longer be limited to amphibian forms but will take in all embryos from the lowest to the highest phyla.

It would be impossible for the author to properly acknowledge all of the help that he has received in organizing this book. He has enjoyed universal and wholehearted cooperation, often entailing considerable time and effort on the part of contemporary investigators. Where figures, graphs, or photographs have been reproduced, and where specific investigators have constructively helped to organize certain sections, specific acknowledgements are made. It is with pleasure that the author acknowledges here the permission granted by The Wistar Institute of Anatomy to reproduce items from papers appearing in their various journals. It must be emphasized again that this book is made possible by the efforts of many experimental embryologists, many deceased, many active, and an increasing number "presumptive". If biology students who attempt these various procedures are thereby stimulated to make further contributions to the field of experimental embryology, all the effort expended in its compilation will have been justified.

Roberts Rugh

New York
September 1948

INTRODUCTION TO THIRD EDITION

This third edition of the Experimental Embryology appears simply as a direct response to the persistent demands on the part of a small but select group of advanced experimentalists who found the previous edition so useful that they began to make photostatic copies when the supply was exhausted.

Many of the old references have been deleted, and new ones added. New sections include the disassociation and reaggregation of cells; basic tissue culture techniques; and use of the mouse embryo. For the last decade the author has been studying the effects of ionizing radiations on the rodent (mouse and rat) embryos and he is convinced that the mammalian embryo can now be made available to all advanced biology students for important, rewarding, and exciting research. This chapter on the mouse embryo is merely an introduction to the possibilities ahead.

Most publishing houses are conducted on a cold and closely calculating business basis. For Burgess of Minneapolis to republish a book such as this, which can never have wide adoption because of its very specialized subject, is a generous gesture which, the author hopes, will stimulate many biologists to research in embryology.

Roberts Rugh

New York
February 1962

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"When it is recalled that the 9,200,000,000 cells in the human cerebral cortex are the nervous elements of this organ and that they collectively constitute rather less than a cubic inch of protoplasm, it seems almost incredible that they should serve us as they do. They are the materials whose activities represent all human states, sensations, memories, volitions, emotions, affections, the highest flights of poetry, the most profound thoughts of philosophy, the most far-reaching theories of science, and, when their action goes astray, the ravings of insanity. It is this small amount of protoplasm in each of us that our whole educational system is concerned with training and that serves us through a lifetime in the growth of personality."

- G. H. Parker

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I. TECHNIQUES IN EXPERIMENTAL EMBRYOLOGY

Equipment and Procedures in Experimental Embryology

GENERAL INTRODUCTION

"Biologically Clean": These two words should be the most frequently emphasized in any laboratory of experimental embryology, even as they are in any tissue culture laboratory. Glassware, instruments, solutions and hands must be "biologically clean" before any experimental results may be considered valid. The term means that, barring any experimental conditions imposed which might alter the situation, there is no possible contamination of the living material either by chemical substances, by living parasites or by harmful organisms such as bacteria or viruses. The experimental conditions should be such that any embryo, introduced into that environment, would be expected to survive. The following precautions, in the interest of biological cleanliness, are suggested:

1. Glassware: Regardless of the source of the glassware used, it should be thoroughly scrubbed with hot soap and water, rinsed in running tap water for at least 2 hours, rinsed in distilled water and air-dried either by or under the direct supervision of the person planning to use it. If the glassware is cleaned with the usual cleaning fluid (potassium dichromate saturated in 10% sulfuric acid) it must be thoroughly washed and rinsed for a longer period in order to remove the tenacious chemicals (Richards '36). Properly cleaned glassware may be wrapped in clean paper towelling and heat sterilized for 1/2 hour at 100° C. As long thereafter as the glassware remains wrapped it may be considered as sterile.

2. Hands: A surgeon usually spends as much time scrubbing his hands as in operating, and such cleanliness in experimental embryology will result in more dependable and reproducible results. Formaldehyde, osmic or hydrochloric acid fumes, adherent to the hands, will contaminate instruments and ultimately the embryos. Thin white surgeons rubber gloves are advised in tissue culture experiments.

3. Instruments: If the instruments have never before been used, they may be thoroughly washed, rinsed, and sterilized in the autoclave at 15 pounds pressure for 30 minutes; cooled; immersed in 95% alcohol until used. Dissecting instruments from other laboratories should never be "trusted". Each student should provide himself with a new set of stainless steel instruments (never chromium plated) and should keep them in a plastic tube, or in a cotton-filled box, to be reserved exclusively for his operational procedures with living embryos.

   It is often necessary to preserve eggs or embryos but the operating instruments should never come into contact with any fixatives. The student may use an unsterile or even contaminated set of instruments for the handling of such material to be preserved. Often eggs, embryos or even tissues may be introduced into a fixative with the flat end of a toothpick, thus avoiding the contamination of instruments. Once an instrument has come into contact with a fixative, it should thereafter be regarded as "contaminated" and not "biologically clean."

4. Embryos: Dead or dying embryos are probably the most common source of contamination of cultures, because they are infested with bacteria and necro-toxins. Ailing embryos should be isolated, and crowding should be avoided. The culture medium should, at all times, appear to be clear. Healthy embryos may be passed through several changes of sterile medium in order to free them of adherent bacteria. Some stages of development, particularly of aquatic forms, may tolerate brief immersion

in hypertonic salt solutions or dilute potassium permanganate, either of which appears to be bacteriocidal. Generally such treatment is unnecessary, and should be avoided, if other precautions are taken. Also, if the embryos are kept at the lowest but tolerable temperatures, and the dishes are covered, there is less likelihood of infections killing them. (See special directions for chick and mouse embryos)

The sooner the student insures personal and operative "biological cleanliness" the sooner will he obtain reproducible experimental data, and enjoy the experiences of experimental embryology. It is a complete waste of time and talent to allow contamination to invalidate experimental data.

THE EXPERIMENTAL PROGRAM

Every experimental program is built upon a foundation of facts, or theories, well supported by demonstrated facts, and must follow a very definite course if the results are to be reproducible and acceptable. It should help the beginning investigator to outline such a research protocol in order to instill in him the habit of rigid and complete planning. This does not imply that anyone can forsee the results of an experiment. Indeed, the research worker must at all times avoid the pitfall of prejudicial planning, of a program designed to prove rather than investigate some pre-conceived concept. A research worker, true to his opportunities, will be entirely open-minded to the consequences of his research. His planning should be designed to investigate an unexplored area, and his reward will be discovery, often in unpredictable directions.

The unplanned, or the unintelligently-planned research program, usually results in wasted materials, effort, and talent. The uninitiated investigator too often finds, at the conclusion of his program, that he has omitted some vital procedure or has tragically by-passed some fruitful corollary. Thus, as experience is accumulated, the initial research program will become much more specific, but as the research is in progress the ramifications become more varied. With experience, the results of genuine experimentation are more subject to prediction and more apt to open up new and wider vistas of challenge. The research worker does not, therefore, put his program into a machine and await the end results but rather designs his program on the basis of facts and theories, and is ever alert during the investigation for new challenges for an inquiring mind. The program should never be final, unalterable, but pragmatic and subject to change as new facts are revealed.

In light of the above, an outline is suggested which might be useful not only in planning an experiment but in reporting it for publication in a scientific journal. Again, this is only suggestive, but is offered as a protocol for complete research thinking and planning.

THE RESEARCH PROTOCOL

PURPOSE: Here the major purpose of the investigation should be succinctly stated. For example, it could be: "To determine the effect of temperature on the growth rate of the tadpole of the frog, Rana pipiens."

Such a statement gives immediately the one major variable, temperature and the test object or situation, growth rate, and, most important, the form used, Rana pipiens tadpoles. There can be no doubt by anyone that if the purpose is achieved, there will be established some correlation between temperature and growth rate.

But, one cannot always anticipate that results will be positive so that the purpose might be stated: "To investigate the possible relation between temperature and

growth rate of Rana pipiens tadpoles". Thus, if the investigation proves that no relation exists, even the negative data will be significant.

Further, one cannot anticipate that the results will be encompassed by the original statement of purpose. But the more specific and succinct is the original statement of purpose, the more clear-cut will be the entire research program.

MATERIALS: There are two major classes of material that may be used in experimental embryology, or any experimental procedure in biology, namely (a) Biological and (b) Physical and/or Chemical.

One should list species or strain of animal used, as well as age, sex, and numbers. If it is an embryonic stage, that should be properly defined.

One should list all of the chemicals and apparatus that are to be used so that anyone attempting to repeat this experiment can do so under identical conditions.

EXPERIMENTAL PROCEDURE: In this section one must outline exactly what he intends to do.

a. The controls.
b. The number, age, sex stage of development, or other conditions of the animals or embryos.
c. The single experimental variable imposed.
d. The method of collecting data.

THE CONTROLS: Every experiment, every research project, must include a "control" or "controls. It is difficult to conceive of a situation in which a control is not possible, and most experiments are not valid without adequate controls.

The control is the standard, the normal, the untreated organism or situation with which experimentally induced results are to be compared. One simply cannot evaluate experimental data without controls, without organisms or embryos exactly identical but without the imposed experimental variable. Only by direct comparison of an experimental situation with a control situation can we evaluate any deviations caused by the experimental variable.

The ideal control, in any animal experiment, is a genetically identical individual, if such is obtainable. Identical twins, derived from the same zygote by cell separation at the two-cell stage, would therefore be ideal in the sense that one could be regarded as the exact (genetic) biological duplicate of the other. One could be kept protected from the single variable of interest while the other could be subjected to it, to determine the effect. Isolation cultures among the protozoa or bacteria, resulting in clones which may be separated and followed, all derived from a single parent, would constitute ideal experimental material. This is because these unicellular forms have asexual reproduction, the progeny resulting from binary fission and therefore being identical with each other as well as with the single parent.

Among multicellular forms, and all embryos, this ideal situation is generally impractical so that we usually satisfy the condition of a control with litter mates, derived from the same parents and at the same time. Certainly these are not genetically identical, but nonetheless, they are more closely identical than any other possible combination of paired individuals.

Thus, while one's interest may be directed toward the effect of some experimental variable, he cannot even recognize his results if he does not have proper and adequate controls with which to compare. The CONTROL is absolutely essential to every experiment.

The novice might ask about controls for extirpation or transplantation experiments. Obviously, if an extirpation results in a specific organ loss, the control would be an extirpation from another region to determine whether there results a similar organ loss. Likewise, in transplantations there is the donor tissue and the location in the host field, either condition which must have a control to specifically

circumscribe potentialities. In these qualitative experiments large numbers of controls are not necessary, but similarly placed transplants of other donor material, or varied locations within the host area of the same anlagé, would constitute adequate controls. The function of the control is to prove positively that the results obtained could not have been obtained with any other set of circumstances.

THE NUMBER, AGE, SEX, ETC. OF ANIMALS USED: The results of many experiments are the more convincing the greater the numbers of experimentals and controls used, within certain limits. If every experimental animal shows a particular response never seen among the controls, then 10-20 controls and experimentals should suffice. However, if the differences between experimentals and controls are (calculated to be) small, then, on a purely statistical basis, larger numbers are necessary. In the latter instance the results are primarily quantitative, even though they may be concerned with qualitative differences. Thus, it is important to plan on a sufficient number of animals all used at one time. There are, for instance, seasonal differences even within a species, and also diurnal differences (cycles) in certain rhythmic functions, so that in order to obtain large numbers it is not safe simply to accumulate data from small numbers over an extended period.

The purpose of any experiment is generally to study the effect of a single variable, e.g., temperature. It is important therefore that all other physical and biological variables be eliminated between the experimental and controls, or at least be reduced and recognized. One variable among animals that is not always recognized is sex, though the differences are usually obvious! There are few biological experiments in which sex does not play a part, though sometimes a negative one. In general the female is the more hardy, the more resistant to trauma, etc. Another variable that must be recognized is age. Certainly the embryos of different ages are very different, so that one cannot put together data from temperature observations on the blastula, gastrula, and neurula stages of the frog tadpole, for instance. Another area of variation is in physiological activity, the more difficult to recognize. Thus, one must try to obtain a large number of identical animals or embryos; half of which may then be subject to the one experimental variable in order to determine the valid biological response.

THE SINGLE EXPERIMENTAL VARIABLE IMPOSED: The above statements emphasize the necessity of preparing the animals or embryos so that the results obtained are clearly due to the single variable imposed. If one is experimenting on temperature effects he must be absolutely certain that no other variable is involved. Aside from the few biological variables above mentioned, among the physical variables would be light, agitation, salinity, number of organisms per unit of space (concentration), etc. any one of which could easily becloud the results of the intended variable of temperature. Both the biological and the physical and chemical variables must be eliminated or so controlled that there remains but a single variable between the control and the experimental animals.

METHOD OF COLLECTING DATA: This will depend entirely upon the material, and the nature of the experiment. In any case it should be systematic and complete, so that anyone else could step in and complete the experiment at any point of progress. Dates should appear by every set of data collected, and every measurable or detectable bit of information should be recorded in a book which never leaves the laboratory. The practice of collecting data on scraps of paper, later to be transposed to the record book, is to be discouraged.

Sketches, accompanied by photographs, are very useful in qualitative experiments. But in every case the illustration must be fully labelled. No one can remember accurately such minute data for any length of time. When the data are all available it is well to reduce it to a table and then, if possible, to a graph.

Brief mention must be made here of the necessity in all scientific research for absolute honesty, to the minutest detail. One false entry or record, one faked operation or even incomplete data will ruin forever the reliability of all data collected by that individual. Science must be a body of knowledge based upon fact, as truly as the human mind can perceive it. One quickly discovers that facts in science are more exciting than any concoction of the human imagination.

DISCUSSION: In this section will appear all references to related or pertinent experiments and objective discussion of their meaning with respect to the findings of the data presented. The discussion should never be exhaustive, should never be a means to "padding" the paper, and only those papers of recent date should be included. It is presumed that intelligent readers will be familiar with the earlier works in the field, and through references included, other and earlier references become available. The author should freely admit disparities, and point toward further work that would be instructive.

SUMMARY AND CONCLUSIONS: The summary may be a part of the discussion, although some authors offer a single paragraph summary as the first of the items in the conclusion. In a proper discussion the points of a summary would naturally appear. The conclusions, if no summary is offered, should start with a very succinct statement of the project, animals used, and the general findings. Succeeding conclusion statements would then relate the newly proven facts. It should be remembered that often a reader will read the introduction and the conclusions, possibly look at the illustrations, in order to decide whether the material is of particular interest to him. This lends importance to these sections. However, it is a cardinal error to include in any summary or conclusions any statements not born out by the data of the paper.

REFERENCES: There are many ways of presenting this information and one should consult the Journal to which he intends to send the manuscript. In any case, a complete reference should include the names and initials of all authors, the date of publication, the complete title, and the detailed reference including the volume of the Journal and the inclusive pages. This is a very important section of any scientific report.

The general outline, therefore, for a manuscript would be as follows:

Statement of purpose
Materials and methods
Experimental data
Discussion
Summary and conclusions
References

EQUIPMENT AND INSTRUMENTS NEEDED BY EACH STUDENT IN EXPERIMENTAL EMBRYOLOGY

OPTICAL EQUIPMENT

Standard microscope with usual lenses; oil immersion optional.
Dissecting microscope (binocular):
Objectives 1.7 x and 3.5 x most useful, with 10 x oculars.
The U-shaped base should be removable for indirect lighting.
Microscope lamp: Spencer diaphragm most useful.
Heat absorbing flask: A 250 cc. round bottom Pyrex flask filled with distilled water and supported on vertical stand by screw clamp. This device will absorb the infra-red heat rays and also provide a means of concentrating the cold light.

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