Embryology Course Materials 1950

EMBRYOLOGY COURSE STAFF, 1950

I. Instructors

Donald P. Costello, Kenan Professor of Zoology, University of North Carolina, in charge of course.
Arthur L. Colwin, Assistant Professor of Zoology, Queens College
Charles B. Metz, Assistant Professor of Zoology, Yale University
James A. Miller, Professor of Anatomy, Emory University
S. Meryl Rose, Associate Professor of Zoology, University of Illinois
Albert Tyler, Associate Professor of Embryology, California Institute of Technology

II. Research Assistant

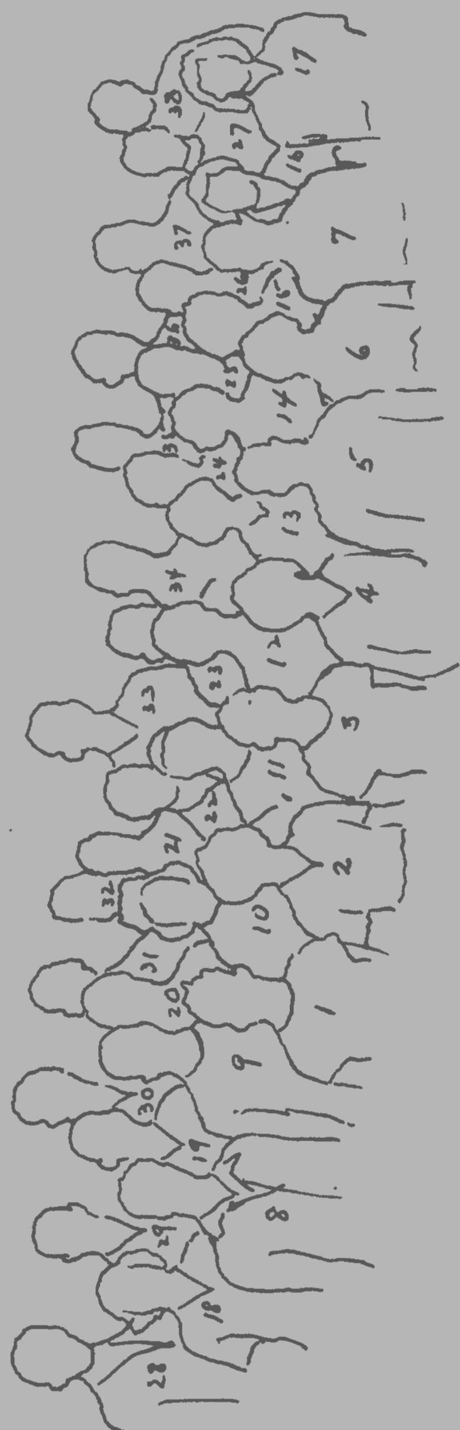
Margaret E. Davidson, McGill University

III. Laboratory Assistants

J. Bruce Guyselman, Northwestern University
Donald E. Kent, University of North Carolina

EMBRYOLOGY CLASS, 1950 - M. B. L.

Anderson, Irene Louise	Brown University (B.A., University of Toronto; M.A., McMaster University)
Babbott, Elizabeth	Connecticut College
Bernstein, Paul William	Wesleyan University (A.B., University of Massachusetts)
Carpé, Lestra	Sarah Lawrence College
Dunnebacke, Thelma Hudson	Smith College (A.B., M.A., Washington University)
Feightner, Lawrence Edward	University of Illinois (B.S., North Central College)
Fowler, Ira	Louisiana State University (B.S., Louisiana Polytechnic Institute; M.S., Louisiana State University)
Gamero-Reyes, Alonso	University of Michigan (M.S., University of Michigan)
Gravett, Howard L.	Texas A. and M. (A.B., James Millikin University; M.A., University of Illinois; Ph.D., University of Illinois)
Greengard, Paul	Johns Hopkins University (B.A., Hamilton College)
Jacobson, Eugene Donald	Wesleyan University
Konigsberg, Irwin Raphael	Johns Hopkins University (A.B., Brooklyn College)
Lansche, James McLaren	Washington University Medical School (A.B., Washington University)
Leavitt, Earle E.	Norwich University (B.S., University of New Hampshire)
Lindberg, Robert Gene	U.C.L.A. (A.B., U.C.L.A.)
Mabel, Judith	Goucher College
Magner, Bertha Ardys	Duke University (B.S., University of Miami)
McCullough, Kirk W.	Washington and Jefferson College (B.S., M.A., Washington and Jefferson College)
Neff, Ruth Hensley	University of Missouri (A.B., M.A., University of Missouri)
McKibben, Juliet Nancy	Carnegie Tech (B.S., Grove City College; M.S., University of Pittsburgh)
Pepper, Max Philip	Amherst College
Renzi, Alfred Arthur	Syracuse University (B.S., Fordham University; M.S., Syracuse University)
Sexton, Owen James	Oberlin College
Small, Jean Elizabeth	University of Massachusetts
Smithberg, Morris	University of Rochester (B.A., Brooklyn College)
Spiroff, Boris E.N.	Northwestern University (B.S., Loyola University; M.S., University of Chicago)
Van Breeman, Verne	State University of Iowa (B.S., Kletzing College; M.S., State University of Iowa)
Vogel, Philip H. (S.J.)	Loyola University (B.S., Xavier College)
Volz, Ruth	Brothers College, Drew University



Key

1. Pepper
2. Magner
3. Barnstein
4. Volz
5. Smithberg
6. Mabel
7. Greengard
8. Miller
9. Guyselman
10. McKibben
11. Feightner
12. Carpé
13. Neff
14. Anderson
15. Dunnebacke
16. Small
17. Babbott
18. Davidson
19. Rose
20. Colwin
21. McCullough
22. Renzi
23. Spiroff
24. Van Breeman
25. Gravett
26. Vogel
27. Gamero-Reyes
28. Kent
29. Tyler
30. Costello
31. Metz
32. Jacobson
33. Lansche
34. Leavitt
35. Fowler
36. Lindberg
37. Konigsberg
38. Sexton

EMBRYOLOGY COURSE, 1950

Schedule

Wed.	June 14	7:30 PM	Introductory Lecture	Costello
Thurs.	June 15	9:00 AM	Teleosts	Rose
Fri.	June 16		Teleosts	Rose
Sat.	June 17		Teleosts	Rose
Sun.				
Mon.	June 19		Teleosts	Rose
Tues.	June 20		Squid	Rose
Wed.	June 21		Squid	Rose
Thurs.	June 22		Fertilization	Costello
Fri.	June 23		Fertilization	Costello
Sat.	June 24		Cell lineage	Costello
Sun.				
Mon.	June 26		Echinoderms	Metz
Tues.	June 27		Echinoderms	Metz
Wed.	June 28		Echinoderms	Metz
Thurs.	June 29		Class experiments - echinoderms	Metz
Fri.	June 30		Class experiments - echinoderms	Metz
Sat.	July 1		Annelids	Miller
Sun.				
Mon.	July 3		Annelids	Miller
Tues.	July 4		Mollusca	Colwin
Wed.	July 5		Mollusca	Colwin
Thurs.	July 6		Class picnic	
Fri.	July 7		Coelenterata	Miller
Sat.	July 8		Coelenterata	Miller
Sun.				
Mon.	July 10		Tunicates	Colwin
Tues.	July 11		Tunicates	Colwin
Wed.	July 12		Chemical embryology	Tyler
Thurs.	July 13		Chemical embryology	Tyler
Fri.	July 14		Chemical embryology	Tyler
Sat.	July 15		Experimental period	Staff
Sun.				
Mon.	July 17		Experimental period	Staff
Tues.	July 18		Experimental period	Staff
Wed.	July 19		Experimental period	Staff
Thurs.	July 20		Experimental period	Staff
Fri.	July 21		Preparation of reports	
Sat.	July 22		Presentation of reports on group experiments.	

EMBRYOLOGY COURSE LECTURES

1950

Wednesday, June 14	Introductory remarks. Dr. Costello
Thursday, June 15	Teleosts: Introduction and normal stages. Dr. Rose
Friday, June 16	Teleosts: Localization. Dr. Rose
Saturday, June 17	Teleosts: Differentiation and organization (I). Dr. Rose
Monday, June 19	Teleosts: Differentiation and organization. (II). Dr. Rose
Tuesday, June 20	Squid: Normal development. Dr. Rose
Wednesday, June 21	Squid: Specializations. Dr. Rose
Thursday, June 22	Fertilization. I. Nereis limbata. Dr. Costello
Friday, June 23	Fertilization. II. Dr. Costello
Saturday, June 24	Cell lineage. Dr. Costello
Monday, June 26	Normal echinoderm development. Dr. Metz
Tuesday, June 27	Determination in the sea urchin egg. I. Dr. Metz
Wednesday, June 28	Determination in the sea urchin egg. II. Dr. Metz
Saturday, July 1	Normal development of the annelids. Dr. Miller
Monday, July 3	Remarks on the early history of the Laboratory. Dr. E. G. Conklin
Tuesday, July 4	Normal development of the molluscs. Dr. Colwin
Wednesday, July 5	Experimental embryology of annelids and molluscs. Dr. Costello
Friday, July 7	Factors influencing reconstitution in the coelenterates. Dr. Miller
Saturday, July 8	Patterns of coelenterate development. Dr. Miller
Monday, July 10	Normal and experimental analysis of development of the Tunicate egg. Dr. Colwin
Tuesday, July 11	a) Differential retardation and acceleration in Tunicate development. b) Asexual development. Dr. Colwin
Wednesday, July 12	Chemical embryology. Part I. Dr. Tyler
Thursday, July 13	Chemical embryology. Part II. Dr. Tyler
Friday, July 14	Chemical embryology. Part III. Dr. Tyler
Tuesday, July 18	Amphibian gastrulation and the "organizer". Dr. J. Holtfreter

SEMINARS

Monday, June 26	A study of the metabolism of amphibian neural crest cells during their migration and differentiation <u>in vitro</u>. Dr. Reed A. Flickinger, Jr., University of Pennsylvania.
Wednesday, July 12	Studies on the changes occurring in the protein systems of the sea urchin egg upon fertilization. Dr. A. Monroy, Rockefeller Institute for Medical Research
Wednesday, July 19	Embryonic induction in Amphibia. Dr. Johannes Holtfreter, University of Rochester

EMBRYOLOGY COURSE EXPERIMENTAL GROUPS - 1950

1. Developmental capacities of isolated blastomeres of the egg of Ilyanassa, with special reference to the effect of the polar lobe. Dr. Colwin

   Carpé   Volz
   McKibben

2. Effect of "conditioned" water and Cu on Ascidian tadpole metamorphosis. Dr. Colwin

   Small   Pepper

3. Development of isolated blastomeres of Nereis. Dr. Costello

   Gravett   Vogel
   Gamero-Reyes   Fowler

4. Studies on sperm extracts - action of antifertilizin and basic proteins on eggs and sperm. Dr. Metz

   Babbott   Neff

5. Effect of chemicals on animal and vegetal differentiation in the echinoderm egg. Dr. Metz

   Dunnebacke   Mabel

6. Experiments designed to test whether substances circulating in the coelenteron are necessary for reconstitution in Tubularia. Dr. Miller

   Feightner   Sexton

7. A study of apyrase activity in Tubularian reconstitution. Dr. Miller

   Anderson   Konigsberg

8. Tubularia: Test for circulation factor in dominance of distal over proximal regenerating regions. Dr. Rose

   Magner   McCullough

9. Fundulus (if available): Attempt to influence position of shield region by raising temperature locally just after fertilization and during cleavage. Dr. Rose

   Lansche   Bernstein

10. History of polar bodies and chromosomes in artificial parthenogenesis of Chaetopterus, Nereis and Mactra. Dr. Tyler

    Lindberg   Leavitt

11. Production of double embryos by KCl.

    Renzi   Smithberg
    Spiroff   Jacobson

12. Action spectrum of photosensitive shedding substance of Hydractinia. Dr. Miller

    Greengard

13. Muscle development in Styela. Dr. Colwin

    Van Breeman

EMBRYOLOGY CLASS - FINAL SEMINAR

9:00 AM, Saturday, July 22, 1950

1. The development of isolated blastomeres of Ilyanassa. Iestra Carpé

2. Studies on the effect of copper and other factors on ascidian tadpole metamorphosis. Max E. Lepper

3. A study of the technique of isolation of blastomeres in Nereis. Alonso Gamero-Reyes

4. Studies in basic protein and its action on sperm. Elizabeth Babbott

5. Effects of lithium-and sulfate-free sea water on animal and vegetal differentiation in Echinarachnius parma eggs. Judith Mabel

6. An attempt to determine whether circulating substances are necessary for reconstitution in Tubularia. Lawrence Feightner

7. Monophosphoesterases in reconstitution. Irwin R. Konigsberg

8. The circulation factor in dominance of distal over proximal regenerating regions in Tubularia. Kirk McCullough

9. Attempt to influence the location of the embryonic shield in the developing Fundulus egg by the local application of heat. James M. Lansche

10. The suppression of polar bodies and their relation to cleavage in artificial parthenogenesis of Mactra, Nereis and Chaetopterus. Earle E. Leavitt

11. Production of double embryos in KCl. Boris E. N. Spiroff

12. Action spectrum of photo-sensitive substance respnsible for maturation and shedding of the eggs of Hydractinia. Paul Greengard

13. The effect of colchicine and urethane carbamate on the viscosity of the Echinarachnius egg. Verne Van Breeman

Development of
Non-Pelagic (Demersal) Eggs
Type - Fundulus sp.

Breeding Season: Material is best and most abundant during the first three weeks of June but small numbers of fertilizable eggs can be procured through July 15.

Equipment:
Living Material-Fundulus heteroclitus and/or Fundulus majalis

Glassware, etc. -
General Class Equipment- 3 large aquaria
Individual Equipment - 3 clean finger-bowls (4 by 2")
3 glass plates to cover finger-bowls
2 syracuse dishes
3 ordinary pipettes and bulbs
1 fine-tipped pipette and bulb
paper toweling or filter paper
lens paper
2 syracuse dishes
hair loop
culture slide- 1.7-1.8 mm. depression
plain glass slides
thin sheet of mica
cover slips

Solutions - stock 0.5% Neutral Red solution
Dilute sea H_2O (70% sea H_2O, 30% fresh water)

Additional Reagents needed if eggs are to be fixed for sectioning or total preparations:
1- Total preparations:
Stockard's solution: formalin- 5 parts
glacial acetic - 4 parts
glycerine - 6 parts
distilled water - 85 parts

2- For Sectioning:
Bouin's or Zenker's Fluids
Graded series of Alcohols
amyl- acetate
56-58 degree paraffin

Technique of Preparing and Handling Material:
A. Care of Adults
Although fish are usually brought to the laboratory in mixed lots of males and females it is advisable to segregate the sexes to prevent spawning. Males and females should be placed in separate tanks until needed and after stripping removed to a discard tank. The sexes of both species of Fundulus are easily identified. The mature female F. heteroclitus is pale olive in color and usually possesses no distinct bars or spots, although the young females have indistinct, dark, transverse bars on the sides. The dorsal fin is non-pigmented. The adult male of this species is a dull, dark-green, the sides bearing narrow, ill-defined transverse bars

composed of silvery spots. The dorsal fin possesses black pigment arranged in a mottled pattern. The body markings of F. majalis are more conspicuous. The pale olive female has a pattern of heavy black longitudinal stripes on the sides and a non-pigmented dorsal fin. The sides of the slightly darker male bear a dozen broad, dark, transverse bars. The black patch on the dorsal fin is striking.

B. Procuring Gametes

Both eggs and sperm are procured by stripping. The fish is held firmly with the left hand while gentle pressure is applied to the abdomen using the thumb and forefinger of the right hand. As these fingers are drawn towards the anus the pressure forces out the gametes. If the fish is held against the light while stripping the eggs may be seen passing through the oviduct which runs along the anal fin.

C. Preparation of Cultures

Strip eggs into a clean finger bowl which has been moistened with sea water. Strip milt into a small amount of sea water, mix with eggs, and allow to stand in 1" of salt water. Neither eggs nor sperm should be allowed to stand before fertilizing. After 30-45 minutes change the sea water and leave the eggs in $\frac{1}{4}$-$\frac{1}{2}$". Keep bowl covered with a glass plate. Do not allow eggs to clump or accumulate in one spot. Label each lot with the exact time of fertilization. Change the water at least twice a day.

D. Methods of studying eggs

The eggs should first be studied in the condition in which they are spawned, but for many purposes it is desirable to prepare them for microscopic study as follows: roll the eggs on a piece of filter paper or paper towel until the jelly and the outer fibres are removed leaving the surface of the outer membrane smooth and clean. The same procedure should be followed for day-old stock cultures in order to prevent clumping of the eggs.

For experimental work where absolutely normal development is essential, eggs are usually examined uncovered in shallow depression slides and manipulated with hair loops. For laboratory study where eggs are to be observed over long periods of time and specific orientation is desired, either of the following methods is suggested: Place the eggs in sea water in culture slides having a 1.7-1.8 mm. depression (slightly less than the diameter of the egg). The egg may now be rotated by moving the cover slip. If these special slides are not available eggs may be placed in a drop of sea water on an ordinary glass slide and covered with a very thin, flexible sheet of mica. Water is then withdrawn with lens paper until capillary attraction causes a pressure on the egg and it may be rotated as in the previous method.

E. Permanent Total Preparations

Fix the eggs in Stockard's solution. This turns the protoplasm white but leaves the yolk transparent. The fixative may be used as a preservative or the material may be transferred to 10% formalin after 2 days.

F. Preparation of Eggs for Sectioning

Eggs must be dechorionated before fixation to allow fluids to penetrate the interior. (For details of this process see Nicholas, '27). The following method of embedding is that of

Barron '34, with some modifications of timing suggested by J. Oppenheimer.

1- Fix in Bouin's or Zenker's solution 12-24 hours.
2- Run up in ordinary manner through the alcohols leaving eggs in each through 95% for one hour.
3- Place in absolute for 2 hours-running through several changes.
4- Place in equal parts absolute and amyl-acetate for 2 hours.
5- Place in amyl-acetate for 24-48 hours.
6- Place in equal parts of amyl-acetate and paraffin and incubate at about 30 degrees for about 12 hours.
7- Transfer through three changes of paraffin (15 minutes in each) and embed in 56-58 degree paraffin.

Observations of Normal Development

1- The Unfertilized Egg: Strip the eggs from a female into diluted sea water (70% fresh water, 30% sea water). Keep them in this solution to retain the morphological characteristics observable at time of extrusion. Note the details of structures of the unfertilized ripe ovum. These include platelets, oil drops, protoplasm, membranes, micropyle, etc. (The micropyle must be observed before removal of chorionic jelly). If young ova are present compare with ripe ova.

2- The Sperm: Sperm may be stripped into sea water and a drop of the suspension examined under a cover slip under high power. Note the general structure and the enormous size difference between eggs and sperm.

3- Fertilization: Prepare a culture of fertilized eggs according to the method outlined in part C of the section on technique. Be sure to record exact time of insemination. Be prepared to transfer eggs immediately to a slide for observation. Record time of fading of platelets, of the formation of the the perivitelline space. What are your conclusions in regard to the rapidity of activation of the egg? If practicable, place a number of newly-extruded unfertilized eggs in a depression slide and partially cover the depression with a cover slip leaving uncovered a space large enough to permit the introduction of a fine pipette. Rotate eggs until the micropyle of one comes into view. Introduce a drop of sperm suspension into the depression without disturbing the cover slip and watch the entrance of the sperm and the spread of the fertilization reaction from the locus of the micropyle. Since polar bodies have not been seen in Fundulus it has not been determined at what stage of maturation the sperm enters the egg.

4- Formation of the Blastodisc: Note the gradual accumulation of the protoplasmic cap. This is the blastodisc or germ disc. Compare polar and lateral views. What is the relation of the pole of the egg to gravity? How does this compare with the condition in the frog egg; with the chick egg? Do any processes take place in the unfertilized egg similar to those in the fertilized egg?

5- Cleavage: Watch for the appearance of a groove on the surface of the blastodisc- the indication of the first cleavage plane. This usually occurs within 2-3 hours after fertilization. Note the geometric relation, and time sequence of the subsequent cleavages. Do the cleavage planes divide the entire ovum? The entire blastodisc? During interkinesis the nuclei are sometimes visible. Distinguish between central and marginal cells. Follow cleavage carefully to the 32 cell stage. Note irregularities. When do horizontal cleavage planes first occur? Does the blastodisc increase in size or alter in form? Note that cleavages continue for a considerable period without much change in form from that of the original blastodisc. This is called the period of the high blastula. When does the change of form to the flat blastula occur? (See Oppenheimer '37 for chronological terms).

6.- Time Table of Development: The rate of development varies with temperature and other external environmental conditions. The approximate developmental stages which may be expected under various conditions of temperature and salinity may be seen in chart 1. The stages are numbered according to the chronology established by Oppenheimer '37.

7.- The Periblast: (Appears 16-24 hours after fertilization) The uncleaved protoplasm around the margin of the group of blastomeres is called the marginal periblast; that beneath the blastodisc (only visible in sections) the central periblast. In the late blastodisc (16-20 hours) observe particularly the behavior of the marginal cells and distinguish between circular and radial cleavages. The large pinkish nuclei of the periblast are easily visible. Note how the nuclei of the marginal row of cells become free of cell outlines, continue their divisions and migrate into the marginal periblast, converting it into a nucleated but non-cellular structure. Follow the periblast structure in later stages.

8.- The Germ Ring and the Extension of the Blastodisc (18-48 hours) Subsequent to the nucleation of the periblast note the change in form and size of the blastoderm. The embryo is now referred to as a bastula. Soon the margin of the disc appears relatively thicker. This thickening is termed the germ-ring and is due both to an actual peripheral increase in cells and to a thinning of the central part of the disc. This germ ring can best be observed in F. majalis. During the next few hours the germ ring grows completely over the surface of the yolk mass. The uncovered portion of the yolk is the blastopore. The final covering of the yolk or the closing of the blastopore occurs after the first stages of the formation of the embryonic axis. Under favorable conditions the beginning of gastrulation may be observed in the appearance of a slight indentation at the edge of the germ ring at the time when the yolk is about ¼ covered. Staining with neutral red will aid in identification of the germ ring and periblast. (Add 1 or 2 drops of stock solution to a syracuse dish of sea water).

9.- The Formation of the Embryo (Begins in 24-36 hours) While the germ ring is extending around the yolk the embryonic axis is being established. Its first indication is a cellular thickening known as the embryonic shield caused by a more active movement of cells in one part of the germ ring. This formation is initiated when the blastoderm has covered from one-quarter to one-third of the surface of the yolk. By the time that the blastoderm has covered about one-half of the yolk the embryonic shield has become a bluntly triangular area extending from the margin of one portion of the blastoderm to near the center of the blastoderm. The shield can best be identified in profile view. As the blastoderm spreads over the surface of the yolk the embryo grows rapidly in length.

Observe the whole egg in profile view, so that the embryo is seen in sagittal optical section, when the yolk is 1/3, 1/2, 2/3, and 3/4 covered. What proportion does the length of the embryo bear to the diameter of the blastoderm and to the length of the germ ring in each of these successive stages?

After the yolk is 7/8 covered, look for a large clear vesicle near the hind end of the embryo. (Do not confuse this with a cluster of small oil drops frequently found in a similar position). This is Kupffer's vesicle.

During this period the embryo becomes segmented. This segmentation is confined to the mesoderm which lies on each side of the axis of the embryo forming mesoblastic somites. How many somites are present at the time of the closure of the blastopore?

Look for the notochord. Study it in longitudinal and transverse optical sections. Where does it terminate anteriorly and posteriorly?

10. Later Development Obtain series of embryos of 2,3,4,5,6 days and make a detailed comparative study. It is suggested that drawings be made at 24 hr. intervals and that a chart be made showing the first appearance and later development of the organ systems. The following method of chart construction may be used:

	Days after fertilization				
	2	3	4	5	6
Somite Number					
Nervous System					
Brain Divisions					
Eye					
Otocyst					
Olfactory Pit					
Circulatory System					
:					
:					
:					
etc					

If you are unfamiliar with the form of chronological charts good examples may be found in the following texts:

Lillie, F. R. 1940 The development of the chick. New York: Holt. (opposite page 68)

Arey, L. B. 1940. Developmental anatomy, Philadelphia and London: Saunders. (opposite page 148)

It is suggested that embryos be removed from their chorions in the later stages, for better observation of structural details. Although this is difficult to perform in early stages and requires special instruments (See experimental section, page...) in later stages the loose chorion may be torn off with sharpened forceps, or with the beading needles. Be careful to avoid injury to the yolk sac.

The following developmental features should be observed and incorporated into drawings and chart;

a. Somites. Note first appearance and determine numbers formed on successive days.

b. Nervous sytem Find in early stages the optic vesicles, lens neuromeres, mid-, fore-, and hind-brain regions and trace the development into cerebrum, optic lobes, cerebellum, medulla, etc. Study the development of the eye, olfactory pit and otocyst. How is the lens of the eye formed?

c. Circulatory system. Note the extra-embryonic body cavity; formation of the pericardium; first blood vessels (and especially their mode of formation from wandering mesenchyme cells); the first action of the heart; form and position of the heart. Compare the course of circulation on the 4th and 6th days. Consult chart No. 2 for an outline of the development of the circulatory system.

d. Mesenchymal cells. Note wandering mesenchymal cells, especially abundant beneath the posterior part of the embryo on the 2nd, and 3rd days. Can you distinguish various types? By successive observations at brief intervals, the change in form, migration, and division of these cells may be noted.

e. Note first appearance of fins; of the urinary vesicle or bladder (a bi-lobed outgrowth of the hind-gut); the liver posterior to the left fin bud.

11. After Hatching. The young fish may be studied just after hatching by anaesthetizing with chloretone. Consult paper by Oppenheim '37 for further details of developmental stages.

Bibliography
DEVELOPMENT OF THE TELEOST

This bibliography contains only a few of the older papers. (for more complete references see general works and bibliographies in papers here listed)

1. General Reference Works

Brachet, A. 1935 Traite d'embryologie des vertebres. 2nd ed.
Oppenheimer, J. M. 1936 Historical introduction to the study of teleostean development. Osiris, 2, 1936.

11. Special References on Descriptive Embryology

Armstrong, P. B. 1936 Mechanism of hatching in Fundulus heteroclitus Bio. Bull. 71.
Sumner, F. B. 1900 Kupffer's Vesicle and its relation to gastrulation and concrescence. Mem. N. Y. Acad. Sci., 2.
-------- 1904 A study of early fish development, experimental and morphological. Arch. Entw. 17 92-119.
Stockard, C. R. 1905 A study of wandering mesenchymal cells on the living yolk sac. Am. Jour. Anat. 18.
Wilson, H. V. 1891 The embryology of the Sea bass (Serranus atrarius) Bull. U. S. Fish Com., 9.

111. Stage Series

Oppenheimer, J. M. 1937 The normal stages of Fundulus heteroclitus Anat. Rec. 68
Solberg, A. N. 1938 The development of a bony fish (Fundulus) Progr. Fish. Culturist, and Dept. of Comm., Bureau of Fish. Washington, D. C.

1V. Reproductive Cycle and Breeding Habits

Craig-Bennett, A. 1931 The reproductive cycle of the three-spined stickle-back, Gasterosteus aculeatus, Linn. Phil. Trans. Roy. Soc. Lond., B 219, pp 197-279
Newman, H. H. 1907 Spawning behavior and sexual dimorphism of Fundulus heteroclitus and allied fish. Biol Bull. Vol 12

V. Circulatory System

Armstrong, P. B. 1931 Functional reactions in the embryonic heart accompanying the ingrowth and development of the vagus innervation. J. E. Z., 58.
Stockard, C. R. 1915 An experimental analysis of the origin of blood and vascular endothelium. Mem. Wistar Inst. Anat. Biol. No. 7 Also Am. Jour. Anat. 18.

V1 Germ-Cells

Okkelberg, Peter 1921 The early history of the germ cells in the brook lamprey, Entosphenus wilderi (Gage) up to and including the period of sex differentiation. Jour. Morph., vol 35 (This paper has a complete bibliograph of work on germ cells in other groups)

Richards, A. and J. I. Thompson, 1921 Migration of primary sex cells of Fundulus Biol. Bull. vol. 40

Vll Experimental work

Child, C. M. 1943 Oxidation-reduction pattern in development of a Teleost. Physiol. Zool., vol. 16

F[illegible]in, A. N. 1939 Regional determination in the development of the trout. Arch. Ent.-Mech. 139

Kagan, B. M. 1935 The fertilizable period of the eggs of Fundulus heteroclitus and some associated phonomena, Biol. Bull. vol. 69.

Luther, W. 1936 Potensprufungor an isolierten Teilstucken der Forellenkeimscheibe. Zool. Anz., Suppl. 9

__________ 1936 Austausch von prasumptiver Epidermis und Medullarplatte bein Forellenkeim. Arch. Ent. Mech. 135

Nicholas, J. S. 1927 The application of experimental methods to the study of developing Fundulus embryos. Prof. Nat. Acad. Sci 43: 695-698

Nicholas, J. S. 1942 Regulation and reconstitution in Fundulus J. E. Z., vol 90, 127

Oppenheimer, J. M. 1936a The development of isolated blastoderms of Fundulus heteroclitus. J. E. Z., vol. 72

__________ 1936b Transplantation of experiments on developing teleosts (Fundulus and Perca) J.E.Z., vol. 72.

__________ 1936c P rocesses of localization in developing Fundulus J. E. Z. vol. 73.

__________ 1938 Potencies for differtiation in the Teleostean gorm ring J. E. Z., vol. 79

__________ 1939 The capacity for differentiation of fish embryonic tissues implanted into amphibian embryos J. E. Z. vol. 30

Pasteels, Jean 1936 Etudes sur la gastrulation des Vertebres mero-blastiques l. Teleosteens, Arch.Biol. vol. 47.

__________ 1940 Un apercu comparatif de la gastrulation chez les Chordes. Biol. Rev., vol. 15.

Spek, J. 1935 Die biplare Differenzierug des Protoplasmas des Teleosteeneies und ihre Entstehung. Protoplasma, vol. 18.

Stockard, C. R. 1910 The effects of alcohol and other anesthetics on embryonic development. Am. Jour. Anat. 10.

Vandebrock, G. 1936 Les Mouvements morphogénetiques au cours de la gastrulation chez Scyllium Canicula Cuv. Arch. de Biol., 47, pp 499-584

Weissenberg, V. R. 1934 Untersuchungen ueber den Anlagephlan beim Naunaugenkeim; Mesoderm, Rumpfdarmbildung und Ubersicht der centralen Anlagezonen. Anat. Ans., 79.

Vlll Hybridization

Bancroft, F. W. 1912 Heredity of pigmentation in Fundulus hybrids. J. Exp. Zool., vol. 12

Morris, Margaret 1914 The behavior of chromatin in hybrids between Fundulus and Ctenolabrus. J. Exp. Zool., vol.16

Newman, H. H. 1918 Hybrids between Fundulus and mackerel. J. Exp. Zool., vol 26 (See this for reference to other paper by same author)

IX Technique

Barron, Donald H. 1934 Amyl Acetate: a useful solvent for embedding masses. Anat. Rec., 59 (1), and Supp.: 1-3.

Technique of Handling Pelagic Eggs

Type - Tautogolabrus adspersus, the American cunner or Chogset

Whenever possible, observations should be made on pelagic eggs as well as on the demersal eggs of Fundulus. Many structures such as the germ-ring, embryonic shield, and Kupffer's vesicle will be easier to see in the former because they have fewer oil globules and a less granular protoplasm. The formation of polar bodies may also be seen in this type of egg. Pelagic eggs are far more sensitive, however, in their oxygen requirements, so require careful handling.

Pelagic eggs may be obtained from the scup (Stenotomus chrysops Linn.) and the mackerel (Scomber scombrus, Linn.), but must be stripped and fertilized as the fish are taken fresh from the live car. The cunner will prove far more useful, particularly for the study of early stages, for it may be brought to the Laboratory aquaria and stripped as needed.

Cunners should be caught the same day as needed; females are ordinarily obtained only after 12 M. The male has a somewhat brighter green color and can also be distinguished by its bright red cloacal lining epithelium. Milt is stripped into a large finger bowl which contains sufficient sea water to cover the bottom. Eggs are stripped into a separate bowl containing a small amount of sea water. It will prove helpful to use a cloth towel for holding the fish while they are being stripped, because they a re extremely slimy and have sharp spines in the dorsal fin.

As soon as possible after stripping, the sperm-suspension should be poured into the egg dish and the time recorded. Let the mixture stand for half a minute; then dilute with sea water and decant into smaller finger bowls, or pour into a cylinder or Erlenmeyer flask and add sufficient sea water to fill. Good eggs will float to the top and collect principally at the edge of the meniscus. They should be pipetted off and placed in covered finger-bowls containing $\frac{1}{4}$" of clean sea water, and s et in the sea water table where they will keep cool.

Only glass-clear eggs are suitable for study; if the eggs show the slightest opacity they are either immature or dead. If bits of tissue are clinging to the egg, it is immature and should be discarded. The perivetelline space appears immediately after fertilization; time should not be wated in observing eggs which do not develop such a space within a few minutes. For observing the formation of polar bodies, the blastodisc, and early cleavage, it is advantageous to place the miscroscope in a horizontal position so that the blastodisc may be observed in profile. It is difficult to see the polar bodies by any other method. They appear 5 to 10 minutes after fertilzation as small clear beads on the surface of the blastodisc. Clevage is rapid, occuring approximately once every 20 minutes at a temperature of 16-18°C. The cleaving eggs are crystal-clear, there are no obscuring oil droplets, and the nuclei appear as pinkish objects for a short time between divisions. If neutral red is used for outlining the cleavage spindles, it should be extremely dilute. Too much stain will stop cleavage or make the pattern irregular.

Cunner embryos may be kept alive for several days if care is taken not to crowd them and the water is changed frequently. It is suggested that not more than 3 to 6 eggs be placed in each finger-bowl, that they be transferred to clean sea water morning and night, and that the bowls be covered and kept in the sea water table. Opaque (dead) eggs should always be removed. The embryos usually hatch within 4 or 5 days after fertilization.

Stage of Development

	11	12	13	14	15	16	17	18	19	20	21	22	23
15°C													
Sea water	1			2			3			4	5	6	
1/2 sea 1/2 distil.	1			2		3			4	5	6	7	8
Tap water		1				2			3			4	
Dist. water	1		2		3		4	5	6		7		9
20°C													
Sea water		1							2			3	
1/2 sea 1/2 distil.		1						2		3			4
Tap water				1				2			3		
Dist. water		1					2			3			4
25°C													
Sea water					1						2		
1/2 sea 1/2 distil.				1					2				3
Tap water						1						2	
Dist. water					1						2		
[illegible]°C													
Sea water								1					2
1/2 sea 1/2 distil.								1					2
Tap water								1					2
Dist. water								1				2	

A table to show the number of days (24 hrs) required by *Fundulus heteroclitus* embryos to reach different stages of development from the blastula to the formation of the swim bladder just before hatching under varying conditions of temperature, salinity, etc.

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24	25	26	27	28	29	30	31
	8	9	10	11	12	13	16
[illegible]	10	[illegible]	12	13	17	20	23
	5	6	7	9	10	11	13
10	11	12	14	16	19	23	
4		5	6	7	8	10	12
	5		6	7	9	12	14
4		5		7	8	10	11
	5	6		7	9	11	14
		3	4	5	6	7	8
		4		5	6	7	8.5
	3		4	5	6	7	8
3		4	5	6	7	8	9.5
		3			4	5	6
		3		4		5	6.5
			3		4	5	6
		3		4	5	6	7

1947

Development of the Cephalopod Egg

Type - Loligo pealii, the common squid

Breeding Season: Females with mature eggs are available at Woods Hole from June through September, although the majority are spent by September.

Living Material - Loligo pealii, mature males and females.
Egg strings of Loligo pealii containing all the developmental stages

Demonstration Specimens- Dissected specimens of adult male and female squid which show the adult structures.

Equipment:

General Class Equipment -	Aquaria in which to keep adults and egg strings.
Individual Equipment -	clean finger bowl 2 watch glasses 1 large glass dish or finger bowl several syracuse dishes pair of coarse scissors 2 pair forceps hair loop 2 fine beading needles annealed into glass handles pipettes and bulbs, fine-tipped and wide-mouthed several depression slides ordinary microscopic slides and coverslips
Solutions, etc. -	concentrated (40%) formaldehyde Ehrlich's Triacid Stain distilled water vaseline

Technique of Preparing and Handling Material:

A. Care of Adults

If adults are transported without undue disturbance from fish traps in live cars with large amounts of water and transferred to large aquaria with a good supply of running water they will live for a few days in the laboratory.

The sexes are distinguishable. The testis of the male shows white through the mantle at the posterior end. The females are usually smaller than the males, and are shorter and broader. The females often have a small pocket of white sperm in the collar between the head and the free edge of the mantle. The accessory nidamental glands of the female are red during the breeding season and can usually be seen through the mantle.

Adult squid are used for obtaining gametes for studies of artificial fertilization, the formation of polar bodies, and cleavage. The male also provides spermatophores for subsequenty study.

B. Procuring egg strings

Stages showing organ formation are more readily obtained from naturally laid egg strings. These strings can be collected at low tide along the sandy beaches of Nonamessett Island. The clumps of strings are found attached to submerged objects in the shallow water. They can be kept in aquaria with running sea-water. The egg strings containing the older stages are usually darker and more weathered in appearance.

C. Procuring Gametes

Open male and female by making a longitudinal section (use large scissors) through the mantle from the siphon to the tip, cutting along the posterior (funnel) side. Remove the ink sac.

In the female - tear the thin wall of ovary with forceps and shake all transparent eggs into a finger bowl of water. If eggs are fully mature they separate readily from the ovary and appear as beautifully transparent as glass. Immature eggs are not transparent and will not develop.

In the male - pick up the bundles of spermatophores at the opening of the sperm duct, transfer them to a watch glass. The spermatophores will explode when placed into sea water; a concentrated sperm suspension will thus be obtained.

Note - If males are not available the sperm in the sperm receptacle of the female may be used.

D. Preparation of Cultures

Obtain gametes as directed in section above. Place eggs in a finger bowl and add several drops of sperm suspension. After 20-30 minutes transfer to a large dish filled with clean sea water and do not disturb for 2 1/2 - 3 hours. Keep in sea water table and change water at least twice a day.

E. Removal of Embryos from strings

Remove an egg string to a syracuse dish. Using the beading needles in the manner of knives cutting against each other, cut it in half. Place left hand needle so that the pressure forces several embryos clear of the jelly at the open end of one of the halves. Keeping this needle in place, puncture the chorion of one of the eggs with the tip of the right hand needle. Tear the chorion with a sharp jerk. The pressure of the enclosed fluid will pop the embryo from the membrane. When the exposed row of embryos has been removed, cut off the empty jelly and repeat the process. If the eggs are not first forced clear of the jelly they are difficult to remove without injury. This method can be used on all embryos, though the younger stages are more difficult to remove.

F. Methods of Studying Artificially Fertilized Eggs

For short observational periods eggs may be studied in depression slides and manipulated with the hair-loop. To obtain a polar view of the cytoplasmic cap which alone will undergo cleavage it is necessary to mount the eggs in an upright position. Place a small amount of vaseline in a depression slide, fill the latter with water and mount the eggs with a hair-loop so that they stand up.

G. Preparation of Intact Spermatophores for Study

1) Transfer some unexploded spermatophores quickly into conc. (40%) formaldehyde; fix for 10 minutes. (They will explode in a weaker solution).

2) Rinse with distilled water several minutes. The transfer from formaldehyde to distilled water must be gradual.
3) Stain with Ehrlich's Triacid for 5-10 minutes. The stain is made by diluting the stock solution: 6 drops to 8 cc of distilled water (8 cc. fills a syracuse dish about half full)
4) Rinse stain off with distilled water and put the spermatophores on slide under cover slip.

D. Preparation of Whole Mounts and Sections

Because of the large amount of yolk which they contain, squid embryos have a tendency to be friable and difficult to section especially in younger stages. The amyl acetate technique may be used (see Teleosts) or the dioxan technique as outlined below:

1. Fix the embryos in Bouin's solution. (If the embryo is highly motile it should first be anesthetized in sea water containing chloretone before being dropped in the fixative).
2. Transfer the embryos from the fixative into pure dioxan. Change to fresh dioxan at hourly intervals (2 changes) until they have been in dioxan for 3 hours.
3. Transfer to pure paraffin for 1 hour; change to a fresh paraffin bath for a second hour, and then to a mixture of paraffin containing 8 to 10% bayberry wax for a third hour.
4. Embed in paraffin-bayberry wax.
5. Section at 5 or 8 microns and stain with Heidenhain's haematoxylin or with Arnant's triple stain.

Observations of Normal Development

1. The Unfertilized Egg. Study mature eggs taken from the ovary of the squid. The mature egg is surrounded by a transparent chorion which is closely applied to it. At the pointed end, find a depression and a minute canal extending entirely through it. This is the micropyle. Note the polarity (blunt and pointed poles) and the bilaterality of the egg by turning it over. The more convex side of the egg is the future "anterior" or mouth side of the embryo. Note the thin cytoplasmic cap covering the yolk at the pointed pole. Study the extent of the cytoplasmic cap by rolling the egg. This cytoplasm will give rise to the embryonic structures.

Make a drawing of the egg and the chorion.

Study a normally laid egg string. The eggs are embedded in a gelatinous matrix which is produced by glands of the oviduct, and covered by a jelly membrane produced by the nidamental glands. Are the eggs wound spirally around a central core? Compare with the structure of the sperm mass below (2).

2. The Spermatophore. The excellent papers of Drew (1911 and 1919) and their illustrations should be consulted for all details.

Obtain spermatophores (see section C of Technique) and watch their explosion and the ejaculation of sperm.

Prepare some intact spermatophores for study (Section C of Technique). Observe the following structures:

1. The opaque sperm mass in the center, surrounded by the inner tunic.
2. The flask-shaped cement body in front of it.
3. The sprial filament in front of the cement body.
4. The outer tunic is the outermost layer of the entire envelope.

5. The cap and cap thread at the smaller tip end.
6. The middle tunic which may be slightly swollen.
7. A liquid space around the sperm mass, lined by middle and inner tunics.
8. Of the three "membranes" which are formed around the ejaculatory apparatus and inside the "tunics", the middle membrane can be most easily identified. It is relatively thick and extends from the cement body to the cap. It is fastened to the outer tunic at the cap end. This fusion will never break during the process of explosion; but the entire contents of the capsule will evaginate at this point. (cf. diagrams of Drew, 1919).
9. The outer membrane begins also at the cement body. It is so closely applied to the inner tunic that it is difficult to distinguish between them. The oral end of the inner tunic and outer membrane can be easily identified as a thickened ring around the middle membrane, at a short distance from the cap.

The evaginated inner tunic and outer membrane will form the sperm reservoir after the explosion. The sperm reservoir is closed at one end by cement from the cement body and open at the other end (see under 9). The sperm, mixed with a gelatinous mass, will ooze out slowly in a cloud; this will continue for hours or days. All other structures are left behind after explosion.

3. Fertilization and Maturation. Artificially fertilized eggs are more favorable for the study of the first phases of development than are those laid by the female because they lack the jelly envelopes. Fertilization and cleavage can be readily observed in this way. However, these eggs are very sensitive and must be kept in a large volume of water. Inseminate a watch glass of eggs and transfer a few eggs immediately into a depression slide (see sections C, D, and F of Technique). Observe the fertilization under high power. Note the penetration of a sperm through the micropyle. After a short time, the cytoplasmic cap will withdraw from the chorion, and a clear perivitelline space will appear, which indicates that fertilization is taking place.

The first polar body appears about 20 minutes after fertilization. Observe and time the appearance of the second polar body and the further divisions of the polar bodies. (See Hoadley, '30).

4. Cleavage. Mount 6-12 eggs in an upright position in a depression slide (See section F of Technique). Use eggs which have both polar bodies formed, and observe them at short intervals.

Observe the first cleavages and note their relation to the axes of symmetry of the egg. The first cleavage plane coincides with the median plane of the future embryo. (Consult the figs. of Watase, I, 1891). The cleavage is meroblastic, and not spiral in contrast to other molluscan eggs.

5. Time Table of Development. There is considerable variation due to temperature differences, and the following table gives only a rough approximation of the times at which certain stages are reached.

Time after Fertilization	Stage
20 min.	1st polar body
1 hour	2nd polar body
3 hours	1st cleavage
12 hours	Blastoderm over top of egg
24 hours	"Gastrula", thickened peripheral ring
2 days	Blastoderm half way over the egg
3 days	Blastoderm nearly covering the egg
3 1/2 days	Appearance of shell gland and eye stalks
5 1/2 days	Siphonal folds and arms appear, eyes project
6 1/2 days	Siphonal folds fused into a tube, eye stalks prominent
11-12 days	Hatching

C. Spreading of the Blastoderm. Study eggs about 24 hours after insemination. Later blastodermal stages may be obtained from egg strings. Note the gradual extension of the blastoderm about the yolk. The "blastocones" which are supposed to give rise to the yolk epithelium are not very distinct in Loligo. Note the thickening of the margin of the blastoderm - the formation of the antomesoderm (gastrulation).

D. Organ Formation. It is convenient to call the pole where the shell gland and mantle appear "dorsal" and the yok pole "ventral" The siphon side "posterior" and the mouth side "anterior", although these designations are not correct from a comparative anatomical point of view. Study a sequence of at least 6 stages as represented on chart... Study embryos from all sides. Make drawings of different stages. Note:

A) Early Stages (Chart figs. 1 and 2). cf. also text books of MacBride and Korscholt.
   1) Shall Gland at dorsal pole
   2) Mantle Primordium, an ectodermal concentric fold beneath the shell gland. (fig. 2)

   On the anterior side:
   3) Mouth
   4) Eye primordia - ectodermal invaginations.

   On the posterior side
   5) Anterior and posterior siphonal folds. The former are the primordia of the siphon; the latter will form its retractor muscles.
   6) Statocysts.
   7) Gill primordia

   At the boundary of blastoderm and yolk
   8) Primordium of the anus
   9) Note the rhythmical contractions of the yolk epithelium. They serve the purpose of circulating the liquefied yolk material in the yok-sac vessels. The material is carried into the embryonic tissues in this way. (See Portmann, 192 6).

B) Medium Stages (Chart figs. 3 and 4). Note the gradual constriction of the yolk sac. The latter continues into the embryo which is thus formed around a core of yolk-mass,

1) Observe growth of mantle and fins. (The shell gland is meanwhile invaginated and not visible.)
2) The eye-stalks are prominent.

On the posterior side

3) The formation of the siphon by concrescence of the anterior siphonal folds. The posterior siphonal folds continue as ridges to the anterior side.
4) The anus, between the gill primordia.

Note the further growth of other primordia; contractions of the yolk sac, etc.

4. Old Stages. (Before hatching - Chart figs. 5 and 6)

1) Eye-stalks are very prominent. They contain the primordia of the optic and cerebral ganglia, the so-called "white bodies", also a separate mass of yolk.
2) The lens. The inner sector which is formed by the outer part of the optic vesicle (not like Vertebrates(is clearly visible as a club-shaped rod extending into the eye vesicle.
3) The mantle has overgrown the anus and gills. It is contractile. In the older stages it is beset with
4) Chromatophores. Note different types and colors: observe their contraction and expansion. They are equipped with muscle and are innervated.
5) The statocysts lie close together.
6) The feather-like gills can be observed through the mantle.
7) The branchial hearts will be found at the bases of the gills and the systemic heart between them. All three pulsate.
8) The rectum and the ink-bag
9) Trace the outline of the internal yolk mass and note the gradual decrease in size of the external yolk sac.
10) Observe the locomotion of an old embryo after it has hatched.

References on Development of Cephalopods

W. K. Brooks — 1880 — Development of the Squid. Memoirs Boston Soc. of Nat. Hist. (Good figures of all stages).

G. A. Drew — 1911 — Sexual activities of the Squid. (Loligo Pealii) 1. Copulation, egg laying and fertilization. J. of Morphology. vol. 22.

__________ — 1919 — 11. The spermatophore; its structure, eja[illegible]tion and formation. J. of Morphology, vol. [illegible].

(Both papers are recommended for collateral reading; consult figures in #11 for details of the structure of the spermatophore).

L. Hoadley — 1930 — Polocyte formation and the cleavage of the Polar Body in Loligo and Chaetopterus. Biol. Bull. vol. 58 (data on artificial insemination).

[illegible] Forschelt — 1892 — Entwicklung des Darmkanals und Nervensystem der Cephalopoden. Festschrift für Leuckart (good figures of development of intestine)

[illegible]erschelt — 1936 — Vergleichende Entwicklungsgeschichte der Tiere vol. 2 pp 968-1009. (Contains Bibliography)

E. W. MacBride — 1914 — Textbook of Embryology. 1. Invertebrata.

A. Naef — 1928 — Die Cephalopoden, vol. 2; Embryologie. Fauna e Flora del Golfo di Napoli.

(Complete series of figures of development of Loligo on plates 1-V11; seriation of stages. Consult particularly plate 7, figs. 4 and 4a for newly hatched squid. Note that Naef describes the Mediterranean species, L. vulgaris, which develops more slowly than L. P.)

A. P ortmann — 1926 — Der embryonale Kreislauf und die Dotterresorption bei Loligo. Zeitschrift f. Morphol. u. Okol. vol. 5. (good figs. of yolk sac circulation).

A. P ortmann and Bidder — 1928 — Yolk absorption in Loligo and the function of the embryonic liver. Quart. Journ. Micro. Sci. vol. 72

S. Ranzi — 1931 — Sviluppo di parti isolate di embrioni di Cefalopodi. Pubbl. della Stazione Zool. di Napoli. vol. 11

__________ — 1931 — Duplicitas cruciata in embrioni di Cefalopodi. ibid. vol. 11.

<u>J. Spek</u> 1934 Ober die bipolare Differenzierung des Cephalopoden und Prosobranchier - Eies. Roux Archiv. vol. 131

<u>S. Watase</u> 1891 Studies on Cephalopods. 1. Cleavage. J. of Morphology vol. 4. (See figures)

L. W. Williams The Anatomy of the Common Squid. (Excellent Figures of the structures of the adult squid).

FERTILIZATION

Use only the pipettes at the stock dishes to obtain gametes. Carelessness will result in contaminating the stock of unfertilized eggs with spermatozoa. The stock dish of eggs will be kept at the front of the room, the stock dish of spermatozoa, at the rear. Care must be taken to avoid inseminating heavily. Too many spermatozoa often cause polyspermy which results in abnormal cleavage and development. Polyspermic eggs of some forms develop more rapidly than normally fertilized eggs. The polyspermic eggs of Nereis, however, usually fail to cleave.

Use the ordinary low power of the compound microscope (approximately 100 diameters magnification, i. e., 10X eye-pieces and 10X objective) for observation and study. This permits maintaining the eggs in a considerable volume of water in a Syracuse watch glass so that concentration of sea water by evaporation is not rapid. Most phenomena can be readily seen with this magnification, which affords excellent definition. Higher powers may be used if one desires to observespermatozoa in detail under a coverslip. After sperm penetration in Nereis has been followed by observing the eggs in the watch glass for at least 95 minutes, it will be instructive to inseminate a second bath, and periodically mount some of these under a coverglass to observe under higher power. Remember that they remain normal but a short time under these conditions.

NEREIS:

Obtain a few unfertilized eggs in sea water in a Syracuse dish. They are approximately 140 microns in diameter as seen from above and 100 microns high in side view. Because of their shape, they tend to orient on a flat surface with the animal pole either above or below, rarely to the side. Observe the large immature nucleus (germinal vesicle or nucleus of the primary oocyte), and the oil droplets and yolk spheres in the cytoplasm surrounding the nucleus. Note also the thick cortex of the egg.

After becoming familiar with the unfertilized egg, inseminate by adding a drop of freshly prepared sperm suspension. Stir the eggs at once by a circular movement of the dish and observe changes. These first changes will begin a few <u>seconds</u> after insemination. At 21°C., the following schedule applies to 50% of a population of eggs (time after fertilization). 1st polar body, 45 minutes; 2nd polar body, 60 minutes; 1st cleavage 95 minutes (ref. #22). The laboratory willprobably be warmer than 21°C., so that development will proceed more rapidly. There are many changes in the egg to observe before the first polar body forms. Observe and record the following: laboratory temperature, time of breakdown of the germinal vesicle, time of final penetration of sperm head through membrane, time of first polar body formation, time of second polar body formation, time of cleavage. The time schedule in the descriptive text below should hold approximately if the air temperature of the laboratory is about 24°C.

If possible, arrange to observe two or more eggs which are touching immediately after fertilization. 2 - 3 minutes after fertilization they will begin to be pushed apart by transparent jelly secreted by the eggs external to the vitelline membrane. By 20 minutes, the zone of jelly around each egg will be as wide as the egg diameter. The margin of the jelly can often be made out by observing supernumerary spermatozoa and other particles at the edge of the jelly. 5 or6 minutes after fertilization the vitelline (fertilization) membrane will be noticeable due to the formation of the narrow perivitelline space upon jelly extrusion. At 7 or 8 minutes, the entrance cone begins visibly to form. Find an egg showing a profile view of the entrance cone and the sperm which is to enter. Form 8 to 12 minutes or longer, the sperm is clearly visible outside the vitelline or longer, the sperm is clearly visible outside the vitelline (fertilization)membrane at the tip of the conspicuous entrance cone. In the course of the next 8 to 10 minutes, the vitelline membrane is indented slightly at its point of contact with the entrance cone. This tends to obscure the sperm from view to some extent, and at about 20 minutes after fertilization the egg wrinkles, becoming distorted and quite irregular in somewhat amoeboid fashion. The entrance cone has already flattened considerably, but is still present, and although the sperm is partially obscured from view, the entrance of its head into the egg is not completed until some time later (ref.#12, 15, 16). The final penetration of the sperm head through the membrane, leaving the middle piece and tail outside, may also be observed. At about 30 minutes, the egg rounds up again, but as the time approaches for 1st polar body formation, the egg elongates in a direction perpendicular to the polar axis. If no eggs lie so that the forming polar body is on the horizon, the dish should be shaken. The 1st polar body may form at about 36-40 minutes, and it lies in the space between the egg and the vitelline membrane. This space is wider in the region of the animal pole than elsewhere. The second polar body often forms at about 50 minutes and commonly does so immediately under the first polar body, which is thus lifted into perivitelline space. At perhaps 80 minutes the eggs will begin to divide into two unequal blastomeres. Observe 2nd and 3rd cleavages also, if time permits. The 3rd division, from 4 to 8 cells, produces 4 micromeres by spiral cleavage (ref. #24).

Place some very recently fertilized eggs of Nereis in a drop of fresh, thick chinese ink suspension (made up by rubbing a piece of ink on a Syracuse dish moistened with sea water) in the center of a Syracuse dish. As the jelly is secreted, the attached sperm causes a canal to form in the secreted jelly into which particles of ink will penetrate. This is due to inhibition of jelly outflow at the point of sperm attachment. The ink thus marks the entrane point of the sperm. After the canal has filled with ink, add sea water and, if time permits, observe and record for a number of eggs the relation of the first cleavage plane to the polar bodies and the entrance point of the sperm as marked by the ink (ref. #12, 16, 19). (Caution, do not leave the piece of chinese ink in a dish of sea water; it will disintegrate).

NEREIS: Exaggerated Entrance Cones:

Place some Nereis eggs insominated 5 to 8 minutes earlier in a Syracuse watch glass containing alkaline NaCl (pH 10.3-10.5. Observe immediately. The vitelline membrane will elevate due to a sudden inhibition of jelly release through the membrane and a subsequent accumulation of the jelly in the perivitelline space (ref. #9). The vitelline membrane remains permeable to water which enters the perivitelline space as the jelly swells. The elevation of the membrane stretches out the sperm entrance cone between membrane and egg surface, forming a long filament which frequently causes marked indentation of the membrane. If the eggs have been kept in the ice box they may become polyspermic upon insemination and show numerous exaggerated entrance cones upon treatment with alkaline NaCl. About ten minutes after treatment the sperm head may be seen moving across the perivitelline space to fuse with the egg surface, at which time the membrane indentation is relaxed. If these eggs are carefully removed from the alkaline NaCl to sea water, and washed, some will develop normally within the raised membranes. If left in alkaline NaCl the optimum length of time before washing, and if the alkaline NaCl has been changed once or twice to remove most of the sea water, the eggs may be completely freed of their membranes. These "naked" eggs have been used for experiments on the development of isolated blastomeres (ref. #8).

Nereis: Centrifuged eggs:

If time permits, centrifuge some unfertilized Nereis eggs in the Emerson electric centrifuge (cover off) for 60 minutes. A layer of 0.95 molar sucrose (undistilled water) at the bottom of the centrifuge tubes prevents injury to the eggs. This is somethat hypertonic, but provides an adequate support for the Nereis eggs without injuring them in any way. This amount of centrifuging separates the various formed components of the egg into several strata (ref. #7). Inseminate the centrifuged eggs after washing off the sucrose with sea water, and observe asymmetrical jelly-extrusion. Is more jelly extruded at the centripetal or centrifugal pole?

If eggs stratified in an ultracentrifuge are available, compare these with the Nereis eggs centrifuged in the Emerson electric centrifuge at about 10,000 X gravity (ref. #10).

Breading habits of Nereis limbata

Nereis swarm in Eel Pond about an hour after dark at certain phases of the lunar cycle. (See Lillie and Just, 1913). On each of two appropriate evenings, about an hour after sunset, half of the class willgather on the floating stage behind the Supply Department to observe this interesting phenomenon.

CHAETOPTERUS:

The chaetopterus egg is rather dark and granular. It is slightly more than 100 microns in diameter, but before fertilization the eggs are often not quite spherical. The polar bodies are larger than in Nereis, and the egg divides to form two unequal cells by means of a polar lobe at the vegetal pole (ref. #14, 18).

When the egg is taken from the female it contains a large immature nucleus (germinal vesicle), as does the Nereis egg, but unlike the egg of Nereis, it spontaneously undergoes partial maturation when placed in sea water, even if not fertilized. A number of species of eggs partly mature when they enter sea water and Pasteels (ref. #21) has shown that this is dependent upon the presence of Calcium in the sea water. Chaetopterus eggs develop quite rapidly. If eggs are fertilized just after the partial maturation in sea water has been completed, they develop as rapidly as eggs inseminated 12-15 minutes earlier when first placed in sea water (ref. #23). (time counted from fertilization): 1st polar body 14.5 minutes; 2nd polar body, 27 minutes; "pear" shaped stage, 46 minutes; polar lobe bulge, 52 minutes; cleavage with polar lobg attached, 58 minutes; completed cleavage with polar lobe resorbed into one blastomere, 62 minutes; 4 cell stage, 82 minutes. If the laboratory air temperature is about 24°C the development will be more rapid, and about at the rate indicated in the descriptive text below.

When eggs are taken from the female, the large immature nucleus is in the center of the egg. After about 15 minutes in sea water, maturation will have proceeded to the metaphase of the first polar spindle at which stage development is arrested unless the egg is inseminated or artificially activated. The spindle cannot be distinguished as such in the living egg, but it will be observed that the relatively clear region of the nucleus and spindle is now located quite eccentrically. It reaches the surface of the egg in the polar region, where the polar bodies will be given off.

If eggs are now lightly inseminated and stirred, a few sperm may be seen adhering to the eggs almost immediately. Within 5 or 6 minutes, the vitelline membrane may be seen to separate from the egg surface, after which time it is called the fertilization membrane. It is not conspicuous and does not elevate much above the egg surface. By 10 or 12 minutes, the eggs, which had become almost spherical after fertilization, are seen to elongate in an axis perpendicular to the polar axis. This is preparatory to formation of the 1st polar body at about 12 minutes. In this division the egg thus assumes approximately the shape of a blastomere, although the polar body is a vestigial cell. After the first polar body forms, the egg again rounds up (16-19 minutes), but it elongates again in the same manner to form the second polar body at perhaps 23 minutes. The second polar body often forms under the first, which is thus pushed away from the surface of the egg. The egg again rounds up (26-30 minutes); the egg pronucleus may now sometimes be seen migrating toward the center of the egg, and occasionally the sperm pronucleus may also be detected. By 35 minutes, the clear zone has extended from the polar region toward the equator of the egg, and at 37-40 minutes a typrical "pear shaped" stage is reached. The polar bodies lie at a position corresponding to where the stem attaches to a pear, and the bulge which forms the polar lobe begins quite suddenly at the anti-polar end of the egg (40-43 minutes). When the polar lobe is fully developed, however, it corresponds to the stem end of the "pear", and the polar bodies are opposite.

Observe the extrusion of the polar lobe which contains coarse globular material. By 47-48 minutes the cleavage furrow begins at the animal pole and passes to one side of the polar lobe, which thus comes to be attached to one of the two blastomeres. The resorption of the polar lobe into this blastomere causes it to become larger than its mate, and at about 51 minutes two smooth unequal blastomeres lie against each other. Polyspermic eggs will now often be in an abnormal 3 cell stage. By 60 minutes the two blastomeres are quite fused together. At 67-70 minutes the second cleavage takes place. The large blastomere again forms a polar lobe, and a 4 cell stage results with one blastomere larger than the other three. By 90 minutes, or earlier, the clear nuclei in the 4 cells may readily be made out. At 93-97 minutes the third division takes place, forming 4 relatively large micromeres. A profile view will reveal the macromeres, micromeres, and polar bodies. A polar view will show the rotated displacement of the micromeres, resulting from spiral cleavage, although the displacement is not great or conspicuous in *Chaetopterus*.

CONCENTRATION AND ACTIVITY OF SPERMATOZOA:

Place a small shallow drop of "dry" spermatozoa directly from the testis on a slide. The sperm must not be diluted with any sea water whatsoever. Sea urchin spermatozoa are good for this because it is especially easy to obtain them "dry" directly from the testis. Place a drop of sea water nearby on the slide so that it does not touch the drop of spermatozoa. With ordinary low power (approx. 100X) ex-mine the spermatozoa and note the degree of activity especially at the edge of the drop where they may be seen more readily. Now take a small glass rod, or a match stick, or other object and drag its tip from the drop of sea water into the drop of spermatozoa so that a connecting bridge is established. As the sperm diffuses into the sea water a gradient of concentration is established. Note swimming activity in relation to concentration.

CUMINGIA:

If *Cumingia* eggs are available, observe the migration and fusion of the pronuclei. While *Cumingia* eggs are small (about 60 microns in diameter) they are clear and show the pronuclei in the living state especially well. The cytoplasmic constituents of these eggs are very readily stratified into four zones in the centrifuge.

SPECIAL PROJECTS:

During spare time students may undertake special projects relating to fertilization or cell-lineage. The following problems are suggested, but students may formulate their own projects subject to the approval of the instructor.

1. Fertilization of Platynereis. Collect Platynereis from the Cayadetta Wharf floating stage at the right phase of the moon. Consult E. E. Just's papers on

breeding habits and fertilization. Compare your observations with those on Nereis limbata.

2. Development of Isolated Blastomeres of Nereis. Remove membranes of inseminated Nereis eggs by the alkaline NaCl method previously described. Use semi-sterile technique with all dishes and instruments. Make Spemann glass needles after directions of Horstadius in McClung's Microscopical Technique. Use small watch glasses (preferably Columbia) with a thin layer of filtered agar made up in sea water, as operating and isolation dishes. Separate the blastomeres with the fine tips of the glass needles immediately after the cleavage furrow is complete. Transfer to separate isolation dishes with mouth pipette and permit isolated blastomerest to develop in cool moist chambers. Observe at frequent intervals.

3. Development of Centrifuged Nereis Eggs. Centrifuge Nereis eggs for 60 to 90 minutes in Emerson electric centrifuge with cover off (or in air turbine, if available), with sucrose, as previously described. Wash off surcrose in sea water, inseminate and study cleavage. Statistics as to the number of AB and OD blastomeres forming from centripetal or centrifugal ends of the centrifuged eggs would be of interest. Position of micromeres may also be noted in relation to stratification and in relation to egg polarity.

General References:

1. Gray, J., 1931. A textbook of experimental cytology. Cambridge University Press.
2. Just, E. E. 1939. The biology of the cell surface. Blakiston, Philadelphia.
3. Lillie, F. R. and E. E. Just 1924. Chapter 8 in Cowdry, General Cytology University of Chicago Press
4. Morgan, T. H. 1927. Experimental Embryology. Columbia University Press.
5. Wilson, E. B. 1925. The cell in development and heredity, MacMillan, New York

Further references relating to laboratory work:

6. Costello, D. P. 1939. The columes occupied by the formed cytoplasmic components of marine eggs. Physiol. Zool. 12, 13.
7. Costello, D. P. 1940. The cell origin Nereis limbata, Biol. Bull. 79, 369.
8. Costello, D. P. and R. A. Young 1939. The mechanism of membrane elevation in the egg of Nereis limbata Collecting Net, 14, 210
9. Costello, D. P. 1940. The fertilizability of nucleated and non-nucleated fragments of centrifuged Nereis eggs. J. Morph., 66, 99
10. Headley, L. 1934. Pulsations in the Nereis egg. Biol. Bull. Vol 67, pp 484-493
11. Just, E. E. 1912. The relation of the first cleavage plane to the entrance point of the sperm. Biol. Bull. 22, 239
12. Just, E. E. 1930. The present status of the fertilizing theory of fertilization. Protoplasma, 10, 300
13. Lillie, F. R. 1906, Observations and experiments concerning the elementary phenomena of embryonic development in Chaetopterus. J. Exp. Zool., 3, 153.
14. Lillie, F. R. 1911. Studies of fertilzation in Nereis. J. Morph. 22, 361
15. Lillie, F. R. 1912. Studies of fertilization of Nereis 111, 1V, J. Exp. Zool. 12, 413
16. Lillie, F. R. 1914. Studies of Fertilization V1. J. Exp. Zool. 16, 523.
17. Mead, A. D. 1897. The early development of marine annelids. J. Morph. 13, 271
18. Morgan, T. H. and Albert Tyler 1930. The point of entrance of the spermatozoon in relation to the orientation of the embryo in eggs with spiral cleavage. Biol. Bull. 58, 59
19. Novikoff, A. B. 1939. Changes at the Surface of Nereis limbata after insemination. Jour. Exp. Biol. Vol. 16, 403-8
20. Pasteels, J. 1935. Recherches sur le determinisme de l' entree on maturation de l'oeuf chez divers invertebres marine. Arch. Biol. 46, 229
22. Whitaker, D. M. 1931. On the rate of oxygen consumption by fertilized and unfertilized eggs. 111. Nereis, J. Gen. Physiol., 15, 191
23. Whitaker, D. M. 1933. Ibid 1V Chaetopterus, J. Gen. Physiology. 15, 191
24. Wilson, E. B. 1892, Cell lineage of Nereis, J. Morph. 6, 361

Lunar Periodicity

1. Clark, L. B. and W. N. Hess, 1940. (Atlantic Palolo) Publication No. 524, p. 21, Carnegie Inst. of Washington.
2. Fox, 1924. Proc. Roy. Soc. London. B.
3. Hempelmann, Fr., 1911 (Nereis dumerilii) Zoologica, Bd. 25
4. Izuka, A., 1903 (Japanese Palolo) J. Coll. Sci. Imp. U. Tokyo 17.
5. Just, E. E. 1914. (Platynereis megalops) Biol. Bull. 27, 201
6. Lillie, F. R. and E. E. Just, 1913 (Nereis limbata) Biol. Bull. 24, 147
7. Mayer, A. G. 1908. (Atlantic Palolo worm) Publication 102, Carnegie Institution of Washington
8. Scott, J. W. 1909. (Amphitrite) Biol. Bull. 17.
9. Woodworth W. Mc. 1907 (Pacific Palolo) Bull. Mus. Comp. Zool. 51

Cell Lineage

Maturation, Fertilization and Cleavage through the 25-cell Stage in Crepidula

Due to the opacity of the living eggs, the details of maturation, association of germ nuclei, and cleavage can best be studied from prepared slides. The eggs are not sectioned but are mounted whole. Number one or zero cover glasses have been used, making it possible to examine the material under high power. Do not, under any circumstances, use oil immersion objectives on these slides. Conklin's two monographs (1897 and 1902) may be used for reference, or the photo copies of the plates from these papers.

Crepidula is a dioecious genus with the males fewer in number and smaller than the adult females. The spermatozoa mingle with ova before the egg capsules are formed around groups of eggs in the oviduct of the female. The mature females are sedentary, the males locomotive, and at the breeding season, orperhaps once for all, the females are visited and inseminated by these motile males. All the ova produced by a given individual during a season are laid within a short space of time.

1. On the prepared slides, make a careful study of various stages in the two maturation divisions. In the same eggs find the sperm nucleus and note its approach to the egg nucleus. Note also the small antipolar lobe. Make drawings.

2. Study the first and second cleavages, noting the direction of the axes of the mitotic figures in the latter. The small antipolar lobe may be visible near the vegetal region of the furrow at the 2-cell stage. Make drawings.

3. Study in detail the formation of the first three quartettes of micromeres and the formation of the derivatives of the first and second quartettes. Indicate which divisions are dexiotropic and which leiotropic. Find and draw examples of the 8, 12, 16, 20, 24 and 25-cell stages. The last is difficult to find and should be checked carefully. The nuclei may be identified by their size characteristics.

Some students, in their spare time, may wish to prepare their own slides of Crepidula maturation and cleavage stages. The following method is suggested:

A. Obtaining Eggs. With a heavy knife loosen a Crepidula shell from its attachment. The egg capsules will either be attached to the substrate or to the foot of the female. Those that are small and light yellow contain eggs in the earlier stages of development; the larger, deep yellow or mud-colored capsules contain older embryos and larvae. Remove the capsules, by means of forceps, to a Syracuse watchglass of sea water. Tear open the capsules with a pair of needles to release the eggs. Discard empty capsules. Examine the eggs under the microscope to ascertain the stage. Dark-field illumination may be helpful. It is best to mix several batches of young stages for slide-making.

B. Fixation. (Do not use fixatives or other reagents in any dishes which are used for living materials. Confine these reagents to vials. Having freed the eggs, wash them by a gentle rotary rinsing with a pipette and then concentrate them in the center of the dish. Change the sea water two or three times. Then take up the concentrated eggs with a pipette, and drop them, with a small amount of sea water, into a vial three-quarters full of Kleinenberg's picro-sulphuric fixative. The eggs should be fixed at least 15 minutes.

Remove the fixative, using a pipette of small diameter equipped with a syringe bulb, and fill the vial with 70% alcohol. Wash in 70% until the eggs are white. It is advisable to avoid washing too long in 70%, since the stain employed is best when it does not penetrate the macromeres. These latter should therefore be left slightly acid. Thus the eggs are removed from 70% immediately after the last wash which removes no picric acid from them, hydrated in 50%, 35% and washed thoroughly in 2-3 changes of water.

C. Staining. After washing with water, fill the vial with undiluted Mayer's haemalum, and stain for 5-10 minutes. For the polar body stages, 5-7 minutes is usually sufficient. After staining, wash thoroughly in water, dehydrate, and clear in xylol. Remove the xylol used in clearing and replace it with a small amount of thin damar.

D. Mounting. Cover glasses must be supported. For this purpose it has been convenient to use paper squares the size of 7/8" cover glasses. A hole is punched in the center of each square with a paper punch. In mounting, the squares are cleared in xylol, and fixed to the centers of slides by adding three or four drops of thin damar before the evaporation of the xylol. When the paper mounts have dried, the eggs are removed from the vial in which they are stored by the use of a pipette drawn out to a long taper and having a small diameter at its tip. The eggs are allowed to settle toward the tip of the pipette, and one drop of the egg-damar suspension is placed in the center depression of each paper mount. The damar is allowed to dry to the point of formation of a thin film in order that the eggs may remain dispersed and with the macromere quartette adjacent to the slide when mounted. Apply thick damar to the edge of the paper mount, immerse a #0 cover glass in xylol and apply it to the slide over the paper mount.

References Specifically for Crepidula:

Conklin, E. G. 1897 Embryology of Crepidula. J. Morph., vol. 13, pp. 1-226

Conklin, E. G. 1902 Cytokinesis and Karyokinesis. J. Acad. Sci. Phila., Ser. 2, vol. 12, part I

Moritz, C. E. 1938 The anatomy of the Gastropod, Crepidula adunca. Univ. Calif. Publ. Zool., vol. 43, pp. 83-92

Moritz, C. E. 1939 Organogenesis in the Gastropod, Crepidula adunca Sowerby. Univ. Calif. Publ. Zool., vol. 43, pp. 217-248

General References on Cell-Lineage:

Huxley and DeBeer 1934 Elements of Experimental Embryology. Cambridge Univ. Press

McBride, E. W. 1914 Textbook of Embryology. Vol. 1, MacMillan, London.

Morgan, T. H. 1934 Embryology and Genetics. ColumbiaUniv. Press, New York

Richards, A. 1931 Outline of Comparative Embryology. Wiley and Sons, NewYork

Special References on Cell-Lineage:

Child, C. M. 1900 The early development of Arenicola and Sternaspis. Roux Arch. f. Entwickl., Bd. 9, S. 587-723

Heath, H. 1899 The development of Ischnochiton. G. Fischer, Jena, pp. 1-90

Lillie, F. R. 1895 The embryology of the Unionidae. J. Morph., vol. 10, pp. 1-100

Lillie, F. R. 1899 Adaptation in cleavage. Biological Lectures at Woods Hole, 1898, pp. 43-67

Mead, A. D. 1897 Early development of marine Annelids. J. Morph., vol. 13, pp. 227-326

Meisenheimer, J. 1901 Entwicklungsgeschichte von Dreissensia polymorpha Pall. Zeit. f. wiss. Zool., Bd. 69, S. 1-137

Newby, W. W. 1932 The early embryology of the echiuroid Urechis. Biol. Bull., vol. 68, pp. 387-399

Newby, W. W. 1940 The Embryology of the Echiuroid Urechis caupo. Mem. Amer. Phil. Soc., 16, pp. 1-219

Treadwell, A. L. 1901 The cytology of Podarke obscura Verrill. J. Morph. vol. 17, pp. 399-486

Whitman, C. O. 1878 The embryology of Clepsine. Q. Jour. Micr. Sci., vol. 18, pp. 215-314

Wierzejski, Anton 1905 Embryologie von Physa fontinalis L. Zeit. f. wiss. Zool., Bd. 83, S. 503-706

Wilson, E. B. 1892 The cell lineage of Nereis. J. Morph., vol. 6, pp. 361-480

Wilson, E. B. 1898 Considerations on cell-lineage and ancestral reminiscence, based on a re-examination of some points in the early development of Annelids and Polyclades. Ann. N.Y. Acad. Sci., vol. 11, pp. 1-27

Woltereck, R. 1903 Beiträge zur praktischen analyse der Polygordius-Entwicklung nach dem "Nordsee" und dem "Mittelmeertypus". I. Der für beide Typen gleichverlaufende Entwicklungsabschnitt: vom Ei bis zum jüngsten Trochophora Stadium. Roux. Arch. f. Entwikl., Bd. 18, S. 377-403

BIBLIOGRAPHY

EXPERIMENTAL EMBRYOLOGY OF ANNELIDS AND MOLLUSKS

TEXTBOOKS

Huxley and de Beer	1934	Elements of Experimental Embryology, Cambridge (MacMilian)
Morgan, T. H.	1927	Experimental Embryology, Univ of Columbia Press
Needham, J.	1942	Biochemistry and Morphogenesis. Cambridge (MacMillan)
Schleip, W.	1929	Die Determination der Primitiv-Entwicklung. Akad. Verl. Leipzig
Weiss, P.	1939	Principles of Development Henry Holt

SPECIAL REFERENCES

Conklin, E. G.	1917	Effects of centrifugal force on the structure and development of the eggs of Crepidula. J. Exp. Zool. 22
Conklin, E. G.	1938	Disorientation of development in Crepidula plana produced by low temperatures. Proc. Amer. Phil. Soc. 79
Costello, D. P.	1938	Studies on fragments of centrifuged Nereis eggs. Biol. Bull. v. 75
Costello, D. P.	1945	Experimental studies of germinal localization in Nereis. 1. The development of isolated blastomeres. J. Exp. Zool. 100.
Costello, D. P.	1945	Segregation of ooplasmic constituents. Jour. Elisha Mitchell Sci. Soc., 61
Crampton, H. E.	1896	Experimental studies on Gastropod development. Arch. f. Entw. 3.
Hatt, P.	1932	Essais experimentaux sur les localisations germinales dans l'oeuf d'un Annilde (Sabellaria alveolata L.) Arch. d' Anat. Micr. T. 28
Hörstadius, S.	1937	Experiments on determination in the early development of Cerebratulus lacteus. Biol. Bull. 73
Lillie, F. R.	1902	Differentiation without cleavage in the egg of the annelid Chaetopterus. Arch. F. Entw. 14
Lillie, F. R.	1906	Observations and experiments concerning the elementary phenomena of embryonic development in Chaetopterus. J. Exp. Zool. 3
Morgan, T. H.	1910	Cytological studies of centrifuged eggs. J. Exp. Zool. 9
Novikoff, A. B.	1938	Embryonic determination in the annelid. Sabellaria vulgaris. 1. The differentiation of ectoderm when separated through induced exogastrulation. 2. Transplantation of polar lobes and blastomeres. Biol. Bull. 74

Novikoff, A. B. 1940 Morphogenetic substances or organizers in annelid development. J. Exp. Zool. 85

Penners, A. 1938 Abhängigkeit der Formbildung vom Mesoderm im Tubifex-Embryo. Zeit. f. wiss. Zool. 150

Schleip, W. 1925 Die Furchung dispermer Dentalium-Eier. Arch. f. Entw. 106

Tyler, A. 1930 Experimental production of double embryos in annelids and mollusks. J. Exp. Zool. 57.

Wilson, E. B. 1903 Experiments on cleavage and localization in the nemertine egg. Arch. f. Entw. 16

Wilson, E. B. 1904 Experimental studies on germinal localization . 1. The germ-regions in the egg of Dentalium. 2. Experiments on the cleavage mosaic in Patella and Dentalium. J. Exp. Zool. 1

Wilson, E. B. 1904 Mosaic development in the annelid egg. Science 20.

Wilson, E. B. 1929 The development of egg fragments in annelids. Arch. f. Entw. 117

Yatsu, N. 1904 Experiments on the development of egg fragments of Cerebratulus. Biol. Bull. 6.

Yatsu, N. 1910 Experiments on germinal localization in the egg of Cerebratulus. J. Coll. Sci. Tokyo. 27

Zeleny, C. 1904 Experiments on the localization of developmental factors in the Nemertine egg. J. Exp. Zool. 1

ECHINODERM DEVELOPMENT

Arbacia punctulata

This species is usually ripe from mid-June to mid-September in the Woods Hole region. When stored in laboratory aquaria they may maintain their ripe condition even beyond the breeding season and supply apparently normal eggs and sperm. In general, however, it is safest to use animals within a few days after they are collected.

While in some species of echinoids the sexes may be distinguished externally (see Marx, 1931), no differentiating characteristics have, as yet, been described for *A. punctulata*. The sexes are readily identified after the animals are opened by the deep-red or purple ovaries and the yellowish-gray testes, or, if unopened animals shed spontaneously, by the red eggs and the white sperm.

Obtaining the gametes:- Any of the following methods may be used. a)Cut around the peristome and remove the Aristotle's lantern. Pour out the body fluid and place the animal, aboral side down in a dish containing a small amount of sea water. The animals then frequently shed thru the gonopores. After 10 minutes remove any eggs that have been shed to a finger bowl (or other large flat dish)containing ca. 200 ml of sea water. Sperm should be kept in concentrated suspension or "dry" (ie. as it exudes from the testes). b)Cut around the teste about half way between the mouth and the equator and proceed as in a. Shedding is more frequently obtained by this method, but there is also more likelihood of cutting the gonads. c)Cut as in b, pour out body fluid and remove gonads (at gonoduct end) with blunt forcep, spatula or spoon. The ovaries should be placed in about 200 ml of sea water in a finger bowl and allowed to shed. If undisturbed the eggs are extruded in compact clumps or strings and may be readily removed to a fresh dish without ovarian tissue by means of a wide-mouth pipette. If large quantities of eggs are desired the ovaries should be allowed to shed for about ½ hour with occasional stirring, then poured gently thru washed (and sea water soaked) cheesecloth or bolting silk. d)Inject about 0.2 ml of 0.5 KCl into the peristomial cavity. Ripe animals will begin to shed within 2 minutes. The eggs can be collected by inverting the animal in a dish of sea water or by washing the eggs gently from the surface of the animal with a pipette. The sperm should be removed "dry" or in concentrated suspension.

Spermatozoa:- Upon dilution with sea water the sperm become temporarily intensely active. They lose their motility sooner in concentrated than in dilute suspension, due presumably to the more rapid accumulation of CO_2. On the other hand their ability to fertilize eggs is lost more rapidly in dilute than in concentrated suspension. (See F. R. Lillie, 1915; Cohn 1918; Hayashi 1945). When kept in the cold 2°C.) "dry" sperm may remain good for several days. At room temperature dilute sperm suspensions may lose their fertilizing power in an hour or less. It is advisable, then, to use freshly diluted sperm for fertilization.

The head of the sperm is comprised of acrosome, nucleus and midpiece that are roughly 0.3 and 1 microns respectively in length and 0.3, 1.3 and 1.2 microns in greatest width. The tail is about 45 microns long and 0.1 micron in greatest width. Its axial filament protrudes a short distance beyond the end of the sheath. Examine under oil immersion and sketch a spermatozoon. Examine moderately active spermatozoa under high-dry and describe their mode of swimming.

Unfertilized Eggs:- Arbacia eggs complete both meiotic divisions while still in the ovary and the polar bodies very seldom remain attached when the eggs are shed. Occasionally, especially from relatively unripe animals or after macerating ovaries, eggs may be found that are in the germinal vesicle (diakinesis of primary oöcyte) stage recognizable by the large clear nucleus (about one-half egg diameter) and nucleolus. Such eggs may exhibit some surface response to sperm but they do not develop upon insemination. The ripe egg (75 microns diameter) has a small clear nucleus. It contains uniformly dispersed pale yolk granules and slightly larger red granules containing a pigment called echinochrom which is a substituted naphthoquinone related to the K. vitamins. (Ball, 1936, Hartmann et al. 1939, Tyler 1939). Upon centrifugation mitochondria and oil sph rules are also distinguishable. The nucleus is generally located excentrically. Since the polar bodies are not usually present the position of the nucleus with respect to the polar axis is not readily determined. Occasionally however, batches of eggs are obtained in which the polar bodies are attached . In these, observations (Hoadley, 1934) have shown that the nucleus may lie in any part of the cytoplasm between the cortex and the center. In the transparent gelatinous coat (about 30 microns wide) of the egg there is a funnel-shaped space which generally lies in the polar axis. The funnel is rendered visible by staining the jelly with Janus green or by placing the eggs in a suspension of Chinese ink. For this purpose the eggs should be taken immediately after shedding since the micropyle (funnel) may disappear as the jelly swells. Examine and sketch some unfertilized eggs under high power noting features described above.

Centrifuge a sample of unfertilized eggs at about 10,000 g for ½ hour and sketch one in "side view" noting the following five layers of stratified material:- oil cap (centripetal end), hyaline zone, mitochondria, yolk zone and pigment layer (centrifugal end). Where is the nucleus located? Have tch granules in the cortex of the egg (see second paragraph below) been displaced?

Fertilization:-Inseminate a sample of eggs, using one drop of freshly diluted 1% sperm (one drop of "dry" sperm in 5 ml of sea water) for each 10 ml of freshly washed dilute egg suspension (containing about 5,000 eggs per ml). Stir the dish immediately after adding the sperm and observe the process of membrane elevation. How soon does it begin? when is it completely separated from the surface of the egg? When does the perivitelline space attain its maximum width? Measure the diameters of an unfertilized egg and an egg at 10 to 15 minutes after fertilization. Is there any appreciable difference in volume apart from that of the perivitelline space?

The spermatozoön enters the egg within a few seconds after attachment. To observe the process place a drop of eggs in the center of a vaseline-ringed slide and add a drop of sperm of just sufficient concentration to fertilize all of the eggs. Add a coverslip and locate as quickly as possible an egg that shows only one spermatozoon on its surface. Note the changes that occur upon penetration of the sperm. Where does membrane elevation first begin? In the cortex of the unfertilized egg there is a single layer of granules which disappear (Moser, 1939) upon fertilization and contribute (according to Runnström, 1944) to the formation of the fertilization membrane. These are best seen in the hyaline zone of the centrifuged eggs. Inseminate a sample of centrifuged eggs on a slide, as described above and observe the behaviour of the cortical granules. Dark-field illumination shows a bright reddish "luminous" layer on the surface of the unfertilized egg. The luminosity diminishes and becomes paler upon fertilization (Runnström, 1928; Ohman, 1945) Using the dark-field stop disc for the condenser of your microscope examine a sample of unfertilized and fertilized eggs.

Preliminaries to Cleavage: At 10 minutes after fertilization a hyaline layer (about 1 micron wide) forms on the surface of the egg. This layer later follows the cleavage furrows and is the material by which the blastomeres are held together. In calcium-free sea water the hyaline layer disappears.

The sperm cannot be distinguished in the living egg. At about 15 minutes after fertilization (at 20°C) a sperm aster is visible as a spherical region containing clear rays extending from a clear center. This attains its maximum development at 20 and 30 minutes. Then a clear streak appears in the egg slightly above the equator and at 45-50 minutes this is replaced by two clear areas, the asters of the first cleavage spindle.

Cleavage:- The following figures give the average time for the first three cleavages (after Fry, 1936).

Cleavage:-	First	Second	third
Minutes after fertilization:	-42 (25°); 113(15°)	107(20°)	145(20°)

Different batches of eggs vary slightly (1-2% in average cleavage time) and, while within a batch of eggs most will develop at the average rate, some may vary by about 10%. For any temperature between 15° and 25° the average time of development can be calculated from that at 20° by means of the following formula:-

log (time at temp.6) = log (time at 20°) - (t=20) 10. log 2.6

Temperatures above 30-32° are lethal for Arbacia eggs. The first three cleavages divide the egg into eight equal sized blastomeres. The planes of the first two cleavages are meridional (in the polar axis), that of the third is equatorial or horizontal (at right angles to the polar axis). Follow the progress of the cleavage furrow in dividing eggs. Note that the hyaline layer forms the surface of the furrow, and later, when the cells flatten against one another, that it forms the boundary between them.

At the fourth cleavage the upper four cells divide meridionally forming eight equal cells called mesomeres, while the lower four cells divide unequally and horizontally forming four large cells called macromeres and below them, at the vegetal pole, four small clear cells called micromeres. At the fifth division the eight mesomeres divide equally and horizontally forming two tiers of cells termed an_1 and an_2 cells divide in more or less radial direction while the macromeres divide horizontally forming two tiers termed veg_1 and veg_2. Veg_2 is next to micromeres which have also divided at this time but which do not form distinct layers. Layers of cells are not readily distinguished in later cleavage stages and no special designation is applied to the cells after the 64-cell stage. It has been shown (see Horstadius, 1939) that the an_1, an_2, and veg_1 cells form the larval ectoderm; the veg_2 cells form endoderm and part of the mesoderm (coelom and 2nd mesenchyme); the micromeres form the mesoderm (primary mesenchyme) which produces the skeleton. Sketch the various stages up to the sixth cleavage.

Blastula:- At the eight cell stage there is a very small central cavity which enlarges, as cleavage continues, to form the blastocoel. At about 6 hours after fertilization a smooth-surfaced spherical young blastula is formed, the wall of which is one-cell thick. Cilia soon develop on the surface and the blastula is rotated by their action within the fertilization membrane. At about 10 hours the blastula hatches out of the fertilization membrane. It has been shown (Kopa., 19..) that the blastula releases a "hatching enzyme" at this time that weakens and dissolves the membrane sufficiently for the blastula to break thru. A small tuft of long cilia develops at the animal pole of the blastula which is the forward end when it is swimming. At the base of this apical tuft the blastula wall is thickened, forming the apical plate. At the vegetal pole the blastula wall becomes flattened and the micromeres migrate into the blastocoel, forming the primary mesenchyme which gives rise to the skeleton. Sketch early and late blastulae.

Gastula:- At about 20 hours after fertilization the cells at the vegetal pole invaginate to form a blind tube, the archenteron. This reaches the opposite end of the blastocoel in about five hours. The gastrula contains about 1000 cells and its outer wall as well as the wall of the archenteron has a single layer of cells. The primary mesenchyme cells form a ring around the blastoporal end of the archenteron. Secondary mesenchyme and later, coelom are budded off from the tip of the archenteron. Draw beginning and completed gastrulae.

Prism:- At the completion of gastrulation the tip of the archenteron turns to one side of the gastrula which becomes flattened over an area extending from the animal pole nearly to the blastopore. This is the first sign of bilateral symmetry, the flattened area representing the ventral side of the embryo. The primary mesenchyme cells aggregate in two groups, one on each postero-ventral side, and each group secretes a triradiate spicule, the beginnings of the skeleton. Where the tip of the archenteron touches the ectoderm there is formed a depression which later acquires an opening into the archenteron to become the stomodeum. The archenteron becomes divided by two constrictions into oesophagus, stomach and intestine. The apical tuft disappears, a ciliated band surrounds the oral field, the embryo begins to elongate in the dorso-ventral axis and the direction of swimming changes so that the ventral side is forward. Draw a prism larva.

Pluteus:- After about 48 hours the embryo enters the pluteus stage which is fully developed at the end of the third day. The original apical plate grows out in a ventral direction to form the oral lobe which includes the stomodeum and anterior part of the oesophagus. Two short outgrowths, the oral (antero-lateral) arms are formed on the oral lobe and, at the anal side, two longer anal (aboral or postoral) arms grow out in the same general direction. The original triradiate spicules form skeletal rods which extend into the oral arms (oral rods) the anal arms (anal rods), dorsally through the body (body rods) and laterally (ventral transverse rods). The rods are each made up of three or four parallel parts joined by cross bars. Different species of sea-urchins differ in this regard, so the structure of the skeletal rods is a useful characteristic in hybridization studies. The embryo continues to elongate in the dorso-ventral direction and becomes pointed at the postero-dorsal end where the body rods meet. The axis running thru oesophagus, stomach and intestines becomes J-shaped. The stomach expands to form a spherical structure that fills a large part of the body of the pluteus and sphincter muscles connect it with oesophagus and intestine. The two coelomic sacs extend postero-laterally from the oesophagus. That on the left side becomes larger and later acquires a dorsal opening called the pore canal. The right coelom buds off cells to form the madreporic vesicle but otherwise remains rudimentary. The left coelom undergoes extensive later development in the formation of the structures of the adult sea-urchin. These changes do not occur until the second week when metamorphosis begins in properly fed larvae and will not be sudied here. It should be mentioned, however, that the adult organs are built up in and around a structure terms the Echinus rudiment which is formed by the fusion of an invagination (amniotic invagination) of the ectoderm on the left side with the mid-portion (hydrocoel) of the left coelom. The left side of the pluteus becomes, then, the future oral face of the adult. Draw a 3 day old pluteus in postero-ventral and side view.

References:-

Ball, E. G. 1936 Echinochrome, its isolation and composition Jour. Biol. Chem., 114, p. Vl

Cohn, E. J. 1918. Studies in the physiology of spermatozoa. Biol. Bull., 34: 167-218

Fry, J., 1936. Studies of the mitotic figure. V. The time schedule of mitotic changes in developing Arbacia eggs. Bio. Bul 70:89-99

Hartmann, M., Schartau, O., Kuhn, R. and Wallenfels, K. 1939. Uber die Sexualstoffe der Seeigel. Naturwiss. 27:433

Hayashi, T. 1945. Dilution medium and survival of the spermatozoa of Arbacia. l. Effect of the medium on fertilizing power. Biol. Bull. 89: 162-179.

Hoadley, L. 1934. The relation between the position of the femal pro-nucleus and the polar bodies in the unfertilized egg of Arbacia punctulate. Biol. Bull. 67: 220-222.

Horstadius, Sven, 1939. The mechanics of sea urchin development studied by operative methods Biol. Rev., 14: 132-179

Kopac, M. J. 1941. Disintegration of the fertilization membrane of Arbacia by action of an "enzyme". Jour Cell. Comp Physiol 18: 215-220.

Kuhn, R. and Wallenfels, K. 1940. Echinochrome als prosthetische Gruppen hochmolekularer Symplexe in den Eiern von Arbacia postulosa. Ber. Deutsch. Chem. Gesell., 73: 458-464

Lillie, F. R. 1915. Studies of fertilization. Vll. Analysis of variations in the fertilizing power of sperm suspensions of Arbacia. Biol. Bull. 28:229-251.

Marx, Wilhelm 1931. Zum Problem der Determination der Bilateralität im Seeigelkeim (Nebst einem Beitrag zur Kenntnis des Geschlechts-dimorphismus einiger Seeigel). Arch. Entwicklungsmech. Org., 125: 96-147

Moser, F. 1939. Studies on a cortical layer response to stimulating agents in the Arbacia egg. l. ll. Jour. Exp. Zool. 80: 423, 447. lll, lV. 1940 Biol. Bull. 78:

Ohman, L. 1945. On the lipids of the sea-urchin egg. Ark. F. Zoologi, 36A. N:07, 1-95

Runnstrom, J., Monne, L. and Wicklund E, 1944. Mechanism of formation of the fertilization membrane in the sea urchin egg. Nature 153: 313-314.

Tyler, A., Ricci, N. and Horowitz, N. H., 1938. The respiration and fertilizable life of Arbacia eggs under sterile and non-sterile conditions. Jour. Exper. Zool. 79:129-143.
Tyler, A. 1939. Crystalline echinochrom and spinochrome: their failure to stimulate the respiration of eggs and of sperm of Strongylocentrotus. Proc. Nat. Acad. Sci., 25:523-528.

Development of Isolated Blastomeres of Arbacia and Echinarachinius

Hans Driesch in 1891 demonstrated that whole embryos may be obtained from isolated blastomeres of the sea-urchin egg. The present exercise is essentially a repetition of that classical experiment with some additional features provided by later work.

Equipment: scissors, blunt forceps, 6" square of cheesecloth, 3 finger bowls, 12 syracuse dishes, 3 stender dishes with lids, 6 embryological watch glasses, 1 test tube, (ca. 5/8" x 6"), 2 fine glass needles, eyepiece micrometer, 3 vaseline-ringed slides and cover-slips, 3 ordinary pipettes, 1 narrow long-tipped pipette, 1 fine pipette.

Solutions:- 1 liter filtered sea-water
100 ml hypertonic sea-water (30grams NaCl in 1 liter of sea water)
100 ml of Ca-free sea-water (1000 ml M/2+22 ml M/2 KCl + 195 ml m/3 $MgCl_2.6H_2O$+103 ml M/3 Na_2SO_4, 6 ml M/2 $NaHCO_3$, adjusted to pH 7.9-8.3 (based on Lyman and Fleming 1940).
50 ml of 5% formalin in sea-water

Removal of Fertilization Membrane. For the purpose of isolating blastomeres the fertilization membrane must first be removed. This is accomplished by shaking a suspension of freshly fertilized eggs. Obtain eggs and sperm of Arbacia or of Echinarachinus in the usual way. Inseminate (noting time) a sample of eggs, check for fertilizability and save to determine first cleavage time (necessary in part (a) below) Later (10 to 15 minutes) fill a test tube about four-fifths full with a freshly washed sample of eggs and inseminate, mixing by inverting the test tube once. About one-half minute later pour about ¼ of the eggs from the test tube gently into a syracuse dish and examine for membrane elevation. When the membranes have separated from the surface of practically all of the eggs (about 1 to 2 minutes after insemination), shake the test tube ten times rapidly up and down using a full fore-arm swing and holding long axis of tube in direction of swing with thumb over open end. Pour about ¼ of the eggs into a syracuse dish, immediately shake tube again ten times, remove, another ¼ of the eggs repeat a third time and remove the remaining ¼. Examine the three dishes of shaken eggs and select the one containing the highest percentage of naked eggs. Wash twice with filtered sea-water.

An alternative method of membrane removal uses a pipette with a narrow opening. The opening can be several times the egg diameter. A concentrated suspension of eggs at the time of membrane elevation is rapidly drawn into the pipette, and expelled into an empty dish and examined. If one treatment is inadequate it may be repeated.

Separation of Blastomeres. For this purpose the student may use either the Ca-free sea-water (Herbst, 1900) or the hypertonic sea-water (E. B. Harvey, 1940) method outlined below. Remove fertilization membranes from eggs and use the control sample of eggs fertilized minutes before the experimental set to determine expected time of first cleavage of the membrane-less eggs.

A. Ca-free sea-water. At about five minutes before the time of first division, concentrate the membrane-less eggs in the center of the dish by gentle revolution of the dish (the center of the dish

Specific Ineracting Substances of Eggs and Sperm

F. R. Lillie (1912) demonstrated that ripe eggs of Arbacia and of Nereis give off a substance, called fertilizin, which activates and agglutinates the species sperm. Similar fertilizins have since been reported in many species of invertebrates and they, together with the antifertilizins from sperm with which they react, have been the subject of many investigations, of which most of the more recent are listed below along with some of the older ones. The present exercise includes more tests with this material than can be completed in the time allotted. Only the simpler tests in the first part of each of the following sections should be undertaken during the class period assigned to the work. The additional material is presented as a guide to further work for those who may elect such investigations at the end of the course.

Equipment:- 4 finger bowls, 50 syracuse dishes, 1 graduate (100 ml), 1 graduated pipette (5 ml), 4 ordinary pipettes (droppers), 1 large pipette (25-50 ml cap. - 25-50 ml rubber bulb), 2 centrifuge tubes (15 ml).

Solutions:- 1 liber filtered sea water; 10 ml 1N HCl; 10 ml In NaOH

Fertilizin:- Obtain eggs and sperm of Arbacia by one of the usual methods. Wash the eggs once and concentrate the suspension to about 25% by volume. After about 15 minutes mix 2 drops of the supernatant egg water with 2 drops of a 1% sperm suspension (one drop of "dry" sperm in 5 ml of sea water) and examine with the microscope. Note the agglutination of the sperm and, a few minutes later, the reversal of the clumping. Are the sperm still motile after reversal? To 2 drops of a strong egg-water (in which eggs have stood several hours, or obtained by acidification-see below) and to 2 drops of sea water in a control dish add 2 drops of 1% sperm. After reversal of the agglutination add 2 drops of egg-water to each dish. Do the reversed sperm re-agglutinate?
To 5 ml of a strong egg-water and to 5 ml of a control dish of sea water add 1 drop of 'dry' sperm. Shake the dishes. What difference in behaviour of the drops of sperm do you observe and how do you account for it?
To 1 ml of a strong egg-water and to 1 ml of a control dish of sea water add 2 drops of 1% sperm. After agglutination has reversed add 1,2,4 and 8 drops from each to dishes containing 5 ml of a dilute suspension of eggs (about 100 eggs per ml). Determine the percentage fertilization in each of the 8 dishes. Has the egg-water treatment had any effect on the fertilizing power of the sperm
Titration of fertilizin solutions may be done by testing serial dilutions of the solution with a standard sperm suspension. The dilutions may be prepared with an ordinary pipette (dropper) as follows:-Place 2 drops of sea water in each of a set of dry dishes. Add 2 drops of egg-water to the first dish, rinse the pipette with sea water, mix the drops, draw up most of the mixture, expel 2 drops into the second dish and return remainder to the first dish. Repeat this procedure with the succeeding dishes. Then add 2 drops of 1% sperm to each dish and examine at once. The first dish contains a four-fold dilution of the egg water, the second eight fold, etc. The fertilizin titer can be expressed as the greatest dilution of egg-water that gives a microscopically perceptible agglutination reaction. Titrate your egg water using eight 2-fold dilutions.

Evidence concerning the source of the fertilizin may be obtained from the following tests. Divide about 200 ml of a freshly prepared 10% egg suspension in two equal parts and acidify one part to about pH3 to 3.5 (requires about 1 ml of 1N HCl per 100 ml). A few minutes later draw off 50 ml of supernatant from each dish, neutralize the acid egg-water with 0.5 ml of 1N NaOH and determine the fertilizin titers. Examine the acid-treated eggs with the microscope and note the absence of the gelatinous coat. Neutralize and wash the acid-treated eggs. After several hours determine fertilizin-titer along with that of the similarly aged control. Acidify the control to pH 3 to 3.5, draw off the supernatant, neutralize and compare its fertilizin titer with that of the first acid-egg-water. Is there evidence of secretion of fertilizin by the eggs? What is the apparent source of the fertilizin? To test for activating action of fertilizin allow a dilute (1%) sperm suspension to stand for 1 hour or until motility has decreased considerably; then add 2 drops of a strong egg water to 2 drops of sea water. Examine the two dishes microscopically and note roughly the activity exhibited by the spermatozoa. Absorption tests may be made by adding a concentrated sperm suspension (10% or greater) to an equal volume of moderately strong egg-water, centrifuging after 1 hour and testing the supernatant as well as a similarly diluted sample of the egg-water for agglutinating action on dilute (1%) sperm. Does absence of agglutinating action necessarily mean binding of fertilizin by the sperm? What other tests would be necessary? Specificity may be examined by testing Arbacia fertilizin on sperm of closely related and distantly related animals, that are available in the laboratory. Where reactions are obtained absorb the Arbacia egg-water with the foreign sperm, as described above, and test the supernatant on both species and foreign sperm.

Antifertilizin:- This material may be prepared from a concentrated (10% or greater) sperm suspension by (a) freezing and thawing the suspension, (b) heating to 100°C for one minute or (c) acidifying to pH3. The treated suspension is then centrifuged or filtered and the supernatant or filtrate will be found to contain the active material.

To demonstrate the agglutinating action of antifertilizin add 2 drops of the solution prepared by methods a, b, or c to 2 drops of 1% suspension of freshly washed eggs. Shake the dish several times and examine macroscopically and microscopically after 1 to 5 minutes. Note the formation of a precipitation membrance on the surface of the gelatinous coat of the egg.

Titration of the antifertilizin is performed in the manner described above for fertilizin. Prepare a set of 8 two-fold serial dilutions of 2 drops of the antifertilizin solution and add to each 2 drops of a dilute (ca. 100 eggs per drop) freshly washed, egg suspension. Examine at once and again after ½ hour. Note differences in width of the gelatinous coat and in the precipitation membrance in the different dilutions and at different times. The end point may be taken as that dilution beyond which a precipitation membrane is no longer visible. Determine the titer of your preparation. Does the egg-agglutination reaction revers spontaneously?

To demonstrate neutralization of the fertilizin add 2 drops of strong antifertilizin solution to 2 drops of a moderately strong egg-water. Prepare a control of 2 drops of egg water plus 2 drops of sea water. After ½ hour add 2 drops of a 1% sperm suspension to both dishes. Note the degree and duration of the agglutination reaction. Titrations may be performed with duplicate serial dilutions of the egg water to one set of which is added a constant amount of the antifertilizin solution while the other gets an equal volume of sea water then sperm added after ½ hour; or with duplicate serial dilutions of the antifertilizin plus constant egg-water to one set and sea water to the other, then eggs added after ½ hour.

References:-

Gray, J. S., 1932 Brit. Journ. Exp. Biol., 9:253-263
Cornman, I., 1940. Biol. Bull., 80:202-207
Frank, J. A., 1939, Biol. Bull., 76:190-216
Glaser, O., 1914. Biol. Bull., 26:367-386
Glaser, O., 1921. Biol. Bull., 41:63-72
Hartman, M., 1940. Naturwissenschaften 51:807-813
Hartman, M., R. Kuhn, O. Schartau and K. Wallenfels, 1939. Naturwis 27:433
Hartman, M., R. Kuhn, O. Schartau and K. Wallenfels, 1940. Naturwis 28:144
Hartman, M. and O. Schartau, 1939. Biol. Zentralbl. 59:571-587
Hartman, M., O. Schartau and A. Wallenfels, 1940. Biol. Zbl 60:308-423
Just, E. E., 1915. Biol. Bull., 28: 93-114
Just, E. E., 1919. Biol. Bull., 36:11-38
Just, E. E., 1930. Protoplasma, 10:300-342 (a review)
Kuhn, R. and K. Wallenfels, 1939. Ber. dtsch Chem Ges 72:1407-1413
Kuhn, R. and K. Wallenfels, 1940. Ber dtsch Chem Ges. 73:458-464
Lillie, F. R., 1912. Science, 36:527-530
Lillie, F. R., 1913. Jour. Exp. Zool., 14:515-574
Lillie, F. R. 1914. Jour. Exp. Zool., 16:523-589
Lillie, F. R. 1915. Biol. Bull., 28: 18-33
Lillie, F. R. 1919. Problems of Fertization. Univ. Chicago Press
Lillie, F. R., Biol. Bull., 40:1-23
Lillie, F. R., and E. E. Just, 1924. Fertilization. In Cowdry's Gen. Cytol
Loeb, J. 1914, Jour. Exp. Zool. 17:123-140
Loeb, J. 1915, Amer. Naturalist, 49:257-285
Medem, F. V. 1942. Biol. Zentralbl, 62:431-446
Metz, C. B. 1942. Biol. Bull., 82:446-454
Metz, C. B. 1945. Biol. Bull., 89:84-94
Montalenti, G. and O. Schartau, 1941 Pubblicaz. Staz. Zool. Napoli 19:48-55
Moore, C., 1916, Biol. Bull. 31:137-180
Moore, C. 1917, Biol. Bull. 33:285-295
Nomura, S. 1924 Sci. Rep. Tohoku Imp. Univ. 4th Ser. Biol 1:83-87
Richards, A. and A. E. Woodward, 1915. Biol. Bull. 28:140-148
Runnstrom, J., A. Tiselius and E. Vasseur 1942, Ark. f. Kemi 15A:N:
Runnstrom, J., A. Tiselius and S. Lindvall, 1944 Nature 153 016,1-18
Runnstrom, J., A. Tiselius and S. Lindvall, 1945, Ark. f. Zool. 36a: No 22,1-25 285
Sampson, M. M., 1922, Biol. Bull., 43:267-284
Southwick, W. E. 1939, Biol. Bull. 77:147-156
Taneo, A. 1926. Sci Rep. Tohoku Imp. Univ. 4th ser. Biol. 2:127-132
Tyler, A. 1939, Proc. Nat. Acad. Sci., 25:317-323
Tyler, A. 1939, Proc. Nat. Acad. Sci., 25:523-528
Tyler, A. 1940, Biol. Bull., 78:159-178
Tyler, A., 1940. Proc. Nat. Acad. Sci., 26:249-256
Tyler, A., 1941 Biol. Bull., 81:190-204
Tyler, A. 1942, West. Journ. Surg. Obstet. & Gynec. 50:126-138

Tyler, A. 1942, Prof. Nat. Acad. Sci., 28: 391-395
Tyler, A. and S. W. Fox 1939. Science 90: 516-517
Tyler, A. and S. W. Fox, 1940, Biol. Bull. 79: 53-165
Tyler, A. and K. O'Melveny, 1941. Biol. Bull 81: 364-374
Woodward, A. E. 1918, Jour. Exp. Zool., 26: 459-500

General Review: Tyler, A. 1948 Phys. Rev. 28: 180-219

Production of Exogastrulae in Arbacia and Echinarachnius

Curt Herbst in 1892 discovered that treatment of developing sea-urchin eggs with sea water containing lithium salts results in the formation of exogastrulae and other related types of abnormal embryos. This has been the subject of numerous investigations since that time (See Child, 1940, 1941; Lindahl, 1940 and your experimental embryology texts for complete references). The exogastrulating action of the lithium is interpreted as a result of a general vegetalization of the egg in which the vegetal, endodermal and mesenchymal, material increases at the expense of the animal, ectodermal, material. Other agents may bring about this effect but none are, as yet, known to give as consistent results as lithium.

Equipment:- Scissors, blunt forceps, 6" square of cheesecloth, 3 finger bowls, 12 syracuse dishes, 4 stender dishes with lids, 3 slides and coverslips, 1 graduate (100 ml), 3 ordinary pipettes, 1 narrow long-tipped pipette, 1 fine pipette.

Solutions:- 1 liter filtered sea water
50 ml Li-sea-water (20 ml of m/2 LiCl + 80 ml sea water)

Treatment:- Obtain eggs and sperm of Arbacia or of Echinarachnius in the usual way. Inseminate a fairly large sample of the eggs in a finger bowl. At the time of first cleavage transfer a sample of concentrated eggs (with not more than 1 ml of sea water) to a finger bowl containing about 50 ml of the Li-sea-water and, at the same time, a similar sample to a control dish of sea water. After 2, 4, 8 and 12 hours transfer samples (ca. 0.5 ml) through three dishes of 10 ml of sea water and culture in half-filled, covered, stender dishes.

Development:- Observe the eggs at various times during the lithium treatment and compare their rate and form of cleavage with the controls. Examine the cultures twice a day during the next three days, and sketch various types of exogastrulae, noting inhibition of development of arms and ectodermal structures, tripartite structure of archenteron, etc. Determine the approximate proportion of normal to abnormal embryos in the four cultures.

Li-treatment of Isolated Animal Halves. Animal halves of sea-urchin eggs isolated in the 8-or 16-cell stage fail to gastrulate generally forming "Dauerblastulae". Von Ubisch (1929) made the interesting discovering that lithium treatment would enable some of the animal halves to develop into normal plutei. Students who are skillful in micro-dissection may substitute this experiment in place of the above, after discussing details of procedure with the instructor.

References:-

Child, C. M., 1940 Physiol. Zool. 13: 4-42
Child, C. M., 1941. Patterns and Problems of Development. Univ. Chic. Press
Herbst, C. 1892. Zeitschr. wiss. Zool 55:446-518
Lindahl, P. E. 1940, Arch. Entw.-mech., 140:168-194
MacArthur, J. W., 1924, Biol. Bull, 46: 60-87
Von Ubisch, L. 1929, Arch. Entw.-mech., 117: 80-122
Tamini, E., 1943, Reale Instituto Lombardo (Rend. Sci.) 76: 363-392

should follow the circumference of a circle about 1 to 2 cm. in diameter) and transfer, with the narrow long-tipped pipette, a sample of the eggs with less than 0.1 ml of sea-water through three dishes of 10 ml of Ca-free sea-water. Examine, under high power, a sample of the eggs in the Ca-free sea water and compare their ectoplasmic layer with that of the control eggs. After 10 to 20 minutes remove a sample to a dry finger bowl. If the blastomeres have not separated draw the sample rapidly in and out of the pipette several times. Fill the bowl with sea water and transfer to a stender dish half filled with fresh sea water and cover. This will serve as a mass culture of isolated blastomeres along with some whole eggs. To study pairs of blastomeres from the same egg pick out of the Ca-free sea water dish, under the dissecting microscope, eggs in which the blastomeres are still together or are close enough together to be recognized as sister. Transfer each pair along with a whole egg to a separate embryological watch glass containing sea water. If the blastomeres of the pair were not completely separated at the time of selection bounce the egg in the dish a few times or separate the blastomeres by means of a glass needle before transferring to the sea water. After one or two cleavages mount pairs of isolated blastomeres along with a whole egg on each of two or three of the vaseline-ringed slides. To do this place a small drop containing the eggs in the center of the ring, add a coverslip and press it down so that it touches the drop and a continuous seal is made with the vaseline, but avoid having the drop touch the vaseline.

B, Hypertonic sea-water. With this method, the eggs (one drop) are placed in the solution (about 10 ml) when most of them have just completed the first cleavage but before maximal separation of the blastomeres has occurred. Examine, under high power, a sample of the eggs in the hypertonic solution and note the effect on the ectoplasmic layer. Ten minutes later, transfer the eggs with a minimum amount of solution through three dishes of normal sea-water. The treatment, if successful, causes the ectoplasmic layer, by which the two blastomeres are joined, to become thin and gelatinous, and the two blastomeres are often widely separated with only a thin film between them. Such pairs may be cultured along with control whole eggs, in embryological watch glasses and on vaseline-ringed slides as described above. They may be picked out at a later stage of development since the pairs generally remain attached by a thin hyaline strand until they are swimming blastulae. Only a few twin blastulae are ordinarily obtained by this method since the great majority fuse together during early development.

Development. Observe and sketch the isolated blastomeres in their 4-,8-, and 16-cell stages. How many micromeres are formed at what stage? Does the isolated blastomere cleave as though it were still part of a whole egg? At the beginning of gastrulation fix some "half"-embryos and whole embryos in 5% formalin and measure their respective diameters. What approximate ratios are obtained for their respective volumes, surface areas and wall-thicknesses? Examine and sketch the embryos in the completed gastrula, prism and pluteus stages. Do the "half"-embryos develop at the same rate as the controls? Are the "half"-plutei complete in regard to all structures seen in the whole plutei? Determine whether or not both members of the pairs of isolated blastomeres form normal plutei. (See Hörstadius 1939; Tyler, 1942, and experimental embryology texts for further analysis.

References:-

Driesch, H., 1891. Entwicklungsmechanische Studien. 1. Der Werth der beiden ersten Furchungszellen in der Echinodermentwicklung. Experimentelle Erzeugung von Theil - und Doppelbildungen. Zeitschr. f. Wiss. Zool. 53: 160-184.

Harvey, E. B., 1940. A new method of producing twins, triplets and quadruplets in *Arbacia punctulata*, and their development. Biol Bull. 78: 202-216.

Herbst, C., 1900. Über das Auseinandergehen von Furchungs - und Gewebezellen in kalkfreiem Medium. Arch. F. Entw.-mech., 9:424-463

Horstadius, S., 1939. The mechanics of sea-urchin development studied by operative methods. Biol. Rev., 14: 132-179.

Lyman, J. and R. H. Fleming, 1940. Composition of sea water. Jour. Marine Research, 3: 134-146

Tyler, A. 1942. Developmental processes and energetics. Quart. Rev. Biol. 17: 197-212, 339-353

Asterias forbesii (or A. vulgaris)

A) Obtaining gametes, maturation and fertilization:

The sexes are separate in Asterias, but it is not possible to distinguish them on the basis of external characteristics. Only animals with soft, bulging arms are fully ripe, and it is a waste of material to open small, hard-skinned starfish in an attempt to obtain gametes. Fill two 1750 cc. finger bowls wit h clean sea water from a 2-liter flask in which the sediment has been permitted to settle by about fifteen minutes of standing after withdrawal of the sea water from the tap. With large scissors, make a small puncture in one arm close to the disc, and pipette a few drops of cells from the gonad to ascertain the sex of the animal. If the animal is a female, remove this arm completely, and slit it along the mid-dorsal line to expose the bulging pair of ovaries, of a typical pale salmon color. Then with a pair of forceps carefully detach each plume-like ovary by grasping it near its point of attachment at the disc end, closing the gonaduct, and rinse it with as little injury as possible in the first bowl of sea-water, then transfer it to the second bowl. The animal from which the arm has been separated may be returned to a separate aquarium of running sea water, and other arms may be used for gametes later in the day. Such an injured female will not keep indefinitely, however, and gametes are rarely usable at the time the animal begins to show autotomy. Do not cut up to ovaries in the bowl of sea water, merely allow the eggs to exude from the blunt end of the ovaries for a period of five minutes. At the end of this time, remove the ovaries to another container, or discard them. The best eggs are those first shed. Gently stir the water in the large finger bowl and allow the eggs to settle. Settling occurs very slowly. Then pour off the supernatant sea water and carefully replace with an equal volume from a 2-liter flask. Then leave the eggs undisturbed, without shaking or stirring, for about 20 to 30 minutes. During this time small samples may be removed with a pipette for examination under the microscope, and the steps in germinal vesicle breakdown observed. Note the jelly-hull about the eggs. This may be demonstrated more readily in dim illumination or by adding a trace of Janus Green to one slide preparation. Eggs from a ripe female which was kept under proper conditions of coolness and adequate oxygen supply from the moment of collection, and properly manipulated in obtaining gametes, should show 85 to 90% germinal vesicle breakdown at approximately the same time. Retain a good sample of eggs in a small finger bowl to follow the maturation stages through the second polar division in the uninseminated eggs.

If the animal opened is a male, the testes will be white or ivory. Since it is important to use a fresh sperm suspension, this animal may be placed in a dry fingerbowl until the eggs are ready for fertilization. Then a single testis is removed, rinsed in clean sea water, and a small piece from the blunt end cut off and placed in 200 cc sea water. Two or three pipettes of this suspension should be added to a 1750 cc. finger bowl of eggs, with an immediate but not violent rotational movement to ensure complete mixing. The optimum period for fertilization is after the breakdown of the germinal vesicle and before the 1st polar body has been extruded. It is, therefore, convenient to inseminate when the distal end of the first maturation spindle begins to protrude above the previously smooth surface of the oöcyte, in a fair percentage of the eggs showing germinal vesicle breakdown. Eggs inseminated in the stage of the intact germinal vesicle are non-fertilizable. Even tho they may elevate a fertilization membrane they do not develop further. The details of sperm penetration may be readily studied, if the observer examines the eggs without delay. It was in the egg of the starfish that Fol (1879) first observed the actual penetration of an egg by a sperm. Chambers (193 0) has confirmed these early observations. A microscope with clear objectives, clean slides and covers, and good illumination are prerequisites for observing the finer details of this process in the laboratory.

It must be remembered that the egg of Asterias is very delicate as compared with most eggs used for routine laboratory work. Satisfactory results are not obtained without taking adequate precautions. Important precaustions are: (1) to avoid contaminating either type of gamete with perivisceral fluid-it is because of this that the gonads are rinsed; (2) do not overinseminate; (3) do not crowd the eggs: there should be no more than one layer of well-spaced eggs on the bottom of the dish; (4) use only fresh, motile sperm.

Cleavage:

The blastomeres of Asterias are rather loosely connected, because the perivitelline space is wider and the hyaline plasma membrane thinner and weaker than in the Arbacia egg. Note the relation between the first and second polar bodies and the fertilization membrane. Chambers has pointed out that in the absence of fertilization membrane, the blastomeres tend to separate completely. Because of the relative transparency of the yolk of this egg, details of living asters may be seen.

A detailed study of the cleavage of this form is usually not undertaken by students of the course unless they have a special interest in this material, but the later stages are of considerable significance.

Later Stages:

To raise Asterias to late embryonic stages, it is necessary to change the sea water in the culture dishes at about half-hour intervals during early cleavage to eliminate the excess sperm which would otherwise foul the culture. Then, when the first swimming stages (blastulae) appear, the upper half of the culture, containing the more normal top-swimming blastulae, is poured off into a series of tall battery jars which are subsequently filled to the top with fresh sea water. Care must be taken to avoid carrying over dead embryos of unfertilized eggs. Tall jars are superior to shallow dishes, since evaporation in considerably reduced. It is essential that relatively few larvae be placed in a jar. Early bipinnaria may be obtained without special feeding but the cultures of Asterias larvae must be fed diatoms (prepared by Just's methods) to obtain brachiolaria or later stages.

References:

Chambers, R. 1930 The manner of sperm entry in the starfish egg. Biol. Bull 58.
Costello, D. P. 1935 Fertilization membrances of centrifuged Asterias Eggs. Physiol. Zool. 8 65-72
Delage, Y 1904 Elevage des larves parthénogénitiques d'Asterias glacialis. Arch. de Zool. Exper. 43 Ser.2
Fol, H.1879. Recherches sur la fecondation et le commencement de l'hemogonie. Mem. Soc. Phy. et Hist. Nat. Geneve 26
Fry, H. J. 1937. Article (p.547) in Culture Methods for Invertebrate Animals, edited by Galtsoff, Lutz, etc. Comstock Publ. Co.
Gemmill, J. F. 1914. The development and certain points in the adult structure of the starfish, Asterias rubens. Philos. Trans. Roy. Soc. London B. vol 205.
Just, E. E. 1939. Basic methods for experminents on eggs of of marine animals. Blakiston, Phil.
Larson, E. J. Article (p 550) in Culture Methods for Invertebrate Animals edited by Galtsoff, Lutz. etc. Combstock Pub. Co.

Asterias Forbesii

B. Development

Prepare your own cultures for early stages and use those prepared by the assistant for stages from blastula on. Development up to the early Bipinnaria (Dipleurula) can be followed on living material. Older Bipinnaria, Brachiolaria larvae and metamorphosis stages will be studied on stained whole mounts. Whole mounts of early Bipinnaria are also available for comparison. It is advisable to begin with early gastrula stages and follow the development through to the Dipleurula.

Study fertilization, cleavage and blastula later on, using your own cultures. Consult the illustrations in MacBride, Agassiz (1877) and Gemmill (1914) See time table in appendix.

1. Fertilization (see lab directions for "Fertilization")

2. Cleavage. The first two cleavages are meridional, that is, they go through animal and vegetal poles and are perpendicular to each other. The third cleavage is horizontal, the eight cells of this stage are approximately equal in size. In the 16-cell stage, no definite arrangement of cells in rows takes place, and cleavage from now on is irregular. Throughout these early cleavage stages the blastomeres have a tendency to assume spherical shape, resulting in a rather loose arrangement of cells.

3. Blastula. Eventually the cells arrange themselves in an epithelial wall enclosing the blastocoele. The surface cells acquire cilia, and the blastula rotates within the vitelline membrane. The two polar bodies are still visible, either attached to the animal pole, or detached from the embryo. The embryo hatches in the late blastula stage.

4. Early gastrula. The vegetal pole area thickens and flattens and invagination begins. The blastopore is destined to become the anus. The larva elongates along the animal-vegetal axis.

5. Middle and late gastrula. The gastrula becomes pear-shaped. The blind inner end of the archenteron becomes thin-walled and expands. From this end mesenchyme cells wander out into the blastocoele. In a slightly later stage, two outpocketings of the distal end become distinct, the primordia of the coelomic sacs. At the same time, the archenteron bends towards one side which is the future ventral side. This is the first sign of the change of radial into bilateral symmetry. Note the ciliation in the archenteron.

6. Transition to Dipleurula-larva. The blind end of the archenteron bends sharply towards the ventral body wall, makes contact with an ectodermal depression, the stomodaeum, and the mouth breaks through. The two coelomic vesicles have been constricted off from the archenteron. The left one is larger from early stages on. It forms a tubular outgrowth to the dorsal body wall which opens to the outside. This is the pore-canal.

7. Fully formed Dipleurula (early Bipinnaria) larva. This larva represents an early larval type common to Asteroidia, Echinoidea, Ophiuroidea and Holothurea (see Korschelt, vol. 1 p. 499). Study carefully a ventral, dorsal and lateral (preferably left) view

Observe the following:

Shape of larva. Notice convexity of ventral side and mouth opening underneath the overhanging oral lobe.

Locomotion

Ciliation. Small cilia cover the entire surface. The ciliary band is at first continuous, a longitudinal band with two cross bars. The longitudinal band above the upper cross bar loops towards the midline. Eventually the loops meet in the midline, and a frontal field, the pre-oral ciliary band is separated in the upper ventral part of the larva, overhanging the oral field. This separate frontal field is characteristic of Asteroid larvae. Observe carefully the course of the entire longitudinal band. Observe the beat of the cilia in dark field illumination, if available.

Alimentary tract. The three parts, characteristic of Echinoderm larvae: oesophagus (with constriction near entrance into stomach,) stomach and intestine. Observe in lateral view the bend of the intestine. Study ciliation in oral field and different parts of the tract.

Coelom. Study the two coelomic vesicles from all sides. They are clearly visible at the lower end of the oesophagus near its entrance into the stomach. A subdivision of the vesicles is not yet clearly demarcated, but the narrow tube connecting the larger left coelomic vesicle with the dorsal body wall, the pore canal, and its opening, the madreporic pore, can be readily seen. Loose mesenchyme cells are scattered in the body cavity which is the persisting blastocoele.

Vital staining with neutral red is helpful; but study unstained specimens first. Study also stained whole mounts of these stages.

The following stages are rarely found in dredges of plankton and difficult to raise from eggs in the laboratory. Prepared and stained whole mounts will be provided.

8. Fully formed Bipinnaria. Consult figures in Gemmill, MacBride, and Agassiz. This larva is characterized by a number of pairs of lobes or arms which grow out from the margin of the ectoderm and which carry the ciliary band along. They are not supported by skeleton. Pairs of arms follow each other in succession. Young stages may not have all arms developed.

Arms Identify unpaired median dorsal, paired anterior dorsal, posterior dorsal, posterior lateral, postoral and preoral arms. See Gemmill, plate 18, fig. 7, and MacBride, p. 465.

Intestinal Tract

Coelom. The coelomic vesicles have grown out into long tubes and have fused in the anterior part of the larva. No further subdivisions have yet occurred.

9. Brachiolaria and metamorphosis stages. Study ventral and lateral views. Consult the excellent figures in Gemmill, plates 19 and 20.

The Bipinnaria arms are long, hollow tubes.
The three Brachiolaria arms (brachia) are short. They contain diverticula of the coelom. They are not ciliated but their end discs differentiate small papillae and can adhere to the substrate.

A sucker, the gland cells of which secrete a sticky substance, is formed between the brachia. Brachia and sucker serve for attachment of the larva to the substrate in later stages of metamorphosis. (Gemmill, p. 250).

Intestine

Coelom, in different stages of subdivision

Disk, or developing starfish, on left side.

In late stages of metamorphosis, the anterior part of the larva in front of disk shrinks to form the stalk which is attached to the substrate by sucker and branchia and which carries the Asterias anlage at its distal end. For details of metamorphosis consult Gemmill, MacBride, and Korschelt.

Echinarachnius parma

Obtaining gametes

The sexes are separate in Echinarachinus (the sand dollar) but it is impossible to distinguish the male from the female by superficial examination. A cut is made about one-quarter inch from the margin around the entire animal through both oral and aboral calcareous skeletal parts. Then a scalpel is carefully inserted, just beneath the oral skeleton, separating the oral and aboral portions. The oral portion is lifted away and discarded, taking care not to destroy the gonads, which adhere to the aboral portion. The aboral portion is then placed (outside surface down) on a clean, dry Syracuse watch-glass. If the animal is ripe, gametes will ooze from the gonads. Allow the opened male to remain undisturbed until the eggs are to be inseminated. The ovaries of the female are a reddish purple color, and the eggs are usually mixed with an opalescent or milky peri-visceral fluid. From the female carefully pipette the eggs to a small finger bowl of sea water. After allowing the eggs to settle, carefully pour off the supernatant fluid and replace with fresh sea water. If the females are not in good condition (if the eggs do not readily stream from the ovaries), the gonads may be removed with a forceps to a finger bowl of sea water, and the egg suspension strained through clean, washed cheesecloth previously soaked in sea water.

Fertilization

The eggs of Echinarachinus are larger than those of Arbacia (135 microns as compared with 75) and surrounded by a much thicker jelly-hull in which beautiful red pigment granules are suspended. The egg itself, free of the jelly, is pale yellow. Examine the unfertilized eggs under low and high magnification. Then inseminate the eggs as was done in the case of Arbacia, and examine the eggs immediately after adding the diluted sperm suspension. Because of the relatively large size of the egg, the fertilization reaction may be readily followed. Membrane elevation proceeds from the entrance point of the sperm around the egg cortex in a wave. The membrane begins to elevate in from seven to twenty-two seconds after sperm penetration, and is completed in from nine to thirty seconds after it begins. Since sperm penetration occurs from fourteen to forty-five seconds after insemination, both processes (i.e., sperm penetration and membrane elevation) may be completed within about 40 seconds after insemination (Just, 1919).

Cleavage of the egg of Echinarachnius is not markedly different from that of Arbacia, and unless this form is of special interest to the student, detailed drawings of the cleavage need not be made. One culture should be prepared and kept to provide plutei for comparison with those of Arbacia.

References:

Just, E. E. 1919 The fertilization reaction in Echinarachinus parma. 1. Biol. Bull. 36: 1-10
Just, E. E. 1923 VI. Biol. Bull. 44: 1-9

Bibliography on Echinoderm Development

1. Normal development and metamorphosis

Agassiz, A. 1877. Embryology of the Starfish. Mem. Mus. Comp. Zool. Harvard, vol 5 (first publ. in 1864)

Bury, H. 1895. Metamorphosis in Echinoderms. Quart. J. Micr. Sci. vol. 38

Chadwick, H. C., 1914. Echinoderm larvae. Liverpool Marine Biol. Committee Memoirs, No. 22

Gell, H. B., 1948. Echinoderm embryology and the origin of chordates. Biol. Rev. 23: 81

Gemmill, J. F., 1914. The development and certain points in the adult structure of the starfish, Asterias rubens. Philos. Trans. Roy. Soc. London, Ser. B., vol. 205 (excellent figs. of Bipinnaria and Brachiolaria)

Goto, S. 1898. The metamorphosis of Asterias pallida. Jour. Coll. Sc., Imp. Univ. Tokyo, vol. 10

Grave, Caswell, 1900. Embryology of Ophiocoma echinata. Mem. Biol. Johns Hopkins Univ., vol. 12.

Harvey, Ethel Browne, 1939. Arbacia. Collecting Net, vol. 14

------- 1941. The cytology of fertilization and cleavage of Arbacia punctulata. Turtox News, vol. 19, no. 7

Hoerstadius, Sven, 1939. Ueber die Entwicklung von Astropecten auranciacus. Publ. Staz. Zool. Napoli, vol. 17

MacBride, E. W. 1903. Development of Echinus esculentus. Philos. Trans. Roy. Soc. London, Ser. B., vol. 195 (good figs.)

Mortensen, Th., 1931-1938. Contributions to the study of the development and larval forms of Echinoderms. 1-IV. Mem de l'Acad. de Sci. Copenhagen. Sect. de Sci., Ser. 9 vol 4-7

Müller, Joh., 1848-1855. Ueber die Larven und die Metamorphose der Echinodermen. Abh. Königl. Akad. Wiss. Berlin.

Schmidt, H. 1904. Zur Kenntnis der Larvenentwicklun von Echinus microtuberculatus. Verh. Phy.-med. Gesellsch. Wurzburg. vol 36

von Ubisch, L. 1937. Die normale Skelettbildung bei Echinocymus pusillus und Psammechinus miliaris. Zeitschr. Wiss. Zool. vol 149 (good figs. of origin of larval skeleton)

II. Experimental: general reviews:

Brachet, J. 1944. Embryologie Chimique. Masson & Cie., Paris

Child, C. M. 1941. Patterns and problems of development. Univ of Chicago Press.

Dalcq., A. 1928. Les Bases Physiologiques de la Fecondation. Presses Univ. of France, Paris.

Dalcq., A., 1938. Form and Causality in Early Development Cambridge Univ. Press.

Faure-Fremiet, L. 1925. La Cinetique du Development, Presses Universitaries de France, Paris

Horstadius, S., 1939. The mechanics of sea urchin development, studied by operative methods. Biol. Rev. 14: 132-179

Huxley, J. and DeBeer, R. 1934. Elements of Experimental Embryol.

Lindahl, P. E. 1942. Contributions to the physiology of form generation in the development of the sea urchin. Quart. Rev. Biol. 17

Lindahl, P. E. 1941. Physiologisch-chemische Probleme der Embryonal entwicklung. Fortschr. der Zool. 5: 187-359

Morgan, T. H. 1927. Experimental Embryology. Columbia Univ. Press. NY

Needham, J. 1942. Biochemistry and Morphogenesis, pp 476-50?. Cambridge Univ. Press, London

Schleip, W., 1929 Determination der Primitiv-Entwicklung 542 pp Akad. Verlages. Leipzig.

Tyler, A. 1941 Artificial Parthenogenesis. Biol Rev. 16: 291-336

Tyler, A. 1942 Developmental processes and energetics. Quart. Rev. Biol. 17: 197-212 & 339-353

Weiss, P. 1939 Principles of Development, Holt & Co. New York

Wilson, E. B. 1925. The Cell in Development and Heredity. MacMillan Co.

Embryology of Annelida

Nos. 1, 2 and 3 are required, #4 should be done if time permits.

The three forms to be studied are Hydroides (Eupomatus) hexagonus, Nereis and Sabellaria.

Cultures of advanced stages will be prepared. If you wish to prepare your own cultures proceed as follows: Hydroides both male and females will spawn immediately after being removed from their calcareous tubes. Remove several and place them in finger bowls (one worm per dish to keep the sexes separate). Remove the animals after they have spawned. Let sperm stand for about ½ hour. Add a few drops of sperm suspension to a dish of eggs. (Extrustion of polar bodies and cleavage may be easily studied). The blastula stage is reached after 5-6 hours, gastrulation after 8-12 hours; the trochophore stage lasts from 20 hoursto two weeks. The trochophores are best for study when 2-5 days old. Sabellaria may be treated in the same way. Fertilization of Nereis has been studied in a previous lab period.

1.

The Trochophore of Hydroides.

The Trochophore is a typical Annelid trochophore. Consult the excellent figs. in Hatschek ('86) and Shearer ('11). The larvae show positive phototaxis and gather at the window side of the dish. Mount trochophores, 3-5 days old, on a slide on which a few shreds of lens paper have been placed to entangle them and hold them quiet. Narcotics, e. g., a few drops of chloretone or of $MgSO_4$, are not very effective but may be tried. Vital staining obscures rather than clarifies the structures. The larvae are transparent, and proper adjustment of the illumination by moving the mirror and the Abbe condenser will bring out all structures. Study animals in lateral and in polar views (both from animal and from vegetal pole). The apical tuft and the anal visicle are landmarks for the poles, the mouth is on the ventral side; the eye is on the right side. Observe the locomotion first.

Observe:

1. Shape of the trochophore
2. Apical tuft (several long cilia, probably functioning as a sense organ).
3. Apical organ, a thickening of ectoderm at the animal pole; a nerve center and probably the primordium of the cerebral ganglia.
4. The Prototroch, an equatorial band of large cilia. In older trochophores, two rows of cilia will be found; a row of short cilia anterior to the large cilia. The prototroch is the most characteristic structure of the larva, and gave it its name. It is always anterior to the mouth (prooral). It consists of a few large prototroch cells which become pigmented in older stages.
5. The metatroch (paratroch), a circular band of cilia in the middle of the posttrochal hemisphere.
6. A ciliated groove on the midventral line connecting the mouth and anus. This groove is interesting in that it marks the line of closure of the blastopore. The mouth is the remnant of the blastopore; the anus is a secondary opening at the lower end of the original blastopore slit.
7. One eye on the right side of the pretrochal hemisphere. Note the red eye pigment.
8. Two statocysts on the ventral side.
9. The digestive tract, Consisting of: mouth opening, stomadaeum (= oesophagus; ectodermal), enlarged stomach (entodermal), narrow intestine (entodermal except for the end portion which is invaginated ectoderm = proctodaeum), and the anus, an opening behind the vegetal pole. All parts are beset with cilia. Feed india ink and study the mechanism of food intake.

10. The anal vesicle, a large vacuolated cell at the posterior end, not found in other trochophores.
11. The cavity between intestine and outer body wall is not a true coelom but a primary body cavity, the persisting blastocoele.
12. The larval kidnesy (paired) are typical protonephridia with flame cells; they open near the anus. They appear as slender cords near the statocysts, extending between oesophagus and anus. They are best identified in animals with vegetalpole up (consult figs. in Batschek and Schearer)
13. Muscles. Two fine strands will be seen bifurcating at the upper end of the larval kidney. One of them can be traced to its insertion at the apical plate, the other inserts at the oesophagus. These are longitudinal muscles. Other longitudinal muscles extend from the stomach to points of the upper hemisphere. A strong circular muscle is near the metatroch: the constriction of the larva caused by its contraction willbe frequently observed. Note also circular (sphincter) muscles in the digestive tract.
14. Undifferentiated ectomesoderm cells, single or in small groups, will be seen attached to the stomach, to the inner body wall, near the apical organ, etc.
15. The important entomesodermal cells (derivatives of 4d=Teloblasts) which will give rise to the mesodermal structures of the worm body are difficult to distinguish. They are small groups of cells near the lower end of the head kidney.

Draw lateral and polar views.

11.

Metamorphosis of the Nereis Larva

The metamorphosis of an Annelid larva into a segmented worm can be studied best in Nereis, 1 to 7 days old. Nereis has no typical trochophore but an abbreviated, telescoped larval development. The first signs of the adult, segmental organization appear very early. Prepare slides as under 1. Study larvae from all sides. Consult the figs. in E. B. Wilson ('92).

a. Trochophore-like stage 24 hrs. (Wilson, fig. 84)

Observe:

1. The prototroch, composed of 12 very large ciliated cells.
2. The paratroch, near the vegetal pole.
3. A pigmented area at the anal pole; the anal pigment.
4. The mouth and stomodaeum; the latter is a short ectodermal invagination.
5. The large macromeres have not yet differentiated into the entodermal parts of the intestine; their cell boundaries may be seen. No anus is formed as yet.
6. Several "frontal bodies" near the upper end. Circular disc-like structures of unknown function.
7. Two pairs of seta sacs, spherical structures in the postrochal hemisphere. The setae (= chaetae) will be differentiated inside of them. These sacs are the first indication of the first two segments of the worm.
8. Observe the trochophore in locomotion.

B. Advanced trochophore, 2 days old (Wilson, figs. 85-89.

Identify all structures found in A. In addition observe:

1. Eye spots
2. A third pair of seta sacs has appeared behind the first two. All three are lined up in a row indicating the first 3 segments. Setae can be seen in

the process of formation, inside of these sacs; in slightly older stages, they will be seen projecting from the sacs.

3. Pigment appears in the prototroch cell.

C. Metamorphosis, 2½-3 days (Wilson, figs. 90-91)

Notice the change of shape and the gradual demarcation of the first three segments.

5. The hood, a fold overhanging the mouth
6. Very long bristles deveopein seta sacs. Study their fine structure. They appear one pair after another, eventually 10 pairs. They will be replaced at metamorphosis by ordinary setae.

IV. Gastrulation and Formation of the Trochophore in Hydroides

Prepare your own cultures (see p. 1). Gastrulation by invagination occurs approximately 7-10 hours after fertilization. Consult the figs. in Schearer ('11) and Hatschek ('86).

Child, C. M.	1900	Early development of Arenicola. Roux' Archiv. Bd. 9
Fraipont, J.	1887	LeGenre Polygordius. Fauna u. Flora des Golfes von Neapel, Vol XlV.
Hatschek, B.	1886	Entwicklung der Trochophoro von Eupomatus. Arbeit. a.d. Zool. Inst. Wien, Vl. (Hydroides)
Iwanoff, C. P.	1928	Entwicklung der Larvalsegmente bei den Anneliden. Zeitschr. Morph. Oek. Bd. 10
Just, E. E.	1922	On the rearing of sexually mature Nereis from eggs. Am. Nat. vol. 56.
Lillie, F. R.	1902	Differentiation without cleavage in the egg of the annelid Chaetopterus. Roux Archiv ' f. Entw. Bd. 14
---------------	1906	Observations and experiments concerning the elementary phenom. of embryonic develo ment in Chaetopterus (Formative stuff) Jour. Exp. Zool. 111.
----------	1909	Polarity and bilaterality of the Annelid egg. Biol Bull. XVl.
Mead, A. D.	1897	The early deveopment of marine annelids. J. Morph. vol. 13.
Newby, W. W.	1940	The embryology of the Echiuroid worm. Urechis caupo. Mem. Amer. Philos. Soc. vol. 16 1-219.
Novikoff, A. B.	1938	Embryonic determination in the Annelid, Sabellaria vulgaris, Biol. Bull. vol. 74.
Shearer, C.	1911	Development and structure of the trochophore of Hydroides, Quart. J. Micro. Sci. vol. 56.
Wilson, D. P.	1929	Larvae of British Sabellarians. J. Marine Biol. Assoc. N. S. 16.
Wilson, E. B.	1892	Cell lineage of Nereis, Journ. of Morph. v. 6.
Wilson, E. B.	1898	Considerations of cell lineage and ancestral reminiscences. Ann N. Y. Acad. Sci. vol. 11.
Wistinghausen, C. A. von	1891	Utersuchungen Uber die Entwickl. von Nereis dumerilii. Mitt. a. d. Zool. Stat. Neapel, Bd. 10
Woltereck, R.	1904	Wurmkopf, Wurmrumpf und Trochophora. Zool. Anz. Bd. 28.
-------------	1904	Beitraege zur praktischen Analyse der Polygordius-Entwicklung etc. Roux Archiv. Bd. 18.
-------------	1905	Zur Kopffrage der Anneliden. Verh. d. Deutschen Zool. Ges. (His figs. are reproduced in McBride)

EMBRYOLOGY OF MOLLUSCA

Gastropoda

1. The Veliger Larva

Study the typical Veliger larve of Crepidula fornicata. Obtain material by breaking the animal from the substrate to which it is attached. You will find the yellowish eggs (enclosed in transparent capsules) attached to the substrate or in the shell of the mother. Tease the embryos out of the capsules. Obtain swimming larvae in different stages, particularly old ones with the yolk resorbed. They are transparent and show the inner organs. Consult Conklin (1897) figs. 80-82 and the textbooks of Parker-Haswell volume 1 (1928) fig. 657 on p. 734, or MacBride figs. 2?? ff. on pages 301 ff. or Korschelt, 1936, vol.2, pp. 881-891. Study dorsal, ventral and lateral views. Mouth and foot are on the ventral side, the anus is on the right side.

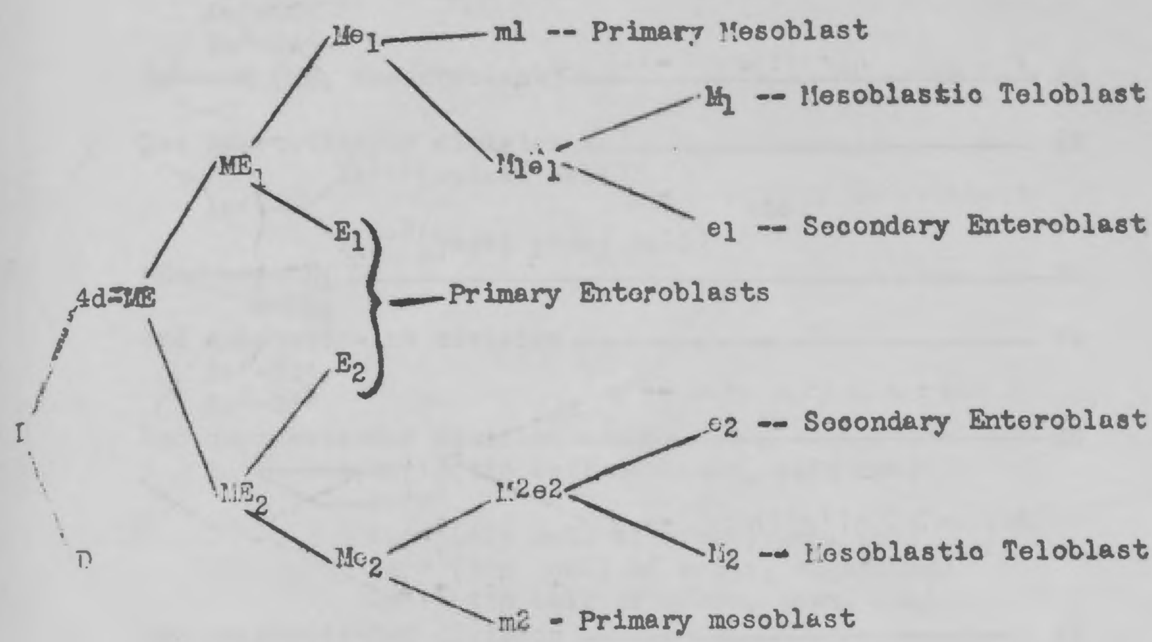
Note:

1. Velum with powerful cilia
2. Head vesicle (dorsal)
3. Eyes with lens (dorsal)
4. Foot (ventral), study it in lateral view
5. Statocysts (ventral, on basis of foot)
6. Mouth with powerful cilia (ventral, above the foot.)
7. Oesophagus (dorsal), stomach, liver. The different parts of the intestine can be distinguished only in older embryos which have resorbed the yolk.
8. Anus (on right side)
9. External kidneys (lateral to foot)
10. Heart (dorsal). Observe the heart beat in older embryos.
11. Transparent shell.

II. EARLY STAGES OF THE VELIGER LARVA

There is no typical trochophore stage in Crepidula. Study stages of direct transformation of the gastrula into the Veliger larva. (Conklin, 1897, figs. 77-79). Observe the gradual development of shell gland, shell, velum and foot.

CREPIDULA



Me1
m1 -- Primary Mesoblast
M1 -- Mesoblastic Teloblast
ME1
M1e1
E1
e1 -- Secondary Enteroblast
4d=ME
Primary Enteroblasts
E2
I
e2 -- Secondary Enteroblast
ME2
M2e2
D
Me2
M2 -- Mesoblastic Teloblast
m2 - Primary mesoblast

CREPIDULA

	No. of cells
A, B, C, D.	4
1st quartette (1a-1d)	8
2nd quartette (2a-2d)	12
1st quartette-1st division	16
$1a^{1}-1d^{1}$ = apical cells	
$1a^{2}-1d^{2}$ = turret cells	
3rd quartette (3a-3d)	20
2nd quartette-1st division	24
$2a^{1}-2d^{1}$	
$2a^{2}-2d^{2}$	
D → 4d (ME, Mesentoblast); D	25
1st quartette-2nd division	29
$1a^{1}$ → $1a^{1.1}$ (apical cell); $1a^{1.2}$ (basal cross cell) etc.	
ME → ME_1; ME_2	30
3rd quartette-1st division	34
$3a^{1}-3d^{1}$	
$3a^{2}-3d^{2}$	
2nd quartette-2nd division	38
$2a^{1}$ → $2a^{1.1}$ (tip cell of cross, left arm); $2a^{1.2}$	
$2b^{1.1}$ (tip cell of cross, ant. arm)	
$2c^{1.1}$ (tip cell of cross, right arm)	
$2d^{1.1}$ (tip cell of cross, post. arm)	
2nd quartette-3rd division	42
$2a^{2}$ → $2a^{2.1}$; $2a^{2.2}$	
ME_1, ME_2 divide	44
divide	49
A, B, C yield 4a-4c	52

CREPIDULA -- DERIVATIVES

1st quartette of micromeres

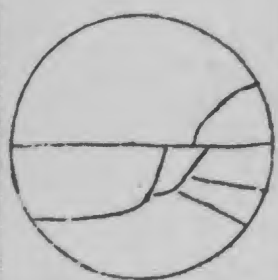
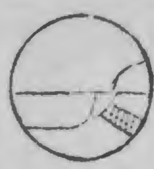
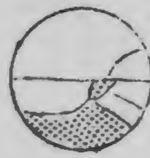
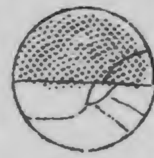
a. all ectoderm cells of head vesicle.
b. apical plate of ciliated cells.
c. posterior cell plate.
d. dorsal portion of functional velum and portion of first velar row on ventral side.
e. supraoesophageal ganglia and commissure.
f. cerebro-pedal connectives.
g. possibly pedal ganglia.
h. an apical sense organ.
i. paired eyes.

2nd quartette of micromeres

a. larger part of velum.
b. shell gland.
c. at least part of foot.
d. larval mesenchyme from derivatives of 2a-2c.

3rd quartette of micromeres

derivatives lie wholly outside of velar area and form a considerable part of lower hemisphere.

Unshaded= cells developing	Based on data in v. Ubisch, 1939									
	CELLS EXTIRPATED									
	2 A7.3 2 A7.7		2 B6.2 2 B6.3 2 B6.4		2 A7.8 2 A7.4 2 a6.5 2 a6.7		2 A6.1 2 A6.3 2 B6.1		8 an / 16	
										
	P	F	P	F	P	F	P	F	P	F
ectoderm	+	+	+	+	+	+	+	+	-	-
cerebral ves.	+	+	+	+	-	-	+	+	-	-
eye	+	+	+	+	-	-	+	+	-	-
otolith	+	+	+	+	-	-	+	+	-	-
nerve tube	+	+	+	+	-	-	+	+	+	(+)
notochord	-	-	+	+	+	#	+	+	+	(+)
muscle mesenchyme	+	+	-	-,+	+	+	+	+	+	(+)
endoderm	+	+	+	+	+	+	+	+	+	(+)
papillae		+		+		+,-		+,-		-

P= presumptive F= formed (+)= tissue not organ differentiated

Lamellibranch (Pelecypod) Development

Type: Mactra solidissima

1. Normal Development:

A. The Unfertilized Egg: Obtain a sample of unfertilized eggs in a stendor dish and transfer a few to a depression slide for study. When shed, the eggs are irregular in shape due to the pressure within the ovary, but they become spherical on standing. They are small (55 micra in diameter) and the center is almost completely filled by the enormous germinal vesicle with its prominent nucleolus. Note the thin layer of clear cortical cytoplasm and the densely packed yolk. Unless the eggs are inseminated, they will retain this appearance for many hours. The eggs are fertilizable until the germinal vesicle breaks down, although the capacity for normal fertilization and development is impaired with long standing.

B. Fertilization and Maturation: Fertilize eggs and immediately transfer a sample to a depression slide. A few minutes after insemination the outline of the germinal vesicle starts to become indistinct, and in 15-20 minutes there is only a lighter area in the center of the egg marking its former position. A thin fertilization membrane is raised, but this is not lifted far from the egg surface, and it is best seen in the region of the polar bodies or spanning the cleavage furrows. The first polar body forms shortly after germinal vesicle breakdown, and the second polar body follows directly beneath the first. Both polar bodies are usually formed by 30-35 minutes after insemination. Note the position of the polar bodies, for they mark the plane of the coming cleavage.

C. Early Cleavage: About 50 minutes after insemination two nuclei will be visible, the male and female pronuclei. Can you see them approach and fuse? Cleavage occurs about 15 minutes after they first appear. The first cleavage is unequal, and the first two blastomeres differ greatly in size. The second cleavage follows in about half an hour. In the case of the larger cell, cleavage is again unequal, resulting in one large cell and three smaller cells. Compare with the four cell stage of Crepidula and Nereis. The following cleavages are rapid, perhaps only ten minutes intervening. between the 4 and 8 celled stages. The cleavage is undoubtedly of a spiral type, but this characteristic is more difficult to detect than in Crepidula due to the size differences of the blastomeres.

D. Time Table of Development: There is much variation in developmental rate depending on temperature and other environmental conditions, but the following table will give some idea of the chronology at 25°C. (Schechter, 1941). Times are recorded from insemination.

10 minutes	Germinal vesicle reaction
30 "	Polar bodies formed
50 "	Pronuclei visible
1 Hr., 5 mins.	First cleavage
1 Hr., 35 mins.	Second cleavage
5 Hrs.	Swimming forms

E. Later Development: The figures of the development of Dreissensia (Meisenheimer, 1900) will prove very helpful, for the embryology of the two forms is very similar.

1. Gastrulation and early Trochophore stages: Remove samples from cultures 4-9 hours after insemination. If forms are moving too rapidly add a drop

of Janus Green to mounts. In the younger stages note that the smaller, more rapidly dividing ectodermal cells are spreading over the larger, yolk-filled endodermal cells. This type of gastrulation is known as epiboly. The uncovered region is the blastopore. When the larva just starts to swim (5-6 hours after insemination) a plate of large cells which will form the shell gland is visible on the future dorsal surface. Internally two large, dark cells, the mesodermal teloblasts, are often visible. By 9 hours the embryos have lost their somewhat barrel-shaped appearance and are pyramidal, the expanded base of the pyramid being the region in which the velum will form. The cilia are not marked at this time. The blastopore visible on the ventral side as a small indentation, and the invaginated shell gland forms a conspicuous concavity on the dorsal surface. By 1- hours the shell gland will have evaginated and this concavity will be no longer visible. The cilia of the velum and the apical flagellum will be visible at this stage.

2. Young Veligers: Obtain samples of cultures about 18-19 hours after insemination. Note:
   a. General shape.
   b. The two-valved shell with its straight hinge line. How much of the body is enclosed by the shell?
   c. The apical flagellum, telotroch, and the long cilia of the developing velum.
   d. The stomodeal invagination on the ventral side just below the velum. The proctodeal invagination appears later (about 23 hrs.)
   e. The internal structures are difficult to make out at this time, for a large, dark mass of undifferentiated endodermal and mesodermal cells fills most of the post-velar area.

PELECYPODA

BIBLIOGRAPHY

Brooks, W. K.	1880	Development of the oyster. Stud. Biol. Lab. Johns-Hopkins Univ. 1.
Fujita, T.	1929	Early development of the Japanese oyster. Jap. J. Zool. 2:353-357.
Heilbrunn, L. V.	1920	III Cortical Change and the initiation of maturation in the egg of Cumingea. Biol. Bull., 38:317.
Horst, R.	1884	The development of the oyster (Ostrea edulis L.) Ann. Report Fish. Com. Wash., 891-911.
Jordan, H. E.	1910	A cytological study of the egg of Cumingea with special reference to the history of the chromosomes and the centrosome. Arch. of Zellforsch., 4:
Kostanecki, K.	1902	Ueber kunstliche Befruchtung und kunstliche parthenogenetische Furchung bei Mactra. Extrait du Bulletin de l'Academie des Sciences de Cracovie, Juillet: 363-387.
________	1904	Cytologische Studien an kunstlich parthenogenetisch sich entwickelenden Eirn von Mactra. Arch. f. mikr. Anat. und Entwick., 64:1-98.
________	1908	Zur Morphologie der kunstlichen parthenogenetischen Entwicklung bei Mactra. Arch. f. Mikr. Anat. und Entwick., 72:327-352.
________	1911	Experimentelle Studien an den Eirn von Mactra. Extrait du Bulletin de l'Academie des Sciences de Cracovie. Classe des Sciences Mathematiques et Naturelles. Serie B, Sciences Naturelles, Mars: 140-161.
Lillie, F. R.	1895	The embryology of Unionidae. J. Morph., 10:1-100.
Meisenheimer, J.	1900	Entwicklungsgeschichte von Dreissensia polymorpha Pall, Zeit. f. wiss. Zool., 69:1-137.
Schechter, V.	1936	Comparative hypotonic cytolysis of several types of invertebrate egg cells and the influence of age. Biol. Bull., 71:410.
________	1937	Calcium and magnesium in relation to longevity of Mactra, Nereis, and Hydroides egg cells. Biol. Bull., 73:392.
________	1941	Experimental studies upon the egg cells of the clam, Mactra solidissima, with special reference to longevity. J. E. Z., 86:461-477.

AMPHINEURA

Chaetopleura apiculata (the Chiton)

The species is dioecious but there is not way of distinguishing the sexes externally.

Suggestions for Observation: Due to the large size and opacity of the egg, the early development is best studied by mounting egg samples in depression slides. Swimming larvae can be mounted in a dilute solution of Janus Green for observation.

Study of Normal Development:

1. The Unfertilized Ovum: The spherical egg measures from 180 to 190 microns in diameter and appears opaque due to the large amounts of Yolk. Although internal processes can not be seen in living eggs, sections show that the ovum is usually in the process of developing the first maturation spindle when it is shed. Surrounding the egg is a tough, bristly chorion. When the eggs emerge from the oviduct they are embedded in a visoid, jelly-like secretion which spreads over the bottom of the dish in a thin film.
2. Fertilization and Cleavage: There are no visible changes at the time of fertilization; a fertilization membrane is not raised and the egg does not change shape. Two transparent polar bodies are given off but no polar lobes are formed. The first noticeable change occurs shortly before first cleavage when there is a slight flattening of the egg at the animal pole. The first cleavage furrow (1 hr. 40 mins. to 1 hr. 50 mins. after insemination) divides the egg in most cases into equal blastomeres. In a small percentage of cases one blastomere is perceptibly larger. The second cleavage is at right angles to the first, and again in some cases the D cell is slightly larger. The cells of the first quartet of micromeres given off by the dexiotropic third cleavage, are distinguishable from the larger macromeres. The further divisions follow the regular pattern of spiral cleavage. Four quartets of micromeres are given off. The first three give rise to the ectoderm, nervous system, and stomadeum, while the fourth quartet except for the 4d becomes part of the endoderm along with the macromeres. The 4d cell gives rise to the mesoderm as well as endoderm.
3. Time Table of Development: The following record, procured from a batch of eggs developing at 23-24 C. is offered as a rough outline of developmental rate. Metamorphosis seemed to occur early in this batch, the usual time being from 7-12 days. Time is recorded from the time of insemination.

1st Polar Body	30 min.
2nd Polar Body	55 min.
1st Cleavage	$1\frac{1}{2}$ hrs.
2nd "	2 hrs.
3rd "	2 hrs. 40 mins.
Gastrulation	about 13 hrs.
Beating cilia	14 hrs.
Rotation in capsule	20 hrs.
Hatching	36 hrs.
Typical free-swimming Trochophores	$2\frac{1}{2}$-3 days
Metamorphosis	4 days

ILYANASSA OBSOLETA

Obtaining eggs and early stages:

1. Best method of obtaining very early stages is to watch snails laying eggs through glass. Eggs are visible at oviducal opening (anterior median part of foot). After egg is fastened to glass, snail may be gently removed and capsule transferred to a watch glass of filtered sea water with a pair of fine forceps. Since snails seem to prefer to deposit the eggs on the wooden sides of the tank in preference to the glass, J. Oppenheimer suggests inserting sheets of glass over these wooden sides. These can be moved from sides for inspection purposes without disturbing the animals.

[illegible] Capsules:

1. The stage of development of the eggs can be determined before removal from the capsule with the aid of a binocular microscope.
2. When proper stage is found, pin down side of capsule furthest from eggs with a dissecting needle. With another needle tear off section that is pinned to dish, making sure that a very large tear is made. If gentle pressure is now applied to capsule, the eggs will flow out in a mass of jelly. Make sure that all of the eggs are free of the capsule and that the capsule is pretty well torn up before releasing the pressure else the [illegible] jelly will rush back into the capsule carrying the eggs with it. All of the operations must be carried out under water, for the eggs rupture on contact with air. The jelly dissolves in the water, and after a few seconds the eggs will settle to the bottom of the dish.

Cleavages:

Approximate Time Table:

1st lobe	50 minutes. (1st polar body coming off)
Disapp. of lobe	59 mins. ≠ 1st polar body off.
2nd lobe appears	1 hr. 12 min.
2nd lobe gone	2 hrs. 12 min.
3rd lobe	2 hrs. 42 mins.
1st cleavage	3 hrs. 42 mins.
4 cells	4 hrs. 52 mins.

Later Stages:

Observe later stages of development through the veliger larva (there is no trochophore larva).

REFERENCES

Clement, Anthony C. 1935 The formation of giant polar bodies in centrifuged eggs of Ilyanassa. Biol. Bull., 69:403-414.

Crampton, H. E. 1896 Experimental studies on gastropod development. Arch. f. Entw'mech., 3.

Morgan, T. H. 1933 The formation of the antipolar lobe in Ilyanassa. Jour. Exper. Zool., 64:433-467.

1935a Centrifuging the eggs of Ilyanassa in reverse. Biol. Bull., 68: 268-279.

1935b The separation of the egg of Ilyanassa into two parts by centrifuging. Biol. Bull., 68:280-295.

1935c The rhythmic changes in form of the isolated antipolar lobe of Ilyanassa. Biol. Bull., 68: 296-299.

1936 Further experiments on the formation of the antipolar lobe of Ilyanassa. Jour. Exper. Zool., 74:381-425.

1937 The behavior of the maturation spindles in polar fragments of eggs of Ilyanassa obtained by centrifuging. Biol. Bull., 72.

4. Later Stages of Development and Metamorphosis:

A. Young Trochophores 40-60 hours old: These larvae are propelled rapidly through the water by the beating of a band of powerful cilia - the prototroch. The body rotates on its longitudinal axis and the course followed is a spiral. Crowning the pretrochal hemisphere (the head vesicle) is a clump of very long cilia - the apical tuft, which is apparently sensory in function. The two lateral, reddish-brown larval eyes give a certain amount of bilaterality to the otherwise radial organism. Although the mouth may be visible just below the prototroch, the other regions of the digestive tract are obscured by the yolk mass.
B. Older Larvae 3-4 Days Old: There is an elongation of the body, especially of the post-trochal hemisphere. The mouth and archenteron are now visible due to the reduction in the quantity of the yolk. The anus plates are beginning to appear on the dorsal surface. Note the contractile foot that develops on the ventral surface just posterior to the mouth. Locomotion is still by way of the prototrochal cilia although older larvae may creep along by means of the foot.
C. Metamorphosing Larvae: Metamorphosing larvae may be procured from the bottom of a culture dish. Note that the prototrochal and apical cilia are lost during metamorphosis and that the larvae now creep about by means of a well-developed foot. The shell plates have increased in number though the full set of the adult is not yet complete. The mantle, a fold of the body wall, develops just dorsal and lateral to the foot.

REFERENCES

Grave, B. H.	1932	Embryology and life history of Chaetopleura apiculata. J. Morph. 54:153-160.
Heath, Harold	1889	The development of Ischnochiton. Zool. Jahrb. 12:
Kowalevsky, A.	1883	Embryogenie du Chiton polii. Ann. Mus. Hist. Nat. Marseilles. 5:

REFERENCES--MOLLUSCA

GASTROPODA

Conklin, E. G.	1897	Embryology of Crepidula. Jour. Morph., 13:1-226.
Coe, Wesley R.	1948	Nutrition, sexuality in protandric gastropods of the genus Crepidula. Biol. Bull., 94:158-160.
Crampton, H. E.	1896	Experimental Studies on Gastropod Development. Arch. of Entw. Mech., 3:1-19.
Erlanger, R. von	1891	Zur Entwicklung von Paludina vivipora. Morph. Jahrb. 17:335-379, 636-680.
Lebour, Marie V.	1945	The eggs and larvae of some prosobranchs from Bermuda. Proc. Zool. Soc. London, 114:462-489.
Mackay, M. E.	1927	A Study of Early development in Cumingia with special reference to Cytology. Proc. Tr. Nova Scotia Inst. Sc., 17:46-60.
Moritz, C. E.	1938	The anatomy of the gastropod Crepidula adunca Sowerby. Univ. Cal. Pub. Zool., 43:83-92.
	1939	Organogenesis in the gastropod Crepidula adunca Sowerby. Univ. Cal. Pub. Zool., 43:217-248.
Patten, W.	1885-6	Embryology of Patella. Arb. ausd. Zool. Inst. Univ. Wien. 6:149-174.
Smith, F. G. W.	1935	The development of Patella vulgata. Philos. Trans. Roy. Soc. London., B:225:95-125.
Wilson, E. B.	1904	Experimental studies in germinal localizations. (Patella and Dentalium). J. Exp. Zool., 1:197-268.

COELENTERATES

Introduction to Hydrozoa

a) Sensitiveness of the material

While working with coelenterates in the laboratory it is essential to remember that the hydroids are very sensitive to environmental conditions. They do not survive well in the laboratory even in aquaria of running water. Do not crowd either the adult stems or the eggs and embryos. In general, your procedure will be to look over a good-sized colony of hydroids under the lowest power of magnification, and to clip off a few pieces containing the best embryological material. These can be rinsed in running sea water, and segregated in plenty of sea water in a dish for more detailed study.

b) Types of Life History Illustrated

There are two phases of embryology in the Hydrozoa. Characteristically, a long series of asexual reproductions (by budding and other methods) is interrupted at irregular intervals by isolated examples of sexual reproduction. This alternation of structure, since zygote production is usually accomplished by medusae and the production of buds by polyps.

In some hydrozoa no medusa-form is known, in others no polyp-form is known, and there are all stages between. The hydrozoa available at Woods Hole illustrate well the structural variability of the medusa-state. The best-known example of a complete medusa with a degenerate polyp stage is the idealized jelly-fish Gonionemus, but it is unfortunately now nearly extinct at Woods Hole. The genera picked for study are arranged in the order of diminishing completeness of the medusa form, the first having free-swimming medusae and the last mere sporosacs. The utter degeneration of the medusa-form is illustrated by the familiar Hydra.

c) Order of Study

Because of seasonal variations and unpredictable fluctuations in the Coelenterate fauna, some of the material described may not be available, and the order of study will have to be announced.

Laboratory Procedure

1. Study of Forms with Perfect Medusa

Examples: Bougainvillia, Obelia, Podocoryne.

Characteristic life history: Zygote shed from medusa; Development to planula larva; Metamorphosis to polyp; Asexual Multiplication of polyps by budding, which produces a colony; Medusae formed by special buds, in a gonosome (Obelia) or separately (Bougainvillia); Shedding of medusae, which mature slowly as separate individuals before forming eggs or sperm.

BOUGAINVILLIA. (June, July, August; not always available in June)
The gonophores are borne singly or in clusters on the main stem and branches, and in this genus develop into complete medusae. The medusa-buds are scattered irregularly thruout the colony, there being no orderly arrangement according to age. Select buds that show various stages of medusa development and mount them under cover slips, and study their unfolding structure. Draw off water from under the coverslip with absorbent paper to produce a slight pressure on the buds. Sketch three stages in medusa development.

When all of its parts except the gonads are fully formed, the medusa breaks loose and swims away. It lives independently one or two months, the gonads gradually mature. Find a well-developed specimen that is swimming actively and sketch its diagrammatic structure. Identify manubrium, radial and circular canals, velum, oral tentacles, 4 groups of marginal tentacles.

Illustrations of medusa in Hargitt, C. W. '01, '04; Nutting '01, of medusa development in Goette '07. Also Hyman ' 40.

OBELIA (June, July, August)

The gonosomes are several times as large as the hydranths. Examine specimens and see if they are located at random along the stalks, or in regular places.

An Obelia gonosome has an enlarged transparent covering, the gonotheca, with a blastostyle extending thru it from base to tip. The outer end, or tip, of the blastostype expands to make a loose plug for the gonotheca when mature.

A cluster of gonophores is borne on the blastostype inside the gonotheca. The gonophores mature as medusae, and break loose, escaping to the outside past the blastostyle plug. They are commonly caught in tow nets. Their free-swimming life lasts two months or so, the gonads maturing slowly.

The older gonophores should show developing tentacles, when pressed slightly under a cover slip. Which gonophores on a blastostyle are oldest?

With needles, press on the gonotheca of a well-matured gonosome and examine under high magnification the gonophores that are released. Those that are oldest may show swimming movements. Younger stages of development may be teased out from the gonosome and studied. Sketch several stages.

If ripe Obelia colonies are kept for an hour or two in a dish of sea water on the desk (remember not to crowd them), swimming medusae can usually be detected in a good light with the naked eye. Examine some of them when available, and note that their structure is slightly imperfect compared with that of Bougainvillia.

The velum is reduced to a narrow and somewhat lobed membrane near the bases of the tentacles. This makes possible an eversion of the bell when the medusa comes to rest, so that the manubrium sticks out from the center of the convex side, like the handle of a pest-hurricane umbrella. Watch the swimming movements, and see how this happens. In the everted condition, the manubrium is still morphologically subumbrellar, though this term has lost its appropriateness.

The newly shed medusa of Obelia geniculate has 24 tentacles, while that of Obelia commissuralis has 16. Both forms may be available in the laboratory. Neither has gonads developed at this stage.

Illustrations of Obelie embryology in Hyman '40, Goette '07.

PODOCORYNE (June, July. 1 or 2 colonies will be collected with each 100 Hydractinia colonies from Sheep-Pen Harbor. None from Pasque.)

The highly specialized colony grows in an encrusting mat on snail shells, etc., and is almost exactly like hydractinia. Both have three types of individuals: feeders, gonosomes, stingers (Cf. descriptions of Hydractinia below, p. 6) Podocoryne is included here because of its startling metagenetic contrast to Hydractinia.

Medusae of Podocoryne are nearly perfect, and may produce several generations of new medusae by asexual budding before getting around to their main business of gameto production. Cf. Goette, '16 (The sporosacs of Hydractinia bear very little resemblance to medusae, being highly degenerate).

Sketch the three types of polyps and show several stages in medusa development. Gonads can be made out along the radial canals of the swimming medusae, very immature but sexually distinguishable. The asexual colony gives off either male or female medusae, not both.

3. Study of Forms with Imperfect Medusae

Examples: Pennaria, Tubularia.
Life Histories: Zygote shed from short-lived imperfect medusa (Pennaria) or retained in reduced sessile medusa form.
(Tubularia): Development to planula larva and metamorphosis to polyp (Pennaria) or development to Actinula larva and growth to polyp (Tubularia): Asexual multiplication of polyps by budding to produce colony; Gonophores formed by special buds on hydranths; Maturation of gonophores (imperfect medusae) and fertilization either in situ (Tubularia) or within the limits of the colony during their detachment (Pennaria)

PENNARIA (July, August, September: begins to ripen middle of July)
Gonophores bud off singly around the lower portion of the hydranth. They form slightly reduced medusae with rudimentary tuftlike tentacles. Before opening out as transparent bell-shaped forms they suggest coconuts. A single colony bears gonophores of one sexonly, but in the living individuals, sex cannbe diagnosed only with difficulty until they mature, when the pinkness of eggs and the whiteness of sperm appear. (Smallwood '89) "Male" and "female" colonies are actually asexual, bearing male and female gonophores respectively.

Mature Pennaria colonies festooned with shedding medusae provide an astonishing and beautiful spectacle that every student should certainly see. The material ripens in the season of warmest water, and a demonstration of the shedding will be made when possible. It starts early in the evening and continues through midnight. It is usually best seen in material brought into the laboratory the preceding day (i.e. the second night).

The ripe medusae gradually start a rhythmic twitching. Those which are males emit puffs of whitish sperm, and those which are female eject with greater travail the three to six opaque pink eggs. In the south, Pennaria medusae generally break loose from the colony and swim about during this discharge, but at Woods Hole they generally remain attached, and the eggs may not be ejected until long after fertilization. The medusae finally drop off, swim very feebly, if at all, shrivel rapidly and die in a few hours.

Put small selected stems from ripe "male" and "female" colonies together in a fingerbowl after careful rinsing, at 3-4 p. m. and leave them overnight. They are extremely sensitive to overcrowding! Next day, remove the stems and look with naked eye for free medusae as evidence of shedding. If they are found, look for developing eggs.

The eggs are very simple and slightly ameboid, with no apparent membrane. Follow their development as far as possible. If the water is changed several times, the planula stage should be reached in 24 hours, and stages in the very simple metamorphosis to the polyp form may be observed. (Hargitt, G. T. 1900, 1909).

Tease apart male and female gonophores and examine the eggs and sperm. Sketch several stages in development of the medusa; sketch the mature male and female medusae; sketch several cleavage stages if found.

Illustrations of medusa development in Goette '07; of cleavage in Hargitt, C. W. '0[illegible] Also Hyman '40.

TUBULARIA (June, July)

The Gonosomes of a well-matured specimen form long racemes or clusters of gonophores growing and drooping from the region between the circles of tentacles on a hydranth. The gonophores quite severely reduced medusae which never become free-swimming, usually have no evident radial or circular canals and develope nothing but buds for tentacles. Male and female gonophores occur in separate colonies. Diagnosis of sex by sight is impossible in the immature, but gonophores that contain embryos are easy to tell from those that are filled with a cloudy mass of sperm. Early stages of developing embryos are found by teasing with needles, those near the hatching stage are visible in situ.

Examine a ripe male gonophore, considering it as a very degenerate medusa, Notice and sketch its mode of attachment, its shape, the structure of its free end and the position of the sperm surrounding the dark red manubrium. Crush it on a slide and inspect the motile sperm under high power.

Examine and sketch a ripe female gonophore. Usually the tentacles at its distal end appear only as four short blunt knobs, but one or more of them are sometimes slightly elongated. The eggs come from favored oocytes that progressively swallow up their neighbors, lying in the space around the spadix (manubrium).

When ripe the egg is very large and somewhat irregular in shape (Allen '00, Lowe '26) After fertilization, cleavage is often chaotic, apparently either a coeloblastula or a morula may be formed (Lowe '26), and gastrulation of the former has been described (Benoit '25), as a mixture of delamination and multipolar proliferation. The embryos are developed up to the "Actinula" stage within the gonophore. The Actinula larva is to be considered as a precociously metamorphosing form, part planula and part polyp,

By teasing some female gonophores open with needles, collect and sketch eggs and larvae in various stages. The larvae just taking shape are flattened with blunt marginal processes. At first these are not symmetrical in outline but radial symmetry is attained later. When the larva has reached the Actinula stage it has a mouth and aboral tentacles, and a rounded aboral body that later becomes attached at its tip.

Study and sketch the structure of a fully formed Actinula larva. Look over some ripe "female" colonies for Actinulae beginning to escape from the gonophores. Actinulae will not develop further without feeding.

Tubularia anatomy and development is illustrated in the texts of MacBride '14, Korschelt '36 and Hyman '40. For cleavage cf. Allen '00, Hargitt, G. T. '09. For gonophore development, cf Goette '07.

C. Study of Forms with Degenerate Medusae.

Examples with blastostyle inside gonotheca: Campanularia, Gonothyrea.
Life Histories:

a) Campanularia, Gonothyrea; Zygote develops into planula larva inside sessile degenerate medusa; Planula escapes, lives free awile, metamorphoses into a polyp; Asexual multiplication by buds; Colony formation; Degenerate medusae (gonophores) formed on a blastostyle; Gonophores mature in situ; Sperm are shed, eggs fertilized in situ.

b) Hydractinia, Eudendrium: Zygote develops into planula larva either inside gonosome (Eudendrium) or after being shed from gonosome (Hydractinia): Planula metamorphoses into polyp; Asexual multiplication by buds; Colony formation; Gonosomes formed from Hydrorhiza (Hydactinia) or by transformation of hydranths (Eudendrium): Gonophores (highly reduced medusae or sporosacs) Borne on gonosomes; Eggs and sperm formed in the sporosacs; Eggs fertilized in situ (Eudendrium) or during shedding (Hydractinia).

CAMPANULARIA (June, July)

It is not safe to try to distinguish this genus from Obelia by the anatomy of the feeding individuals. Even the gonosomes are similar in appearance in the two genera, each consisting of a transparant gonotheca with the blastostyle extending from base to tip and gonophores budding from it.

The striking difference is that Obelia produces nearly perfect free-swimming medusae, whereas Campanularia produces gonophores so utterly degenerate that their medusa-like structure can only be made out in sections. Each gonophore on the blastostyle of a "Female" colony contains a very large irregularly shaped egg which is fertilized in situ, cleaves, forms a morula, gastrulates by delamination and reaches the free-swimming planula stage, still insitu. Campanularia, therefore, releases from its gonotheca not medusae but planulae.

Because the gonophores are so inconspicuous and the embryos so obvious, the colonies which produce female gonophores and later contain embryos are loosely spoken of as "female" colonies, although they are asexual.

Select from a "female " colony a gonosome showing eggs in the basal gonophores. Mount it on a slide, study with various magnifications under the microscope, and sketch it.

Select and sketch another gonosome with planulae showing near the tip. Squeeze or open the gonotheca with needles and liberate the planulae. Notice their ciliated ectoderm and watch their movements. If the planulae are well matured, they are two or three times longer than broad. They show maggot-like movements even while within the gonotheca.

Campanularia is very favorable for the study of planula metamorphosis. Put a few mature planulae aside in sea water (not more than 2 or 3 to a watch glass) and cover them. They should attach to the glads in about 4 to 10 hours. Each should then open a mouth, bud out tentacles, secret hydrotheca and perisarc, and become a full formed individual polyp in two or three days. When the planulae have attached, the water should be changed in the dish at least twice a day. Sketch several stages of attachment and metamorphosis.

The gonosomes of "male" colonies are similar in form to the female. The gonophores, when mature, are rounded and have a thin milky-gray color. The sperm become active when they are discharged into contact with sea water, as may be seen by crushing a male gonophore under a cover slide while watching it under the microscope.

For illustrations of Campanularia gonophore development c. f. Goette '07.

GONOTHYREA (July, August)
As in Abelia, the medusae develop within the gonotheca. When mature, instead of swimming away they remain attached to the end of the blastostyle, projecting outside the gonotheca in groups of three or four, like toy baloons. Within the balls of the medusae the eggs of "female" colonies are fertilized by the shed sperm from "male" colonies. The zygotes develop to the planula stage before being set free, after which the medusae drop off.

Sketch planulae in various stages of development in the projecting attached medusae of a ripe "female" colony. (Wulfert '02).

Gonothyrea cleavage illustrated in Wulfert '02; Medusa development in Goette '07. Cf. also texts of Hyman '40 and Korschelt '36.

HYDRACTINIA (June, July, August)

Colonies of this form are fairly common on Littorina snail shells inhabited by the small hermit crab, Pagurus. There are three types of individuals in the full developed colony: ordinary polyps (feeders), threadlike coiling forms with no mouth and an apical knob of nematocysts (stingers, commonest around the lip of the shell) and gonosomes. The three types all arise from a hydrorhiza network covering a rust-red spine-studdied crust.

a) Gonosomes and Gonophores:

The gonosomes or reproductive individuals are usually without tentacles and have a large knob of nematocysts on the proboscis; each bears a number of gonophores, which are medusa-buds reduced to the status of sporosacs. Ripe "male" and "female" colonies can be told apart with the naked eye since the eggs within the sporosacs are dull green agains the red hydrorhiza, and the sperm when mature are a white mass.

Remove several "male" reproductive individuals showing ripe sporosacs, and crush them slightly under a coverslip on a glass sporosacs, and crush them slightly under a coverslip on a glass slide. This may be done by drawing off some of the water with blotting paper. The sporosacs fall far short of being perfect medusae. Note stages in the production of sperm within them. Burst a mature sporosac and study the sperm under high power.

Simularly, crush several "female" reproductive individuals, and observe the eggs with their large germinal vesicles, in various stages of development. Sketch the "female" gonosome with its female gonophores and contained eggs.

b) Cleavage and Development

If a number of "male" and "female" colonies of Hydractinia are put together in a large dish of sea water (or a pair of prime colonies in a fingerbowl) and left over-night, eggs should be shed and fertilized between 7 and 9 a. m. WST. The shedding can be controlled by light. If fertilization and cleavage stages are needed later in the day or in the evening, the colonies may be kept illuminated during the preceding night, put in the dark for a couple of hours and reilluminated one hour before the time when shedding is desired. Eggs are shed in 55 minutes, sperm in 50 minutes. Cf. Ballard '42.

Materials for the study of the entire development of Hydractinia from egg to polyp will be made available. If possible, observe the shedding of eggs and sperm. Sketch eggs undergoing first three cleavages, elongated gastrula, swimming and attached planulae, metamorphosing form and young polyps.

Eggs are heavily yolky and usually green, but occasionally gray, orange or pink. Maturation takes place during the half hour preceding shedding and polar bodies are lost soon after, a loose jelly being the only covering of the egg.

Cleavage may be irregular or even chaotic, but usually the slightly ameboid egg undergoes three equal total cleavages, each at right angles to the proceeding. The separating pairs of blastomeres tend to retain broad protoplasmic connections with each other on the side opposite to the cleavage furrow, until just before the succeeding cleavages begin. It is soon apparent that there is much variation in the time and degree of shifting of positions of the blastomeres, but the extraordinary and chaotic cleavage patterns commonly seen in the classroom are often the result of drying up and concentration of sea water, or other unfavorable circumstances.

Mitotic sychronism quickly disappears. Gastrulation is said to start even as early as the 16 cell stage, by mixed delamination and multipolar proliferation. The gastrula loses its spherical form and spends a few hours as an irregularly bumpy oblong mass, then returns to the spherical form and gradually lengthens into the planula form.

At the end of 24 hours the embryo is a "preplanula" (Teissier '27) with an elongated ovoid form, recognizable polarity and ciliation which enables it to swim heavily. During several days it lengthens, one end becoming slimmer and slimmer, while it rolls and crawls along the bottom like a planarian. The big end which goes first in this movement is the end which later produces the adhesive disc by which it attaches for metamorphosis. It becomes the aboral end of the polyp.

Following attachment of the attenuated planula, there is a delay of a few hours to several days, and then the tapering free end shrinks down almost to the substrate, where it shortly produces a mouth and a succession of tentacles. The new polyp elongates, its attached and meanwhile actively sending out a number of anastomosing and encrusting hydrorhiza processes from which sprout new polyps.

Illustrations of cleavage in Bunting '94, Beckwith '14; of later planula development in Teissier '37; of developing gonosome in Goette '07 and '16.

EUDENDRIUM (July, August)

The gonosomes of this genus are degenerate sessile medusa-forms or gonophores, strikingly different in the two sexes, borne at the bases of special hydranths which lose their tentacles and degenerate while the gonophores are ripening.

"Female" colonies bear loose irregular tufts of sporosacs attached to the stems, each ripe sporosac being bright orange in color. "Male"colonies bear light pink sporosacs arranged in groups of two to four or more in a line, the lines radiating from a common point on the base of the degenerated hydranth. The ripest male sporosacs occur at the periphery of the cluster and are white with sperm. Sketch both male and female sporosacs.

Eggs are fertilized within the female gonophore or sporsac, and develope to the planula stage before being liberated. (Hargitt, C. W. '04). Tease out embryos from different colonies and study all stages found. The eggs are so rich in yolk that they cleave like insect eggs. The gastrulation is by an extraordinary syncytial delamination. Metamorphosis is simple.

Eudendrium cleavage illustrated in Hargitt '04 (zool. Jahrb.); Gonosome development in Goette '07. Cf. also texts by Korschelt '36 and Hyman '40.

Development of Scyphozoa

SHRELIA OR CYANEA (April, June)

Both these jelly fishes have oral lobes extending downward. In mature specimens granular material will be found entangled on the lobes or contained in small brood sacs in the lobes. Tease off some of this material into a drop of sea water on a slide, and examine under the microscope. Embryos of different stages can be found, from spherical cleaving eggs to oval gastrulating forms and fully formed stocky, active planulae. (Hargitt, G. T. '1?) Hein '00/ Is cleavage regular? Sketch the embryonic stages that are available.

Select a number of active planulae and place them in clean watch glasses of sea water for further study on later days. Their gradual changein form, attachment to the bottom, acquisition of tentacles and elongation into the sessile scyphula stage can be easily followed. The resemblance of the scyphula to a simple polyp is obvious. Attachment plus the formation of 2 to 4 tentacles occurs on the second day, as does the development of an open mouth. There are 8 tentacles at 4 days, 16 at 2 weeks, 24 at 1 month. Sketch the scyphula in side view and in top view.

The scyphula or scyphistoma stage lasts throughout the winter. The animals increase in size and undergo asexual reproduction by transverse fission into ephyrae ("Strobilization"), and by other methods (Percival '23). The tiny ephrae (larval jellyfishes) are liberated and gradually transform into the adult form over a period of many months.

Aurelia life history illustrated in the texts of MacBride '14, Korschelt '36 and Hyman '40.

BIBLIOGRAPHY FOR COELENTERATES

MONOGRAPHS, KEYS, SPECIAL WORKS

Brooks, W. K. 1886 The life history of the Hydromedussae: A discussion of the origin of the medusae, and the significance of metagenesis. Memoirs, Boston Soc. Nat. Hist., 3: 359-430

[illegible], B. 1928 Studien über Geschlecht und Geschlechtszellen bei Hydroiden. Bemerkungen über die Entstehung der Keimzellen und die Entwicklung der Gonophoren bei Clava squamata. Roux' Archiv., 114: 501-510

Goette, A. 1907 Vergleichende Entwicklungsgeschichte der geschlechts-individuen der Hydropolypen. Zeit. f. wiss. Zool., 87: 1-336

Hargitt, C. W. 1901 Synopsis of North American Invertebrates. XIV. The Hydromedusae. Am. Nat., 35: 301-315, 379-395, 575-595

1903 XIV. The Scyphomedusae. Ibid., 37: 331-345

1904 The medusae of the Woods Hole region. Bull. Bur. Fish.. 24: 21-79

Hargitt, G. T. 1919 Germ cells of Coelenterates. VI. General considerations, discussion and conclusions. Jour. Morph. 33: 1-60

Hyman, L. H. 1940 The Invertebrates. 1. Protozoa through Ctenophora. McGra-Hill

Kingsley, J. S. 1910 A synopsis of the fixed hydroids of New England. Tufts College Studies, 3: 13-38

Mayer, A. G. 1910 Medusae of the world. 1. and 11. The Hydromedusae. 111. The Scyphomedusae. Carnegie Inst. Washington 109:

Nutting, C. C. 1901 The Hydroids of the Woods Hole region. Bull. U.S. Fish Commission, 19: 325-386

Sumner, F. G. et al 1913 A biological survey of the waters of Woods Hole and vicinity. Bull. Bur. Fish., 31:

A. GENERAL EMBRYOLOGY, DESCRIPTIVE

Allen, Carrie M. 1900 A contribution to the development of Parypha (Tubularia) crocea. Biol. Bull., 1: 291-315.

Beckwith, C. J, 1914 The genesis of the plasma structure in the egg of Hydractinia echinata. Journ. Morph., 25: 189-251.

Benoit, P. 1925 L'ovogénese et les premiers stades du developpment chez la Myriothele et chez la Tubulario. Arch. de Zool. Exper., 64: 85-326

Bunting, Martha 1894 The origin of sex cells in Hydractinia and Podocoryne, and the development of Hydractinia. Jour. Morph. 9: 203-236

Doflein, F. 1896 Die Eibildung bei Tubularia. Zeit. f. wiss. Zool 62: 61-73

Goette, A. 1916 Die Gattungen Podocoryne, Stylactis und Hydractinia. Zool. Jahrb., Abt. System. Geog. Okol. Tiere 39: 443-510

Gronberg, G. 1898 Beitrage qur Kenntiniss der Gattung Tubularia. Zool. Jahrb. Abth. f. Anat. 11: 61-76

Hargitt, C. W. 1900 A contribution to the natural history and development of Pennaria Tiarella. Am. Nat., 34: 387-415.

1904 The early development of Eudendrium. Zool. Jahrb., Abt. Anat. u. Ont., 20: 257-277

Hargitt, G. T. 1909 Maturation, fertilization and segmentation of Pennaria tiarella and of Tubularia crociea. Bull. Mus. Comp. Zool. Harvarc, 53: 159-212

Lowe, E. 1926 The embryology of Tubularia larnyx. Quart. Journ. Micr. Sci. 70: 599-625

May, A. J. 1903 A contribution to the morphclogy and development of Corymorpha pendula Ag. Am. Nat., 37: 579-599

Smallwood, W. M. 1899 A contribution to the morphology of Pennaria tiarella Am. Nat., 33: 861-870

Smallwood, W.M. 1909 A re-examination of the cytology of Hydractinia and Pennaria. Biol. Bull. 17: 209-240

Teissier, G. 1926 Notes critiques sur la morphologie des gonophores chez les hydraires. Arch. de Zool. Exper. et Gen. 65: 75-86

1931 Etude Expérimentale du développement de quelques hydraires Ann. des Sci. Nat. Zcol. 14: 5-60

Teissier, L and G 1927 Les Principales etapes du developpement d'Hydractinia echinata. Bull. Soc. Zool. Fr., 52: 537-547.

Wulfert, J. 1902 Die Embryonale Entwicklung vcn Gonothyraea loveni. Zeit. f. wiss. Zool., 71: 296-327

B. SPECIAL TOPICS - HERMAPHRODITE GONOPHORES, NORMAL AND EXPERIMENTAL

Bunting, Martha (listed above)

Foyn, B 1924 Kunstlich hergestellter Hermaphroditsmus bei Hydroiden. Zool. Anz., 61: 105-110

1927 Studien über Geschlecht und Geschlechtszellen bei Hydroiden. 1. lst Clava squamata (Müller) eine gonochoristische oder hemmaphrodite Art Roux' Archiv. 109: 513-534

1927 ll. Auspressungsversuche an Clava squamat (Müller) mit mischung von Zellen aus Polypen desselben oder verschiedenen Geschlechts. Ibid., 110: 89-148

Goto, S. 1910 On two species of Hydractinia living in symbiosis with hermit crab. Jour. Exp. Zool., 9: 569-496

Hargitt, C. W. 1906 The organization and early development of the egg of Clava leptostyla, Biol. Bull., 10: 207-232.

C. SPECIAL TOPICS * SYNCHRONOUS SPAWNING AND ITS RELATION TO LIGHT

Baker, E. G. S. 1936 Photoperiodicity in the spawning reaction of Pennaria tiarella. Proc. Indiana Acad. Sci. 45:251-252

Ballard, W. W. 1942 The mechanism for synchronous spawning in Hydractinia and Pennaria. Biol. Bull., June

Brooks, W. K. (listed above)
Hargitt, C. W. " "
Hargitt, G. T. " "
Mayer, A. G. " "

Murbach, L. 1895 Preliminary note on the life history of Gonionemus. Jour. Morph., 11: 493-496.

Smallwood, W. M. (listed above 1909)

Torrey, H. B. 1907 Biological studies on Corymorpha 11. The development of C. palma from the egg. Univ. Cal. Publ. 3: 253-298

D. SPECIAL TOPICS - ISOLATION OF BLASTOMERES

Beckwith, C. W. (listed above)
Hargitt, C. W. " "
Haggitt, C. W. 1911 Some problems of Coelenterate ontogeny. Jour. Morph 22: 493-550

Maas 1895 Experimentelle Beitrage zur Entwicklungsgeschlichte der Medusen. Zeit. f. wiss. Zool., 82: 601-610

Teissier, G. (Listed above)
Torrey, H. B. " "

Zoja, R. 1895 Sullo sviluppo dei blastomeriisolata dalle uova di alcune meduse. Arch. f. Entwicklungsmechenik 1: 578-595, and 2:1-37

E. SPECIAL TOPICS - REGENERATION AND AXIAL GRADIENTS
REGENERATION FROM CELLULAR AGGREGATIONS

Beadle, L. G. and Booth, F. A.
1938 The reorganization of tissue masses of Cordylophora lacustris and the effect of oral cone grats, with supplementary observations on Obelia gelatinosa. Jour. exp. Biol. 15: 303-326

Child, C. M. 1928 Axial development in aggregations of dissociated cells from Corymorpha. Physiol. Zool., 1: 419-461

DeMorgan, W and Drew, G. H. 1914 A study of the restitution masses formed by dissociated cells of the hydroids Antennularia ramosa and A. antennina. Jour. Marine Biol. Assoc. of Plymouth 10:

Föyne, B (Listed above)

Hargitt, C. W. 1915 Regenerative potencies of dissociated cells of Hydromedusae. Biol. Bull. 28: 370-384

Wilson, H. W. 1911 On the behavior of the dissociated cells in hydroids, Alcyonaria and Asterias. Jour. Exp. Zool. 11: 281-338

REGENERATION FROM NON-DISSOCIATED CELLS

Barth, L. G. 1940 The process of regeneration in hydroids. Biol. Rev. 15:405-420

Child, C. M. 1941 Patterns and problems of development. U. of Chicago Press (Contains references to all Child's papers)

Gilchrist, F. G. 1937 The hydroid polyp Corymorpha palma as gestalt and as history Am. Nat., 71: 382-403

Hazen, Annah 1902 Regeneration in Hydractinia and Podocoryne. Am. Nat. 36:193-200

Hargitt, C. W. 1899 Experimental studies upon hudromedusae. Biol. Bull 1: 35-5[illegible]

Lund, E. J. 1921 Experimental control of organic polarity by the electric current. 1. Effects of the electric current on regenerating internodes of Obelia commissuralis. Jour. Exp. Zool. 34: 471-493

1923 111. Norman and experimental delay in the initiation of polyp formation in Obelia internodes. Ibid., 37: 69-88

1924 1V. The quantitative relations between current density; orientation and inhibition of regeneration Ibid 39: 357-380

Morgan, T. H. 1906 Hydranth formation and polarity in Tubularia. Ibid 3: 501-516

Morgan, T. H. 1908 Some further records concerning the physiology of regeneration in Tubularia. Biol. Bull., 14: 149-162

1909 The dynamic factor in regeneration. Ibid., 16: 265-276

Morse, M. 1909 The autotomy of the hydranth of Tubularia Ibid 16: 172-182

Muller, H. C. 1913 Regeneration der Genophoren bei den Hydroiden und anschliessende biologische Beobachtungen. 1. Athecates. Archiv. f. Entwicklungsmechanik, 37: 310-419 11. Thecates Ibid. 38: 388-331

Peebles, Florence 1900 Experiments in regeneration and in grafting of Hydrozoa. Ibid, 10: 435-488

Torrey, H. B. 1910 Biological studies on Corymorpha. 1V. Budding and fission in heteromorphic pieces and the control of polarity. Biol. Bull. 19: 280-301

Zwilling, E. 1939 The effect and removal of perisarc on regeneration in Tubularia crocean. Biol. Bull. 76: 90-103

PAPERS ON SCYPHOZOA

Friedemann, O. 1902 Untersuchungen über die postembryonale Entwicklung von Aurelia aurita. Zeit. f. wiss. Zool., 71: 227-267.

Hargitt, C. W. Hargitt, G. T. 1910 Studies in the development of Scyphomedusae. Jour. Morph 21: 217-262

Hein, W. 1900 Untersuchungen über die Entwicklung von Aurelia aurita Zeit. f. wiss. Zool., 67: 401-438

Korschelt, E. 1936 Vergleichende Entwicklungsgeschichte der Tiere. Jena

MacBride, E. W. 1914 Text-book of Embryology. 1. Invertebrata. MacMillan

Percival E. 1923 On the strobilization of Aurelia. Quart. Journ. Micr. Sci., 67: 85-100

Reconstitution in Tubularia

The processes by which organisms replace lost parts have many points of similarity with those that take place in embryonic development. Thus, in the ontogeny of most species there is a period of cell multiplication with little or no cellular differentiation (cleavage stages) followed by the period in which cellular differentiation occurs. In regeneration the first of these two periods is represented by the formation of the blastema, and the second, by its differentiation.

Coelenterates replace lost parts with great facility but the process differs from true regeneration because no increase in cell division has been demonstrated and there is no blastema from which the new hydranth differentiates. Likewise, there are no special reserve cells to become activated by the injury and give rise to the new structures. Instead, the cut surface is healed by the expansion and migration of adjacent cells, and, without any new growth, transformation of cells of the stem into parts of the hydranth takes place. This type of replacement of lost parts is known as reconstitution instead of regeneration.

The study of reconstitutional development has some inherent advantages over embryonic development, chief of which is that in reconstitution one can study cell differentiation without having cell division as a serious complicating process. Another advantage is that of size. Reconstituting organs are generally considerably larger, and consequently easier to observe and manipulate, than are those in the embryo. Since the analysis of this process has been carried farther in Tubularia than in other forms, we shall study reconstitution in this genus in the laboratory exercises.

The differences in the tubularian stem are so slight that they are readily altered or reversed by alterations in the external or internal environment. If the stem is cut into small pieces each piece will form a hydranth at the distal but none at the proximal end, thus exhibiting polarity. However, if the distal end is inhibited by any means (by being covered by sand, Morgan '03; a glass tube, Barth '38; by being ligatured, Barth, '38; or by deoxygenated sea water, Miller '37) the proximal end will form a hydranth. Indeed, merely removing a piece of perisarc from the middle ofthe stem will permit the cells underlying it to differentiate (Zwilling, '39, Nakamura, '39). Obviously, any cells of Tubularia if exposed to normal sea water will form a hydranth if they are not inhibited from doing so by an earlier developing distal hydranth. This repressive action is known as "Dominance" and the region exerting this influence is known as the "Dominant Region".

Dominance is important embryologically as it allows the formation of a single structure from a mass of tissue which is capable of forming more than one structure. The region which has the greater tendency to form a structure represses adjacent tissue from forming that structure. This might be explained on a nutritional basis with the region of greatest activity drawing materials away from adjacent regions. In a crowded mass of cells as occrins in the gastrula stage there must be a keen competition between various cells for nutriments and a mutual inhibition by cells caused by their excretory products. In both of these phenomena the more rapidly metabolizing cell has the advantage and is most likely to differentiate.

The above situation can be brought about experimentally by taking a mass of cells and placing a barrier to free diffusion on one side of the mass. This is usually accomplished by allowing the mass to settle on the bottom of the container. This has the dual effect of increasing the concentration of excretory products and decreasing the oxygen tension in the region of contact. Differentiation is inhibited on this side while the hydranth forms from the cells of the opposite side. (Goldin and Barth '41, Child '28).

In the case of Tubularia it has been shown that the perisarc around the cells acts as a natural barrier to free diffusion of oxygen (Miller '40, '42), and that the hydranth forms at the cut end because these cells are released from an inhibition caused by both excretory products and low oxygen tension (Zwilling '38, Goldin '38a, '38b, Miller '39).

Collection and Care of Tubularia

Theoretically Tubularia is a solitary form but actually so manyindivuals grow together that a dense tangled mass usually results in the older forms. Young short stems are the best for experimental work and can be obtained from floats and rocks where the current is swift. In general it is best to collect your own stems. Since the stems need running water and a low temperature they do not keep well in the laboratory. In nature the hydranths drop off about the end of July and the stems remain dormant until the water cools down in the fall. At Woods Hole the stems appear in mid June and can be used until August. However, since the waters of Cape Cod Bay on the north shore are much colder, Tubularia may be obtained from the north end of the canal throughout August. The best method of keeping them in the laboratory is to place each bunch in a 3000ml beaker on steps which allow the water to cascade from one beaker to the next below to insure vigorous circulation.

The stems as collected vary in length, thickness and in general physiological condition (some are crushed, some starved, others old with large gonophores). Therefore, we cut the stems off and sort them out in a large finger bowl being careful not to crush them. For most work stems about 10mm in length are suitable. These are selected for uniform diameter and appearance. The hydranth is cut off a few mm from its base. It is necessary to cut off 3-5mm of the stem with the hydranth as this part of the stem does not regenerate consistently, especially in older stems.

Even after selection, the stems show some variability in regeneration and so it is best to pool all the stems for one experiment and select at random for the various parts of the experiment. Thus, if you are treating the stems in 4 different ways you should separate the stems into 5 lots at random using one lot as a control. The number in each lot depends on the nature of the experiment. Many experiments are of the all or none nature and 10 stems in each lot are sufficient. In experiments where rate of regeneration is compared under different conditions it is best to use about 25 stems in each lot. This gives satisfactory accuracy in averaging rates.

The stems are kept in running water and cool by placing them in Syracuse watch glasses which are first submerged in large finger bowls through which sea water is running. Care must be taken that the stems are not washed away.

The instruments used for cutting and handling are a sharp scalpel, a pair of sharp scissors, a pair of forceps and a medicine dropper. In using the forceps care must be taken that only the parts which are finally cut off and discarded are handled. Stems are most easily trimmed to size by cutting them on a glass plate over a black background with a ruler along which the proper length may be measured. After the stems have been cut to size they are transferred with a pipette and must not be handled with forceps.

When the temperature of running sea water gets above 25°C it is necessary to keep the stems in a refrigerated bath or in stoppered flasks with an atmosphere of oxygen.

Experiments to Perform

1. To Demonstrate Dominance

Four lots of 10 stems each will be used.

Lot 1 long stems (12 mm long after cutting)
Lot 2 short stems (6 mm long after cutting)
Lot 3 short stems (6 mm) ligatured in the middle
Lot 4 very short stems (2 to 3 mm long after cutting)

In preparing these lots be very careful to remove 3mm or more of the stem with the hydranth.

With regard to the proximal hydranth this is an all or none type of experiment. If taken from healthy colonies the developing distal hydranth either will completely prevent hydranth reconstitution at the proximal end of the piece or will have little effect upon it.

Place the stems in Syracuse dishes in a large finger bowl through which a current of sea water is flowing gently, or in finger bowls kept on the sea water table. At 48 hours the reconstituted hydranths should have emerged from the perisarc. Record the number of distal and proximal hydranths in each lot.

2 Effects of reducing metabolic exchange through one end of the stem.

Cut 20 10mm long stems in such a way that the two ends of the stems can be distinguished. This may be accomplished very simply by making the distal cut at an oblique angle and the proximal cut at a right angle to the stem.

Insert the distal ends of 10 of these into some washed sand in a finger bowl filled with sea water and insert the proximal ends of the other 10 into the sand. After 36 to 48 hours remove and count the hydranths which have developed at distal and proximal ends (Cf. Morgan '03).

3. Effects of Oxygen upon Reconstitution

Cut 20 or more 6 or 8 mm pieces. Place half of them in a 200cc Erlenmeyer flask filled to the top with oxygenated sea water and stopper tightly so that no air is trapped in the flask. Place the other half of the stems in a similar flask filled with sea water through which nitrogen has been bubbled. After 36 to 48 hours count the number of hydranths reconstituted in each flask. If you wish to continue the experiment, the stems from the nitrogenated flask may now be transferred to the oxygenated flask and their ability to reconstitute can still be elicited (Cf. Barth'38).

4. Effects of Oxygen upon Scale of Organization and upon Bipolarity (Cf. Miller '49)

Cut 60 or more very short pieces (1-1½mm long), select 50 which are most nearly the same size and divide into two lots of 25 each.

Lot 1. Place the pieces of this lot in the flask of oxygenated sea water you prepared for experiment 3. (Since they are very small there is no danger of confusing these pieces with the 6 or 8 mm pieces you already placed in it).

Lot 2. Place these pieces in a covered fingerbowl or Erlenmeyer flask on the water table.

Count the number of complete hydranths reconstituted and also the numbers of various partial hydranths beginning about 48 hours after the experiment was started. Since the partial hydranths are unable to emerge from the perisarc, it will be necessary in most cases to squirt them out by drawing them up into an eye dropper and squeezing the bulb rapidly.

Note that Oxygen increases:

(1) the percentage of pieces that reconstitute.
(2) the number of partial hydranths (a result of its effect upon the size of the primordia when there is not enough tissue in the piece to form a larger hydranth)
(3) the percentage of bipolar types. Can you suggest an explanation for the increased frequency of bipolar types in short pieces as a result of oxygenation, when it decreases bipolarity in longer stems?

5. Demonstration of the liberation of inhibitors of reconstitution by the cut ends of stems.

Fill 10 pieces of 1mm glass tubing 15mm long with oxygen. (This can be done very readily under water). Affix one end of each to a small amount of neutral plasticine attached to the bottom of the finger bowl and insert into 5 the distal end (cut obliquely) of a 10mm piece of stem. Into the other five insert the proximal ends (cut transversely) of similar pieces. After 36 hours record the hydranths reconstituted at the exposed ends and those at the ends inserted in the O_2 filled tubes (Cf. Rose and Rose '41, Miller '42).

6. To Demonstrate Effects of Acidity upon Reconstitution

Tubularia has been found to be very sensitive to acidity. (Goldin '42a, '42b). An external pH of 6 will completely prevent reconstitution. Observe stems which have been injected with phenol red 30 hours earlier and placed in glass tubes which interfere with the release of these pH lowering substances. Compare these with stems which have been injected but have not been placed in long tubes. Draw and color one stem from each lot. After the hydranths have emerged they may be drawn and colored again (Cf. Miller '49).

7. Gradients in Reconstitution

There are quantitative differences along the stem which can be demonstrated in a variety of ways, but perhaps the most significant is the difference in rate and size of the products of reconstitution at different levels of the stem. If there is material and time you may demonstrate this gradient by cutting long stems into thirds and recording the time of appearance of the constriction separating the future hydranth from the neck region (Barth '38). Since this constriction appears from 24 hours after cutting and the still unconstricted stems should be examined every two hours until it appears, it is well to begin this experiment as early in the day as practical.

Select 25mm stems for homogeneity with regard to diameter and appearance and after removal of the hydranth cut each into three pieces of equal length (6mm) and discard what is left. Place them in separate finger bowls marked Distal, Middle and Proximal and keep on a water table until they have been recorded. (If you have an ocular micrometer the length of the hydranth can be accurately measured also). After they all have reconstituted average times(and lengths) should be calculated. What factors can you suggest which might play a role in the differential which your experiments reveal?

RECONSTITUTION FROM NON-DISSOCIATED CELLS

Barth, L.G. 1938a. Oxygen as a controlling factor in the regeneration of Tubularia. Physiol Zool. 11: 179-186.

" 1938b. Quantitative studies of the factors governing the rate of regeneration in Tubularia. Biol.Bull.74:155-177.

" 1940a. The relation between oxygen consumption and rate of regeneration. Biol. Bull. 78: 366-374.

" 1940b. The process of regeneration in hydroids. Biol. Rev. 15: 405-420.

" 1944. The determination of the regeneration hydranth in Tubularia. Physiol. Zool. 17: 355-366.

Beadle, L.G. and Booth, F.A. 1938. The reorganization of tissue masses of Cordylophora lacustris and the effect of oral cone grafts, with supplementary observations on Obelia gelatinosa. J. Exp. Biol. 15: 303

Berrill, N.J. 1948. Temperature and size in the reorganization of Tubularia. Journ. Exp. Zool. 107: 455-467.

Bickford, E.E. 1894. Notes on regeneration and heteromorphosis of Tubularian hydroids. Journ. Morph. 9: 417-430.

Boring, A.M. 1904. Closure of longitudinally split Tubularian stems. Biol. Bull. 7: 154-159.

Child, C.M. 1926a. Studies on the axial gradients in Corymorpha II. Biol. Gen. 2: 609

" 1926b. Studies on the axial gradients in Corymorpha III. Biol. Gen. 2: 771

" 1931. Experimental modification of the scale of organization in the reconstitution of Tubularia. Physiol. Zool. 4: 165-188.

" 1927a. Experimental localization of new axes in Corymorpha without obliteration of the original polarity. Biol. Bull. 53: 469

" 1927b. Modification of polarity and symmetry in Corymorpha palma by means of inhibiting conditions and differential exposure. J. Exp. Zool. 47: 343

" 1929. Physiological dominance and physiological isolation in development and reconstitution. Roux Archiv. Ent. Mech. Organ. 117: 21

" 1941. Patterns and problems of development. Univ. Chicago Press. Pp i-ix, 1-811.

Child, C.M. and Hyman, L.H. 1926. Studies on the axial gradients in Corymorpha palma. I. Biol. Gen. 2: 355

" and Watanabe, Y. 1935. Differential reduction of methylene blue by Corymorpha palma. Physiol. Zool.8:395-416

Davidson, M.E. and Berrill, N.J. 1948. Regeneration of primordia and developing hydranths of Tubularia. Journ. Exp. Zool. 107: 465-477.

Goldin, A. 1942a. Factors influencing regeneration and polarity determination in Tubularia crocea. Biol. Bull.82:243-254

" 1942b. A quantitative study of the interrelationship of oxygen and hydrogen ion concentration in influencing Tubularia regeneration. Biol. Bull. 82: 340-346.

" and Barth, L.G. 1941. Regeneration of coenosarc fragments removed from the stem of Tubularia crocea. Biol. Bull. 81: 177-189.

Hyman, L.H. 1920. The axial gradients in Hydrozoa. III Experiments on the gradient of Tubularia. Biol. Bull. 38:353

" 1926. The axial gradients in Hydrozoa. Biol. Bull.50:406

Miller, J.A. 1937. Some effects of oxygen on polarity in Tubularia crocea. Biol. Bull. 73: 369

" 1939. Experiments on polarity determination of Tubularia regenerates. Anat. Rec. 75 Suppl. 38-39

" 1940. Oxygenation and ciliary rate in regenerating Tubularia stems. Bull. of the Mount Desert Island Biol. Lab. p. 41-44.

" 1942. Some effects of covering the perisarc upon Tubularia regeneration. Biol. Bull. 83: 416-427.

" 1946 Differential reduction of Janus Green in the early development of Tubularia crocea Anat Rec 94:

" 1949. pH of the coelenteric fluid of Tubularia during reconstitution. Biol. Bull.

Moore, J. 1939. The role of temperature in hydranth formation in Tubularia. Biol. Bull. 76: 104

Morgan, T.H. 1903. Some factors in the regeneration of Tubularia. Arch. f. Entw. mech. Organ. 16: 125-154.

Nakamura, N. 1939. Formation of hydranth in Tubularia at the place whence perisarc has been removed. Zool.Mag.51:183-184

Papenfuss, E.J. 1934. Reunition of pieces of Hydra with special reference to the role of the three layers and the fate of differentiated parts. Biol. Bull. 67: 223-243.

Rose, S.M. and Rose, F.C. 1941. The role of a cut surface in Tubularia regeneration. Physiol. Zool. 14: 328-343.

Watanabe, Y. 1935. Physiological dominance in Corymorpha palma in relation to reconstitution and methylene blue reduction. Physiol. Zool. 8: 417-441.

Zwilling, E. 1939. The effect of removal of perisarc on regeneration in Tubularia crocea. Biol. Bull. 76: 90-103.

TUNICATES

A comprehensive survey of developmental processes in the tunicates would include examination not only of eggs and embryos but also of metamorphosing larvae, various types of vegetative reproduction, and regenerating forms. Because these developmental forms must be constantly related to the structure of larvae and adults, it is necessary that the students should first of all review in a standard text the anatomy and life history of the common types of tunicates.

A. SIMPLE ASCIDIANS

DEVELOPMENT TO THE TADPOLE STAGE.

Styela (Old name Cynthia)

Conklin's classic description of the development of Styela eggs (1905) provides the morphological background for the experimental work on the organization of Tunicate eggs and embryos. Conklin's figures should certainly be referred to during the following studies.

a. Methods

Though truly hermaphroditic, Styela is ordinarily self-sterile like several other ascidians (Morgan, 1938). It sheds the eggs and sperm between 4 and 7 p.m., and fertilization takes place when ripe gametes from two different individuals get mixed. It is easy to have eggs shed and fertilized on normal schedule in the laboratory, but this entails the disadvantage of having to study the migrations of yellow pigment within them by artificial light.

The classic method of obtaining eggs and embryos from Styela has been to mince the gonads from a number of large individuals together in a dish of sea water. This liberates all stages in the maturation of eggs and sperm, and usually a few eggs will be fertilized, whatever the time of day or night, and will commence normal development.

S. M. Rose (1939) has developed a method of controlling the natural spawning in the laboratory by illumination, and this is the best way to get fertilization for experimental material. It works well except for a few weeks in mid-summer, when the animals are spent. By such control, the same batch of animals can be induced to shed a number of times on successive days. They are kept darkened in running sea-water until eleven or twelve hours before fertilization is desired, and then an artificial day is started by turning on an electric light. A 40 watt bulb 18" from the animals is sufficient. Eggs and sperm are discharged in clouds at the desired time.

b. Mature Unfertilized Egg.

Sketch the mature, unfertilized egg (diameter ≅ 0.15mm.) which should show the following:

(1) Chorion, a tough membrane with perhaps a few follicle cells adhering to its outer surface.
(2) Small spherical inner follicle cells ("nurse cells") between chorion and egg itself. They contain yellow granules.
(3) Peripheral layer of egg, a clear layer containing minute yellow granules.
(4) Central part, consisting of gray yolk platelets.
(5) Germinal vesicle, a very large eccentrically placed mass.

c. <u>Post-Fertilization Rearrangements</u>

This is a difficult process to observe as most eggs have too little pigment, and few are fertile in "minced" cultures. It is better to omit this section and to concentrate on cleavage and gastrulation. Then, if time is available and if the eggs this year contain sufficient pigment, these rearrangements may be studied.

<u>Procedure</u>
To a small dish containing some unfertilized pigmented eggs, add a drop of sperm. Then quickly transfer some of the eggs to a slide.

Carefully watch for the rearrangement of egg substances starting within 2-8 minutes after fertilization. The clear yellowish peripheral matter streams to the lower pole over the yolk, followed by the clear protoplasm from the animal pole. If the yellow pigment is not visible, try a more brightly colored egg. Use only daylight for illumination, <u>and have the diaphragm of the microscope wide open</u>. Roll the egg around by moving the cover slip with a needle. The yellow inner follicle cells may also be migrating; watch the egg cortex.

The grey yolk rises to occupy the upper pole, all except the space that surrounds the maturation-spindle complex. Soon the yellow substance accumulates on one part of the lower hemisphere, where it assumes crescentic form. Immediately above the broad part of the yellow crescent is a layer formed by the clear cytoplasm.

The most remarkable characteristic of Styela is that now at the conclusion of these movements the position of the future larva is visibly marked on the surface of the uncleaved egg. The broadest part of the yellow crescent is at the posterior pole, and the horns wrap half way around the right and left sides. The animal pole, where the germinal vesicle lay, becomes the ventral-anterior side of the larva. The vegetal pole, where the spermatozoon entered, is the future dorsal side. To avoid confusion, fix firmly in your mind the relationship between egg orientation. and definitive axis of embryo.

d. <u>Cleavage</u>

The following approximate time schedule for the embryology of Styela is from the great monograph of Conklin (1905). Follow the events of cleavage in as much detail as possible. Observe gastrulation and watch the tadpole take shape. Sketch a succession of cleavage stages, showing bilateral symmetry and location of yellow crescent material. Sketch at least 2 stages of gastrulation and 2 of elongating pro-tadpoles. (This schedule is for normally shed eggs. If eggs are obtained from "minced" cultures, cleavage is delayed, the eggs apparently maturing at variable intervals after striking the sea water.)

Fertilization	to	2	cells	after	40	minutes
2nd	cleavage	" 4	"	"	30	"
3rd	"	" 8	"	"	30	"
4th	"	" 16	"	"	20	"
5th	"	" 32	"	"	20	"
6th	"	" 64	"	"	20	"
7th	"	"112	"	"	20	"
8th	"	"218	"	"	20	"

To neural plate stage, 2 more hours
Fully formed tadpole 12 hours after fertilization

(1) First Cleavage. Equal, separating the two horns of the yellow crescent from each other, likewise bisecting the clear protoplasm anterior to the yellow.

(2) Second Cleavage. Nearly equal, vertical, at right angles to the first. The two posterior cells (B3 on the left and B3 on the right) contain little yolk and practically all the yellow crescent substance. The two anterior cells (A3 on the left and A3 on the right) contain much yolk, and practically none of the yellow crescent substance. The clear protoplasm goes equally to the four cells.

(3) Third Cleavage. Horizontal, the upper quadrant (cells a4, a4, b4 and b4) somewhat smaller than the lower quadrant(cells A4. A4, B4 and B4.) The yellow crescent substance is almost entirely confined to the two posterior dorsal cells (B4, B4).

(4) Fourth Cleavage. The planes of cleavage vary in different quadrants, but the new cells do not overlap the sagittal plane of the embryo. Two of the antero-dorsal cells and two of the postero-ventral cells of the 16-cell embryo are crowded a-way from this sagittal plane, but all other cells touch it. The dorsal and ventral hemispheres at this stage are mirror images. The yellow pigment lies in four posterior cells: (B5.1(larger cell), B5.2(smaller cell), B5.1 and B5.2).

(5) Fifth Cleavage. Cleavage in the dorsal (vegetal) hemisphere precedes that in the ventral (animal)hemisphere, and cleavage in the anterior part of each hemisphere precedes that in the posterior part.

At the 32 cell stage, the yellow substance is almost entirely confined to six dorso-posterior cells, three on each side of the midline (B6.2, B6.3, B6.4, B6.2, B6.3, B6.4). They give rise to mesoderm and mesenchyme.

Six yolk-filled cells at the vegetal (definitive dorso-posterior) pole anterior to the yellow mesoderm cells, will give rise to endoderm: A6.1, A6.3, B6.1, A6.1, A6.3, B6.1.

Four cells at the anterior border of the embryo just below the equator (A6.2, A6.4, A6.2, A6.4), and two just above the equator (a6.5 and a6.5) will give rise to the notochord and neural plate.

All the rest of the cells are ectodermal.

(6) Later Cleavage, gastrulation, neuralation. The gastrula passes through disc-shaped, saucer-shaped and cup-shaped stages starting with the 7th cleavage. As it finally becomes egg-shaped, the gastrula's blastopore, found at the small hind end, becomes T-shaped, the stem of the T bordered by the yellow mesoderm-mesenchyme cells.
Thecells overhanging the crossbar of the T-shaped blastopore constitute its dorsal lip. They overgrow it, finally engulfing the yellow cells which from then on are only seen dimly through the translucent ectoderm.
Watch the gradual elongation of the embryo and the appearance of definite tadpole form. (see Berrill, 1929, fig. 10.) Examine older cultures showing metamorphosis to adult. Sketch a 7 day old specimen.

In 1-day cultures note especially: notochord, muscle tail cells, cerebral vesicle with otolith and eye, endostyle, 3 adhesive papillae.
In 3-day cultures metamorphosis is well advanced. Note the well developed 2-4 respiratory ampullae; protostigmata may be present with beating cilia; branchial and atrial siphons; endostyle, developing gut.
In 7-day cultures: the adult form is well shown and organs well developed. The respiratory ampullae no longer functional. Heart beating.

Molgula (egg diameter - 0.11mm.)

The eggs of Molgula follow almost exactly the pattern of development seen in Styela, but they do not have pigment. The animals are self-fertile. A few fertilized eggs may be obtained at any time by mincing the gonads in sea water, although spawning usually occurs at daybreak.

Rose's method for controlling the spawning of Molgula or Ciona is to put a few individuals in a large dish of sea water and store them in the dark, for instance, in a desk drawer, until eggs are needed. Molgula will shed 15 minutes or so after being brought out into the light, Ciona immediately. The spawning of these forms will be demonstrated.

Start some Molgula eggs developing. If developing eggs are isolated in a watch glass, tadpoles and young stages of metamorphosis are obtainable. Tadpoles are fully formed in 8 hours. (Conklin, 1905). Note absence of "eye" but well-developed otolith. Examine older cultures showing metamorphosis. Sketch a 3-day specimen..

In 1-day cultures note the progress of metamorphosis and the appearance of the respiratory ampullae.
In 3-day cultures note one very long respiratory ampulla and several smaller ones (Berrill, 1929, fig. 18b); other structures as for Styela.
In 6-day cultures note the emergence of the adult action system. The renal vesicle may be seen close to the beating heart.

B. COLONIAL ASCIDIANS

1. AMAROUCIUM: TADPOLES, METAMORPHOSIS, EPICARDIAL BUDDING.
(Usually not available until July)
(See Pratt, 1935, Fig. 960 for sketch of adult anatomy)

a) Methods.
The larvae of Styela and Molgula are so small that study of their internal organization is difficult. The compound ascidian Amaroucium is viviparous, and the large tadpoles are easily obtained from parent colonies, provided the material is ripe and has been collected quite recently.

Tadpoles usually leave the parent colony shortly after sunrise. These undamaged and fully developed individuals can be collected in the laboratory, and they are the best material for the study of swimming and of metamorphosis. They collect at the top of the water on the side of the tank nearest the light.

If colonies are kept in a shrouded aquarium the shedding can be postponed until a more seasonable hour. Swarms of active tadpoles usually appear within fifteen minutes of bringing ripe colonies out of the dark. About a third of the tadpoles will emerge within half and hour, if illuminated first at nine o'clock in the morning; if the colony is kept dark until mid-afternoon about three quarters of them will emerge within a half an hour. Nearly all of them will commence metamorphosis within an hour of being shed.

b) Tadpole structure and behavior.
Watch Amaroucium tadpoles swarming in a dish of sea water. What are the reactions to light and gravity? How do they propel themselves? Place a few tadpoles on a slide in a small drop of water and study their anatomy under the microscope. (Grave, 1920, 1921; Scott, 1946)

Not all structures can be seen in a living tadpole. Note the thick test with contained test-cells, the adhesive papillae, the atrial siphon (near the tail), the oral siphon (usually larger); and the sensory vesicle between the two siphons.

The small pigment cup with lens is a light-perceiving organ, and there is a small round black static organ also within the sensory vesicle. In the tail, note the muscle cells and notochord.

c. Metamorphosis.
Place a dozen or so tadpoles in a drop of water on a dry watch glass for study of metamorphosis. When in the course of the next hour the tadpoles have firmly attached to the glass, add more sea water to the dish. After attachment (sometimes before there is time to attach) the tissue of the tail is destroyed by phagocytes, the test swells and metamorphosis is under way. Within a couple of hours, movements of the body may be observed. The results of the extensive and rapid internal reorganization that is going on can be observed best two days to a week later (Grave, 1935). Make several timed sketches of the external aspects of metamorphosis.

d. Later Stages.
Observe and sketch metamorphosed Amaroucium individuals which have been growing for four days or so after attachment. They are fastened to watch glasses which have been stored in frames under water. Gently flush debris from them at the sea-water tap, and avoid tipping off their cover of water. After making your records you will return the specimens, still living in their watch glasses, to the frames from which they were taken.
Examine the specimens under low power first, for orientation, If they are growing upright they may be flattened out be gently lowering a cover glass on them. At one end of this animal are the atrial and oral siphons, at the other is the post-abdomen, with the heart at its tip. Watch the heart for periodic reversal of beat. Identify the epicardium, a usually pigmented strand of tissue running throughout the post-abdomen from pharynx to heart region. It is the agent in asexual reproduction and colony formation.
Below the siphons is the pharynx with its three rows of numerous stigmata (visceral clefts). It opens into a short esophagus which connects with a round yellow stomach marked by muscular bands. The intestine turns sharply after leaving the stomach, and ends near the atrial siphon. The endostyle is sharply indicated on the wall of the pharynx, delimiting the two atrial pouches. These pouches surround the pharynx, and open to the exterior at the atrial siphon.

e. Epicardial Budding.
Asexual reproduction of new individuals may be seen in laboratory cultures about 17 days after attachment of the tadple. Or, swarms of buds in all stages of growth and migration can usually be found at the ba-ses of the tiniest transparent finger-like lobes of a large healthy colony. Demonstrations will be made of them, which should be sketched at low magnification.

Asexual reproduction is accomplished by strobilization, i.e. segmentation of the post-abdomen which contains the epicardial strand. The buds consist at first of inner vesicle (from epicardium) and outer covering (from parent epidermis). All internal organs of the new individuals form from the epicardium tissue, which was a pharyngeal derivative, i.e. endoderm.

This method of asexual reproduction is distinguished from others in Tunicates by being called Pharyngeal or Epicardial Budding (Kowalevsky, '74, Berrill '35). The epicardial buds while differentiating into new zooids move up and take their place around the parent. During the strobilization of the parent's postabdomen, the old heart is isolated and degenerates, and a new heart is regenerated in the parent.

2. BOTRYLLUS: TADPOLES, METAMORPHOSIS, ATRIAL BUDDING
(Sketches will be found in Berrill, 1940, 1941)

Botryllus is another compound Ascidian, which is found encrusting on rocks, wharves and floats. Certain colonies, brought into the laboratory and placed in dishes of sea water, will liberate many tadpoles. Tadpoles and all stages of cleavage and development may also be obtained by mincing the colonies and hunting in the debris. If the normally-shed tadpoles are placed in a little sea water in a watch glass, they soon attach to the dish and commence their rapid metamorphosis (Herman, F.C., 1924).

a. Structure of Tadpole.
The tadpole is not as large as that of Amaroucium, but shows an interesting new feature. Just under the adhesive papillae is a ring of 8 ampullae which are diverticula of the body wall, destined to be parts of the as yet non-functional and incomplete circulatory system (Graves and Woodbridge, 1924). In metamorphosis they unfold like petals of a flower, and spread around the edge of the developing tunicate. As the colony grows these increase in number.

In addition, identify the statolith, a densely black cup suspended in the sensory vesicle by a slender stalk and closely associated with light-sensitive elements (Grave and Riley, 1935); the two siphons; the pharynx with several stigmata; the stomach and intestine. Sketch the tadpole.

Tadpoles attach to a substrate within an hour or two after hatching. The metamorphosis is extremely rapid (Grave, 1935, Grave and Nichol, 1939). They often metamorphose without attaching and attach a day or so later.

b. Two-day Old Form. Atrial Budding.
Tadpoles have been allowed to attach to watch glasses and grow for two days. They are now larger and clearer, and usually so oriented that the observer looks directly down into the atrial and oral siphons. The large pharynx is in the shape of a truncated cone and bears up the three rows of stigmata (visceral clefts) which let water pass out into the atrial cavities of the two sides. The endostyle lies on the under side of the pharynx and thus appears as a rod under the mouth.

The stomach ordinarily appears as a yellow body under the atrial opening. The intestine after turning to one side from the stomach, returns to empty near the atrium. The pulsating heart and its vessels are of particular interest, especially the flow of blood around the mouth and pharynx.

A new bud (First blastozooid) appears from the sexually developed animal (the oozooid) as an evagination of the atrium at one side. It is furnished with a blood supply, and presently the differentiating parts can be distinguished. Coming from the atrium, the whole bud, and all parts of the new individual, are derived from ectoderm. This is the atrial type of budding (Berrill, 1941). Sketch an oozooid showing buds.

c. Week-old form.
In these animals, general structure already studied may be easily seen under low power. The pharynx of the oozooid has developed 4 rows of stigmata, and the first blastozooid may also have three or four rows. Blastozooid buds of the second and third order may be present.

The first blastozooid bud in Botryllus is single, all the later ones are in symmetrical pairs. The same organ structures may be seen in all the individuals, notwithstanding their diverse embryology, with the minor exception that the oozooid does not develop gonads. Through rearrangement of the individuals, the completed colony shows a common atrial pit in the center, with separate pharyngeal openings at the periphery (see wall chart).

Each bud consists at first of a disc, then a sphere. The sphere extrudes sex cells at one or both sides and becomes partitioned into three vesicles, the lateral ones forming atrial chambers, the middle one the pharynx. (Later stages show differentiation of the rest of the organs from the Pharynx-vesicle (Berrill, 1941).

3. PEROPHORA: SEPTAL BUDDING
(For sketches see Berrill, 1935)

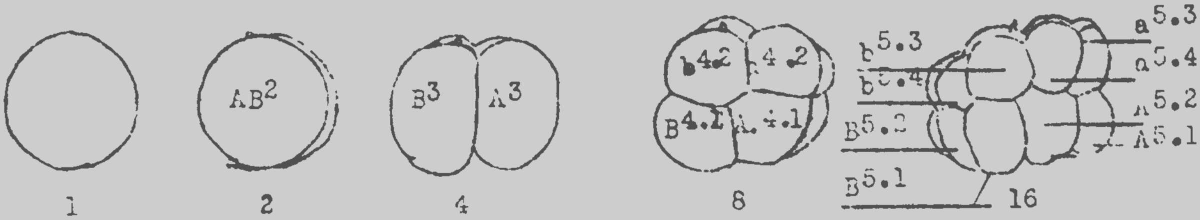
Perophora is a little green-colored ascidian, which by means of stolons forms loose colonies on wharf pilings, etc. Pieces of the colony may be gently stuck to watch glasses with vaseline, and stored in running sea water. After a day or so, stolons will be sent out over the surface of the glass and new blastozooids will be formed at intervals. Examine the watch-glass culture of Perophora which has been growing for two weeks.

Notice the branching pattern of the stolons. The tips show exploratory tendencies like small pseudopodia. The outgoing and incoming blood streams in the stolons are separated by a mesenchyme septum. All stages in the formation of new individuals will be found, arranged like pumpkins on a vine, with the youngest nearest the tips. The youngest buds consist of an outer vesicle derived from the epidorm of the colony and an inner vesicle formed by the splitting of the mesenchymatous stolon septum.

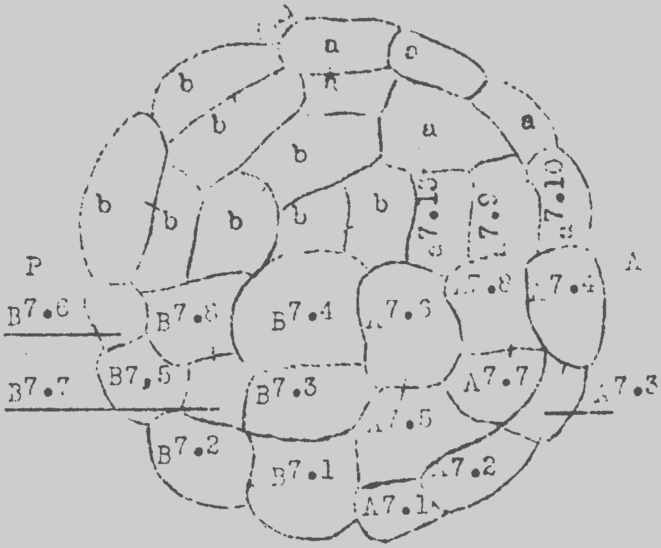
Oogenesis takes place in the inner vesicle, which is derived from mesenchyme (Berrill, 1935). This method of budding is distinguished as the septal type.

Styela

Schematic Representation -- lateral view, left.



an = ventral



veg = dorsal

64

An Hemisphere

Cerebral vesicle = a7.13, a7.9, a7.10

Ectoderm = other a
all b

Veg Hemisphere

Muscle = B7.4, B7.8

Mesenchyme = B7.6, B7.5, B7.7, B7.3, A7.6

Neural Plate = A7.4, A7.8

Chorda = A7.3, A7.7

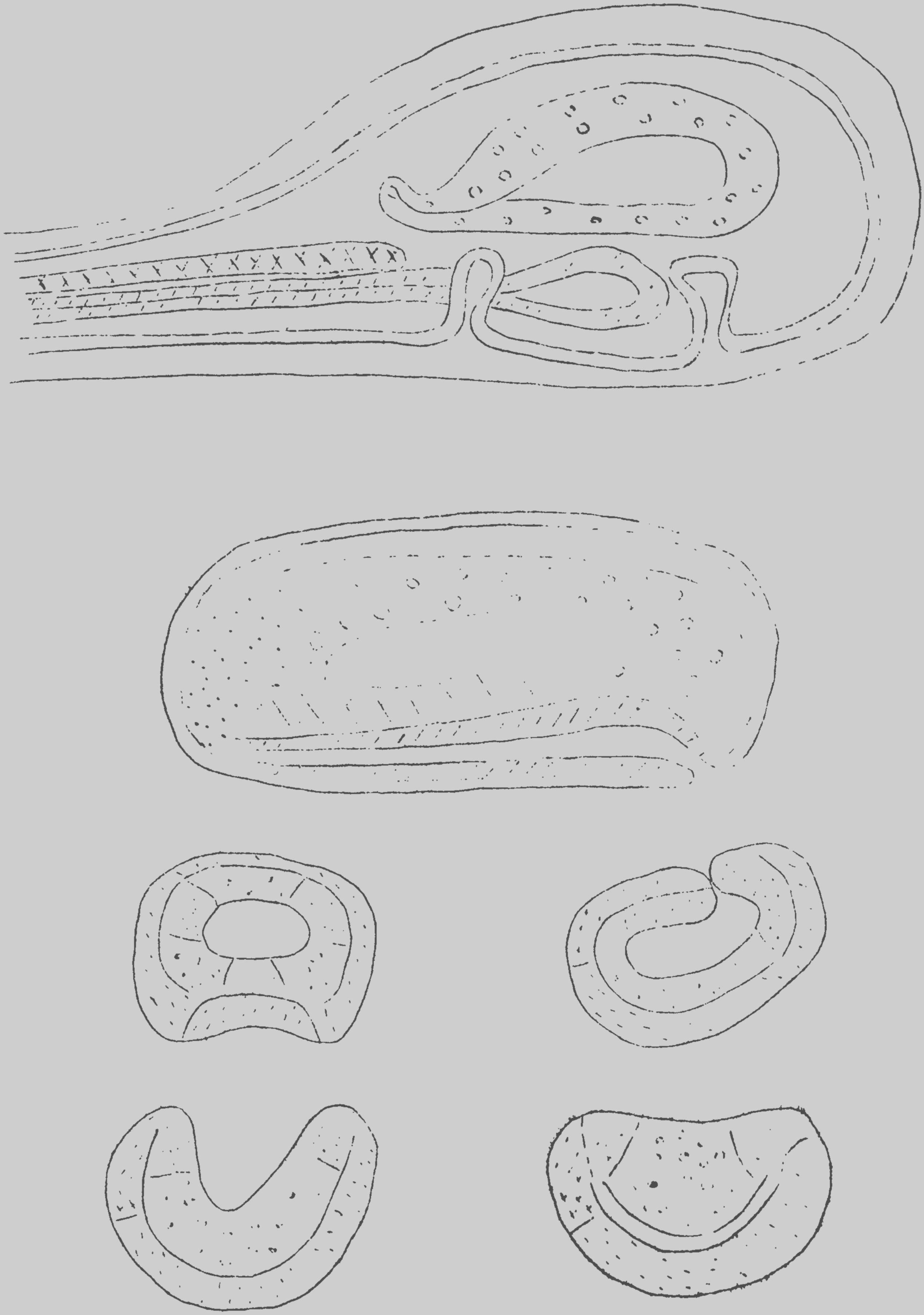
Endoderm = B7.1, B7.2, A7.1, A7.2, A7.5

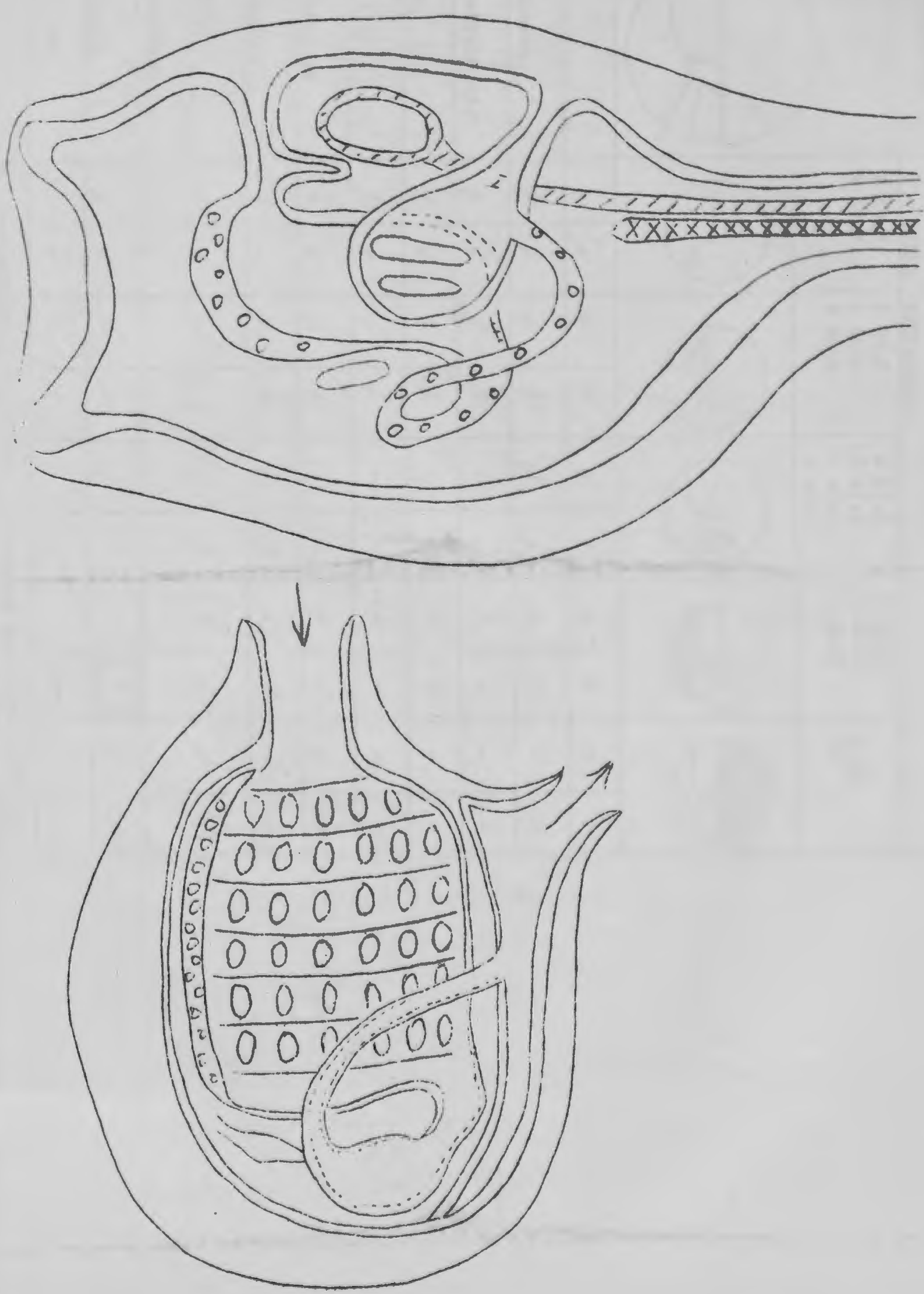
Anterior cells are A or a.
Posterior cells are B or b.

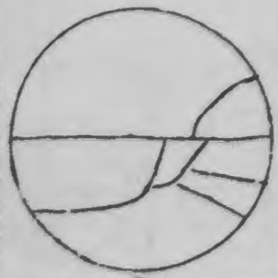
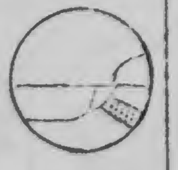
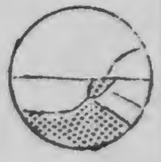
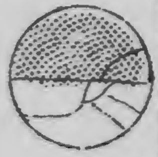
Animal hemisphere cells are a or b) at 8-cell stage
Vegetal hemisphere cells are A or B) and beyond

Left and Right corresponding blastomeres bear same designation except right ones are underscored

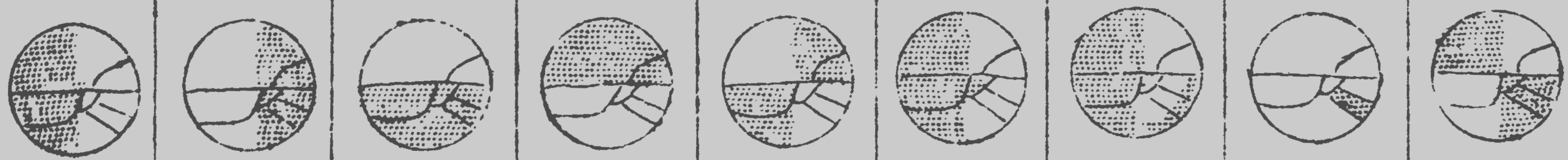
The first number gives the generation of cell, counting the egg as first generation.





Unshaded= cells developing	Based on data in v. Ubisch, 1939									
	CELLS EXTIRPATED									
	2 A7.3 2 A7.7		2 B6.2 2 B6.3 2 B6.4		2 A7.8 2 A7.4 2 a6.5 2 a6.7		2 A6.1 2 A6.3 2 B6.1		8 an / 16	
										
	P	F	P	F	P	F	P	F	P	F
ectoderm	+	+	+	+	+	+	+	+	-	-
cerebral ves.	+	+	+	+	-	-	+	+	-	-
eye	+	+	+	+	-	-	+	+	-	-
otolith	+	+	+	+	-	-	+	+	-	-
nerve tube	+	+	+	+	-	-	+	+	+	(+)
notochord	-	-	+	+	+	#	+	+	+	(+)
muscle mesenchyme	+	+	-	-, +	+	+	+	+	+	(+)
endoderm	+	+	+	+	+	+	+	+	+	(+)
papillae		+		+		+, -		+, -		-
	P- presumptive F- [illegible] ( )= tissue not organ differantiated									

Based on data in ROSE, 1939

Cells isolated								Cells in Transplantation Combinations									
2 A3		2 B3		2 a4.2 2 b4.2		2 A4.1 2 B4.1		2 A4.1 2 b4.2		2 a4.2 2 A5.1		2 a4.2 1 A5.2		all less 2 A6.2 2 A6.4		2 a4.2 2 B4.1	
																	
P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F
+	+	+	+	+	(+)	-		+	+	+	+	+	+	+		+	+
+	(+)	-	-	+	-	-		-	+	+	+	+		+		+	-
+	+	-	-	+	-	-		-	+	+	+	+		+		+	-
+	+	-	-	+	-	-		-	+	+	+	+	+	+	+	+	-
+	(+)	-	-	-	-	+		+		+		+		-		-	
+	+	-	-	-	-	+		+		+		+		-		-	
-	-	+	(+)	-	-	+		-		-		-		+		+	
+	?	+	?	-	-	+		+		-		+		+		+	
+	+	+	(+)	-	-	+	↓			+		+		+		-	
							Poor devel.										

BIBLIOGRAPHY

GENERAL WORKS AND COMPARATIVE EMBRYOLOGY OF PROTOCHORDATES

Berrill, N. J. 1936 Studies in Tunicate Development, V. The Evolution and Classification of Ascidians. Phil. Trans. Roy. Soc. London B. 226:43-70.

Conklin, E. G. 1932 The embryology of Amphioxus. Jour. Morph., 54:69-149.

1933 Development of isolated and partially separated blastomeres of Amphioxus. Jour. Exp. Zool. 64:303-375.

Dalcq, A. 1935 L'Organization de l'Oeuf chez les Chordés. Paris.

1938 Form and Causality in Early Development. Cambridge.

Dawydoff, C. 1928 Traité d'Embryologie comparée des Invertebrés. Paris.

Herdman, E. C. 1924 Botryllus. Liverpool Marine Biological Committee Memoirs, XXVI:

Herdman, W. A. 1899 Ascidia. Liverpool Marine Biological Committee Memoirs, I:

Korschelt, E. 1936 Vergleichende Entwicklungsgeschichte der Tiere. Jena.

Seeliger, O. 1893-1907 Tunicaten. Bronn's Classen u. Ordrungen des Tierreichs, III: Suppl.

Willey, A. 1893 Studies on the protochordata. Quart. Jour. Micr. Sci., 34:295-324.

TUNICATE EMBRYOLOGY

Berrill, N. J. 1929 Studies in Tunicate Development. 1. General Physiology of Development of Simple Ascidians. Phil. Tr. Roy. Soc. London, 218 (B) :37-78.

1931 II. Abbreviation of Development in the Molgulidae. Ibid., 219 (B):281-346.

1935 III. Differential Retardation and Acceleration. Ibid., 225 (B):255-326.

Castle, W. E. 1896 The early embryology of Ciona intestinalis Flemming (L.). Bull. Mus. Comp. Zool. 27:203.

Cohen, A. and Berrill, N. J. 1936 The early development of ascidian eggs. Biol. Bull. 70:78

Conklin, E. G. 1905 Organization and cell-lineage of the Ascidian egg. Jour. Acad. Nat. Sci. (Philadelphia), 13 (2nd series):1-121.

Dalcq, A. and … 1937 On the signi…

Elwyn, A. 1937 Some stages in the develonment of the neural complex in Ecteinascidia turbinata. Bull. Neurol. Inst. New York. 6:163-177.

Harvey, L. 1927 The history of the cytoplasmic inclusion of the egg of Ciona intestinalis (L.) during ovogenesis and fertilization. Proc. Roy. Soc. London. B. 101:

Kingsley, J. S. 1883 Some points in the development of Molgula. Proc. Boston Soc. Nat. Hist., 21:441-450.

Kowalevsky, A. 1871 Weitere Studien uber die Entwicklung der einfachen Ascidian. Arch. Mikr. Anat., 7:101-130.

Scott, F. M. 1945 The developmental history of Amaroecium constellatum. I. Early embryonic development. Biol. Bull. 88:120-138.

Spence, J. and Richards, O. W. 1940 Native Cellulose in the ascidian Phallusia nigra. Carnegie Instit. Papers Tortugas Lab. 32:163-167.

Tucker, G. H. 1942 The histology of the gonads and development of the egg envelopes of an ascidian (Styela plicata Lesueur). Jour. Morph. 70:81-108.

Van Benedin, E. and Julin, C. 1887 Recherches sur la morphologie des tuniciers (the development of Clavelline). Arch. de Biol. 6:237-476.

Vanderbroek, G. 1937 Les mouvements protoplasmique au cours de la fecondation de l'oeuf d'ascidie. Arch. exp. Zellforsch. 19:411-420.

von Ubisch, L. 1938 Eine vergleichende Studie uber die Organization des Keimes von Seeigehn, Amphibion und Aszidien. Biol. Zentralb. 58:370-385.

EXPERIMENTAL ANALYSIS OF DEVELOPMENT

Berrill, N. J. 1932 Mosaic Development of Ascidian Eggs. Biol. Bull. 63:381-386.

Chabry, L. 1887 Contribution a l'embryologie normale et tératologique des Ascidies simples. Jour. Anat. et Physiol., 23:167-319.

Cohen, A. and Berrill, N. J. 1936 The development of isolated blastomeres of the ascidian egg. Jour. Exp. Zool. 74:91-118.

Conklin, E. G. 1905a Mosaic development in ascidian eggs. Jour. Exp. Zool., 2:146-223.

1905b Organ-forming substances in the eggs of Ascidians. Biol. Bull., 8:205-230.

1906 Does half of an ascidian egg give rise to a whole larva? Arch. Entw. Mech. 21:727.

1911 The organization of the egg and the development of single blastemeres of Phallusia mammillata. J. Exp. Zool. 10:393-407.

Conklin, E. G.	1931	The development of centrifuged eggs of Ascidians. Ibid., 60:1-120.
Crampton, H. E.	1897	The ascidian half embryo. Ann. N. Y. Acad. Sci. 10:50-.
Dalcq, A.	1932	Etude des localisations germinales dans l'oeuf verge d'Ascidie par des experiences de morogonie. Arch. d'Anat. micr. 28:224.
	1938	Form and causality in early development. Cambridge University Press.
	1938	Etude micrographique et quantitative de la merogonie double chez Ascidiella scabra. Arch. de Biol. 49:397.
Driesch, H.	1895	Von der Entwicklung einzelner Ascidienblastomeren. Arch. Entw. Mech. 1:398-.
	1903	Uber Anderung der Regulationsfahigkeiten im Verlauf der Entwicklung bei Ascidien. Arch. Entw. Mech. 17:
Morgan, T. H.	1938	The genetic and physiological problems of self-sterility in Ciona. Jour. Exp. Zool., 78:271-334.
	1942	Cross- and self-fertilization in the ascidian Styela. Biol. Bull., 82:161-171.
	1945	Normal and abnormal development of the eggs of Ciona. Jour. Exp. Zool., 100:407-416.
	1945	The conditions that lead to normal or abnormal development of Ciona. Biol. Bull., 88:50-62.
Reverberi, G.	1931	Studi sperimentali sull 'uovo di Ascidie. Publ. Staz. Zool. Napoli, 11:168.
	1933	La segmentazione dei blastomeri iselati dell'uovo di Cione e di Pha-llusia. Pub. Staz. Zool. Napoli, 12:385.
	1937	Richerche sperimentali sulla struttura dell'uovo fecondate delle ascidie. Commentationes Pontif. Acad. Scient., 1:135.
Ries, E.	1939	Versuche uber die Bedentung des Substanzmosaiks fur die embrynale Gewebedifferenzierung bei Aszidien. Arch. exp. Zellforsch., 23:95-121.
Rose, S. M.	1939	Embryonic Induction in the Ascidia. Biol. Bull., 77:216-232.
Schmidt, C. A.	1931	Die Entwicklung der Palpen bei Ascidien-halbeilarven. Arch. Zool. Ital. (Torino), 16:490-.

Tung, Ti-Chow 1932 Experiences de coloration vitale sur l'oeuf d'Ascidiella aspersa. Arch. de Biol., 43:451-469.

1934 Recherches sur les potentialites des blastomeres chez Ascidiella scabra. Experiences de translocation, de combinaison et d'isolement de blastomeres. Arch. d'Anat. micro., 30:381-410.

Tung, T. Ku, S. and Tung, Y. 1941 The development of the Ascidian egg centrifuged before fertilization. Biol. Bull. 80:153-168.

von Ubisch, L. 1938 Uber Keimverschmelzung an Ascidiella aspersa. Arch. Entw. Mech., 138:18-36.

1939 Uber die Entwicklung von Ascidienlarven nach fruhzeitiger Entfernung der einzelnen organbildenden Keimbezirke. Arch. Entw. Mech. 139:438-492.

TUNICATE TADPOLES AND THEIR METAMORPHOSIS

Bradway, W. 1936 The experimental alteration of the rate of metamorphosis in the tunicate, Clavelina huntsmani (Van Name). J. Exp. Zool., 72:213-.

Grave, C. 1920 Amaroecium constellatum. I. The activities and reactions of the tadpole larvae. Jour. Exp. Zool., 30:239-258.

1921 11. The structure and organization of the tadpole larva. Jour. Morph., 36:71-101.

1926 Molgula citrina. Activities and structure of the free-swimming larva. Jour. Morph. and Physiol. 42:453-471.

1935 Metamorphosis of Ascidian Larvae. Papers from the Tortugas Laboratory, 29:209-292.

1940 Studies of larval life and metamorphosis in Ascidia nigra and species of Polyandrocarpa. Carnegie Inst. Papers, Tortugas Lab. 32:1-46.

1941 1 The "eye-spot" and light responses of the larva of Cynthia partita. Biol. Bull. 81:287-.

1944 The larva of Styela (Cynthia) partita. Structure, Activities and Duration of Life. Jour. Morph., 75:173-191.

Grave, C. and Nicoll, P. A. 1939 Studies of larval life and metamorphosis in Ascidia nigra and species of Polyandrocarpa. Papers from the Tortugas Laboratory, 32:1-46.

Grave C. and Riley G. 1935 Development of the sense organs of the larva of Botryllus schlosseri. Jour. Morph. 57:185-211.

Grave, C. and Woodbridge, H. 1924 Botryllus schlosseri; the behavior and morphology of the free-swimming larva. Jour. Morph. and Physiol. 39:207-247.

Holmgren, E. 1933 Ein Beitrag zum experimentellen Studien der Clavelina metamorphose. Arch. Entw. Mech. 129:199-205.

Kowalevsky, A. 1892 Beitrage zur Bildung des Mantels der Ascidien. Mem. Acad. St. Petersburg., 38:

Mast, S. O. 1921 Reactions to light in the larvae of the ascidians Amaroucium constellatum and Amaroucium pellioidum with reference to photic orientation. Journ. Exp. Zool., 34: 149-188.

van Beneden, E. and Julin, C. 1884 Recherches sur le development postembryonnaire d'une Phallusie, Phallusia scabroides. Arch. de Biol. 5:611-678.

TUNICATE ASEXUAL REPRODUCTION

Berrill, N. J. 1935 Studies in Tunicate Development. IV. Asexual Reproduction. Phil. Trans. Roy. Soc., London, 225 (B):327-379.

1935 Cell division and differentiation in asexual and sexual development. J. Morph. 57:353-427.

1940 The development of the colonial organism Symplegnia vivide. Ibid., 79:272-281.

1941a The development of the bud in Botryllus. Biol. Bull., 80:169-184.

1941b Size and morphogenesis in the bud of Botryllus. Ibid., 80:185-193.

1941 Spatial and temporal growth patterns in colonial organisms Growth, Third Growth Symposium, 89-111.

1947 The developmental cycle of Botrylloides. Q.J. Micros. Sci., 88:393-407.

Brien, P. 1927 Blastogènese des Tuniciers. Arch. de Biol., 37:1-46.

1930 Contribution a l'étude de la Regeneration naturelle et experimentelle chez les Clavelinidae. An. Soc. Roy. Zool. Belg., 61:19-112.

Caullery, M. 1909 Contributions a l'étude des Ascidies composees. Bull. Sci. Fr. Belg., 27:1-158.

Deviney, E. M. 1934 The behavior of isolated pieces of ascidian (Perophora vividis) stolon as compared with ordinary budding. J. Elisha Mitchell Sci. Soc., 49:

Kowalevsky, A. 1874 Uber der Knospung der Ascidien. (Didemnium & Amaroucium). Arch. Mikr. Anat., 10:441-470.

Pizon, A. 1905 L'evolution des Diplosomes (Ascidies composees). Arch. de Zool. Exp. et Gen., IV:1-68.

Ritter, W. E. 1897 Budding in compound ascidians, based on studies on Goodsiria and Perophora. Jour. Morph., 12:149-238.

de Selys-Longchamps, M. 1917 Sur le bourgeonnement des Polystyélines. Bull. Biol.-Sci. Fr. Belg., 50:170-276.

Spek, J. 1927 Uber die Winterknospenentwicklung, Regeneration und Reduktion bei Clavellina lepadiformis, und die Bedeutung besonderer "omnipotenter" Zellelemente fur diese Vorgänge. Roux. Archiv. III:119-172.

Watterson, Ray L. 1945 Asexual reproduction in the colonial tunicate Botryllus schlosseri (Pallas) Savigny, with special reference to the developmental history of intersiphonal bands of pigment cells. Biol. Bull., 88:71-103.

TUNICATE REGENERATION AND REDUCTION

Berrill, N. J. and Cohen, A. 1936 Regeneration in Clavelina lepadiformis. Jour. Exp. Biol., 13:352-362.

Birmingham, L. 1941 Regeneration in the early zooid of Amaroucium constellatum. Biol. Bull., 81:287-288.

Brien, P. 1933 Regeneration thoracique chez Archiascidia. Bull. Biol. Fr. Belg., 67:100-124.

Driesch, H. 1902 Die Restitutionen der Clavellina lepadiformis. Arch. Entwicklungsmechanik, 14:247-287.

Huxley, J. S. 1921 Studies in dedifferentiation II. Dedifferentiation and resorption in Perophora. Quart. Journal Micr. Sci., 65:643-697.

1926 VI. Reduction phenomena in Clavellina lepadiformis. Publ. Staz. Zool. Napoli, 7:1-35.

Ries, E. 1937 II. Das verhalten differenzierter und undifferenzierter zellen bei der Regeneration, Reduktion und Knospunguon Clavelina lepadiformis. Arch. Entw. Mech., 137:327-362.

ADDITIONAL REFERENCES

Almagia, Fiorenza	1946	Le sviluppo dei frammenti centrifugati dell'uovo vergine di Ascidie. 20:179-197. Pub. del. Staz. Zool. Napoli.
Bertholf, Lloyd M.	1945	Accelerating metamorphosis in the tunicate, Styela partita. Biol. Bull. 89 (2):184-185
Brien, P.	1946	La branchie des Tuniciers. Ann. Soc. Roy. Zool. Belg. 76:64-72.
Coe, Wesley R.		Nutrition and sexuality in protandric gastropods of the genus Crepidula. Biol. Bull. 94:158-160.
Goldin, Abraham	1948	Regeneration in Perophera viridis. Biol. Bull. 94:184-193.
Reverberi, G. and Minganti, A.	1946	Le potenze dei quartetti animale e vegetativo isolati di Ascidiella aspersa. Pub. del Staz. Zool. Napoli. 20:135-151.
Reverberi, G. and Minganti, Antonie	1947	La distribuzione delle potenze nel germe di Ascidie alle stadie di otto blastomeri analizzata mediante le combinazionie i trapianti di blastomeri. Pub. del Staz. Zool. Napoli. 21:1-35.
Reverberi, G. and Minganti, A.	1946	Fenomeni di evocazione nello sviluppo dell'uovo di Ascidie. Pub. del Staz. Zool. Napoli. 20:199-252.
Reverberi, G.	1939-40	Lo sviluppo dei framenti dell'uovo centrifugato delle Ascidie. Pub. del Staz. Zool. Napoli 18:129-139.
Ubisch, L. Von	1938	Eine vergleichende Studie uber die organization des Keimes von Seeigeln, Amphibien, und Aszidien. Biol. Zentralb. 58(7/8):370-385.
v.Weel, von P.B.	1939-40	Beitrage zur Ernahrungsbiologie der Ascidien. Pub. del Staz. Zool. Napoli. 18:50-79.

CULTURING EMBRYOS ON VASELINE-SEALED SLIDES

AND COVERSLIPS AND IN CELLOPHANE TUBING

It is often desirable to culture single embryos or small numbers of them in a small volume of medium so that they can be readily observed from time to time and so that their swimming activities do not make searching for them too time consuming. For this purpose they may be kept in a small drop in a chamber formed by a slide and coverslip sealed together with vaseline. It is also often desirable to culture large numbers of embryos in rather concentrated suspension. The use of cellophane tubing helps overcome the difficulty of attempting to transfer large numbers of swimming embryos to fresh sea water and permits satisfactory development of rather concentrated suspensions.

Equipment: Vaseline (fairly pure petroleum), slides, coverslips, ordinary droppers, fine-tipped droppers, cellophane tubing (Visking cellulose sausage casings ca. 1" diam.), funnel (6"), Syracuse dishes, finger bowls, and scissors.

Solutions: Filtered sea water, Diatom culture.

Vaseline-Slide Method: Warm a small amount of vaseline in a beaker to just above the melting point and with a warm dropper make a hollow square of vaseline on a clean, dry slide. A bent metal rod can also be used for this purpose in place of the dropper. The outside dimensions of the square of vaseline should be roughly the same as that of the coverslip that is to be used and the height should be about 1 mm. Place a small drop containing the eggs or embryos within the square on the slide. This drop should be of such size that when the coverslip is added, completely sealed to the slide and contact made with the drop, the diameter of the drop will be about 2 to 3 mm. The coverslip should be pressed down sufficiently so that a completely sealed chamber is formed, with no air channels running through the vaseline. Avoid having the drop come in contact with the vaseline. The slide should be kept in a cool place and the embryos transferred to a fresh preparation at invervals of one to four days depending upon the amount of living material present.

Cellophane Tubing Method: The seamless cellophane tubing is usually supplied in flat rolls of about 100 feet. Cut off a piece about one foot in length and tie a knot in one end of it. Soak the piece in sea water for a few minutes, then open the other end by sliding the two sides of the flattened tube against one another. Introduce a funnel into the open end of the tube and pour in about 100 cc. of the suspension of eggs or embryos. Tie off the tube in such a way than an air pocket of about one-fifth of the volume of fluid is left. Then place the tube in a dish or aquarium of running sea water in such a way that the water tends to flow past it. With small embryos of annelids, mollusks, or echinoderms, several thousand embryos per 100 cc. can be kept alive in this manner provided they are changed to fresh tubes once or twice a week. To obtain later developmental stages and growth food materials must be added. For this purpose a small amount of a dense suspension of diatoms (Nitschia) should be added at each transfer.

PREPARATION OF FIXED AND STAINED MATERIAL BY THE FLATTENING METHOD

The following is a brief account of a rapid slide-making that is useful for such purposes as making chromosome counts, determining stages of mitosis, fertilization, etc., on developing eggs and other material. It involves flattening the eggs or embryos betwen two coverslips and is, therefore, unsuitable for any work in which it is desired to retain the normal shape of the cells.

Equipment: - 6 Syracuse dishes, 2 or more clean No. 1 coverslips, 2 Columbia staining dishes (coverslip-size), 1 forceps, 1 dissecting needle, 2 or more slides.

Solutions:- Bouin's fixing fluid, Delafield's hematoxylin, Alcohol series (30%, 50%, 70%, 85%, 95%, and 100%), Acid-alcohol (1% HCl in 30% alcohol), Xylol, Canada Balsam or Euparal.

Procedure:- The method consists simply in joining and later separating two coverslips, one of which contains a drop of the fixing fluid, the other a drop of the egg suspension. The coverslips with adhering flattened eggs are subsequently handled in the same manner as slides of sectioned material. To facilitate later separation the coverslips should be joined crosswise. The following illustrates the procedure. Support one coverslip on the edges of two Syracuse dishes placed next to each other (or some other convenient support) and add a small drop of fixing fluid (Bouin's). Hold the other coverslip in one hand and add a small drop of the egg suspension at the desired stage. Invert the second coverslip over the first in crosswise position (so that the corners do not coincide) and release it as soon as the drops touch. The drops should not be allowed to undergo any appreciable evaporation before joining. The size of the drops should be such that when joined the fluid does not quite fill the space between the two coverslips. The eggs are thus flattened and the degree of this flattening can be regulated to some extent by the size of the drops. The fixing fluid acts rapidly on cells of small diameter, especially when they are flattened. With most marine eggs of 0.05 to 0.2 mm., two to ten minutes usually suffices for good fixation in Bouin's fluid. The joined coverslips are then carefully placed in a Syracuse dish and 70% alcohol is added whereupon the coverslips tend to separate. Lift the upper coverslip off by means of a fine forceps using a needle placed at the opposite edge of the upper coverslip to prevent it from sliding while being removed, and place it egg-side up in another dish of 70% alcohol. Sliding of one coverslip over the other will cause distortion and loss of eggs or fragments thereof. Ordinarily about half of the eggs will adhere to each coverslip. If the fixing fluid had been allowed to act for too long a time before the eggs were actually flattened, many of the eggs would fail to adhere to the coverslips when they are separated. Allow the coverslip to remain in two or three changes of 70% alcohol for a sufficient length of time (usually at least an hour) to remove the yellow color of picric acid (of the Bouin's fluid) from the eggs.

Transfer the coverslips through 50% and 30% alcohol (about one minute in each) to Delafield's hematoxylin for ten minutes or longer. The Columbia staining dishes are convenient for this and subsequent handling. Then wash once in tap water and place the coverslips in the acid alcohol. The latter should be in a Syracuse dish and the progress of the destaining followed under the microscope. When the stain no longer comes out of the eggs in visible clouds (about one-half to one minute after 10 - 15 minutes staining) immerse the coverslip in tap water. After at least three changes of tap water during 5 to 10 minutes run the coverslips up through the alcohols and Xylol and mount on a slide with balsam or omit the xylol and mount in euparal. Counterstaining with eosin or other dyes may be used in the usual way if desired.

References:- Tyler, A., 1946. Rapid slide-making method for preparations of eggs, protozoa, etc. The Collecting Net, vol 19, pp. 40-41.

MEASUREMENT OF OXYGEN CONSUMPTION OF EGGS OF ARBACIA AND OTHER ANIMALS BY THE WINKLER METHOD.

The Winkler method for measuring dissolved oxygen was first applied to the study of the respiratory rates of unfertilized and of developing eggs by O. Warburg (1908) in his classical demonstration of the change in rate resulting from fertilization in Arbacia. While the titrimetric method has now been largely superseded by manometric measurements it still remains useful in many types of experiments and is capable of great sensitivity. Thus in a modification employed by Baron (1942) for experiments on fragments of amphibian gastrulae the determinations were found to be reproducible to within 0.03 cu.mm. O_2. Since, also, many of the students may not in their future work have ready access to the more expensive and elaborate manometric equipment, familiarity with the Winkler method may be of importance. Some of the precautions to be taken for accurate results are discussed by Thompson and Robinson (1939). In the present exercise this method will be used in relatively crude form, but with sufficient accuracy to enable the student to obtain quantitative data on the effects of fertilization and progress of development on rate of oxygen uptake. Supplementary experiments involving the use of various metabolic stimulants and depressants are listed below and may be undertaken by such students who have the time and interest.

Living Material: Eggs and sperm of Arbacia, Asterias Chaetopterus, Mactra, Nereis or Ostrea.

Equipment: - (per two students): Scissors, forceps, bolting cloth (for straining eggs), 1 beaker (1000 ml), 2 beakers (500 ml), 1 beaker (10 ml), 2 medicine droppers, 1 graduated cylinder (100 ml), 4 glass stoppered bottles (100 to 125 ml), 2 heavy rubber bands (to fit lengthwise around bottles), 2 glass stoppered bottles (50 ml calibrated*), 3 pipettes (1 ml with 0.1 ml graduations,) 1 serological pipette (1 ml, wide opening, delivering to tip), 6 marbles or large glass beads (must pass through neck of the glass-stoppered bottles), 1 siphon mounted in a two-hole rubber stopper to fit the 125 ml bottles (the inner arm of the siphon should extend down about 2/3 of the length of the bottle and should have an upturned opening; the outer arm should extend about 4 or 5 inches below the bottom of the bottle; the other opening of the rubber stopper should have a 3 inch length of glass tubing which need not be inserted further than the bottom of the stopper), 1 burette (5 ml or 10 ml graduated in 0.02 ml), 1 ring stand with burette holder, 1 Erlenmeyer flask (125 ml), 1 thermometer.

General Equipment: (for class): 1 slow speed shaker (ca. 5 to 25 round trips per minuted at 2 to 10 inches amplitude, to hold about thirty 125 ml. bottles); 1 balance weighing up to 200 grams to within about 0.2 grams; 2 bunsen burners and tripods with wire screen.

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*The 50 ml glass stoppered bottles should be calibrated by weighing the amount of distilled water they contain when completely filled and with stopper in place and 1 marble or several glass beads inside of the bottle. Note temperature and calculate volume from density of the water.

Solutions (per two students): 10 ml. of 40% $MnCl_2$; 10 ml of 15% "KI" in 36% NaOH; 10 ml of conc. HCl; 100 ml of N/100 $Na_2S_2O_3$; 5 ml of "½%" starch solution in dropping bottle; 2000 ml of filtered sea water; 50 ml of freshly boiled distilled water in stoppered bottle.

Stock Solutions (for class of 15 pairs of students): 250 ml of 40% manganous chloride solution (use iron-free $MnCl_2$, 100 grams made up to 250 ml with distilled water); 250 ml of 15% KI in 36% NaOH solution (dissolve 90 grams of NaOH is some distilled water, cool, add 37.5 grams of KI, make up to 250 ml and keep in dark bottle with rubber stopper); 250 ml conc. HCl (C.P. with no free Cl_2); 2000 to 3000 ml N/100 sodium thiosulphate solution (make up in 1000 ml volumetric flasks if larger sizes unavailable; for each liter dissolve 2.482 grams of C. P. grade $Na_2S_2O_3.5H_2O$ in distilled water to make 1000 ml at calibration temperature of flask; is solution is to be kept several days before use, include 4 ml of 1 N NaOH per liter); 150 ml of "½%" starch solution (emulsify 1 gram of potato starch with 25 ml of water and pour slowly into about 175 ml of boiling water, boil for a few minutes longer, allow to settle and decant clear supernatant; if solution is to be kept longer than a few days add 5 or 10 drops of chloroform).

Manipulation of Eggs: Collect, strain and wash in filtered sea water a large sample of eggs of Arbacia (or other animal available) in the manner described in previous exercises. Allow the eggs to settle in a graduated cylinder for about 10 to 20 minutes and make up (in the liter beaker) approximately 400 to 500 ml of a 1 to 5 percent suspension on the basis of the settled volume. Stir and divide suspension into roughly equal parts in the 500 ml. beakers (pouring rapidly but gently). Inseminate one beaker of eggs (noting time and temperature) with a few drops of a suspension containing just sufficient sperm to fertilize practically all of the eggs (as judged by prior trial on a sample of the suspension) and wash once adjusting to original volume. Allow eggs in both beakers to settle sufficiently to en able siphoning off enough supernatant to fill the calibrated 50 ml bottle to overflowing. The bottles should contain the same marble or glass beads used in calibrating and precautions should be taken to avoid much aeration during the filling. The presence of a few eggs in the supernatant will not interfere seriously with the oxygen-determination but it is best to avoid including eggs in the sample. Immediately after filling, one student should add the Winkler reagents to these "initial reading" bottles, as described below, while the other student proceeds at once with further handling of the eggs.

Stir the suspensions of eggs, fill completely the 125 ml bottles (containing marble or glass beads) with each, insert glass stopper leaving no air space, note time and temperature, place rubber bands lengthwise around bottles to hold stopper in place and place bottles on shaker. Save remainder of suspensions for estimating concentration of eggs as described below. Allow the "respiration bottles" to remain on the shaker for a period of time that is estimated to give a readily measurable oxygen uptake, but not, in any event, for a period that would use up more than 3/4 of the oxygen available in the sea-water or for longer than two hours. The following figures may help the student decide on the respiration time for Arbacia. The instructor should be consulted for data on other animals. Ordinary sea water at 25° C contains about 5 cu.mm.O_2 per ml. One million unfertilized Arbacia eggs (= ca.1 ml of lightly settled eggs of 0.2 ml of centrifuged, packed eggs) consume about 10 cu.mm.O_2/hr (see Whitaker, 1933). So a suspension containing 250,000 eggs per ml will consume half of the oxygen present in the sea water in a period of one hour. As a minimum, 25,000 eggs per ml may be used for a two-hour run. The fertilized eggs consume oxygen at about 5 times the above rate and the respiration time may be estimated accordingly. At the end of the respiration period allow the eggs to settle sufficiently to enable siphoning off at least 55 ml of supernatant (practically free of eggs). Remove stopper, immediately insert siphon and fill the 50 ml calibrated bottle to overflowing, avoiding aeration. Proceed at once with addition of the Winkler reagents as described below.

Counting the Eggs: With the wide mouthed serological pipette remove 1 ml of the stirred egg suspension, remaining in the beaker, to 99 ml of sea water. Stir this diluted suspension and remove 1 ml (or less if more than 500 eggs per ml are present) to a dish for counting. The counting is facilitated if the sample is streaked in the dish in streaks not wider than the field of the microscope at a magnification of 20 to 40x. Alternatively, the eggs may be counted in the pipette placed on the stage of the microscope, preferably in a dish of sea water to facilitate observations.

Determination of Oxygen Content: To the samples in the 50 ml calibrated bottles quickly add 0.2 ml of the $MnCl_2$ solution and 0.2 ml of the NaOh-Kl solution. These should be introduced about half-way down the bottle with 1 ml pipette and need not be measured more accurately than about 20%. Immediately insert stopper, forcing out some water (subtract 0.4 ml from calibration volume) and avoid trapping air bubbles. Shake bottle for about a minute and allow it to stand for a couple of minutes for the precipitate to settle sufficiently to leave at least 1 cm of the upper end of the bottle free of precipitate. Carefully remove stopper and introduce about 0.4 ml of the HCl just below the surface of the liquid in the bottle. Stopper again and shake until precipitate has dissolved. Transfer to the 125 ml Erlenmeyer flask for titration. The mixture now contains free iodine which should be titrated fairly soon to avoid loss due to its volatility. In titrating first add sufficient thiosulphate solution to cause most of the yellow color due to the iodine to disappear. Then add enough starch solution to give a distinct blue color to the solution (4 or 5 drops will probably suffice) and continue the titration until the blue color just disappears. Each ml. of N/100 thiosulphate corresponds to 0.0025 millimoles of O_2 (=0.08 mg or 0.056 ml as of 0°C and 760 mm Hg). Since 50 ml of sea water in equilibrium with air contains about 0.25 ml of dissolved oxygen then about 4.5 ml of the N/100 thiosulphate would be required for a sea water blank, and approximately the same amount for the "initial reading" supernatant. From the difference in titration of the supernatants of the "initial reading" and "respiration bottles", from the calibration volume of the reaction bottles, from the duration of the run, and from the egg counts the student may calculate the rate of oxygen consumption per egg (or per 10^6 eggs).

The reactions involved are as follows. $Mn(OH)_2$ is first formed by reaction of the $MnCl_2$ with the NaOH. As it forms some combines with the oxygen present to form Mn_2O_3, a brown precipitate, while the excess $Mn(OH)_2$ flocculates as a white preciptate. The balanced equations are:

$$MnCl_2 + 2NaOH = Mn(OH)_2 + 2NaCl$$

$$4Mn(OH)_2 + O_2 = 2Mn_2O_3 + 4H_2O$$

The addition of HCl then causes the liberation of free iodine from the KI according to the equations:

$$Mn_2O_3 + 6HCl = 2MnCl_2 + 3H_2O + Cl_2$$

$$Cl_2 + 2KI = 2KCl + I_2$$

So for each molecule of O_2 present two molecules of I_2 are liberated. In titrating the free iodine reacts with the thiosulfate to form tetrathionate and iodide,

$$2Na_2S_2O_3 + I_2 = Na_2S_4O_6 + 2NaI$$

which are both colorless, permitting the end point to be determined by the disappearance of the blue color that forms when iodine reacts with the starch indicator.

<u>Supplementary Work:</u> As additional exercises the student may attempt one or more of the following:

(1) Respiratory rate at various stages of development.
(2) Effect of cyanide
(3) Effect of dinitrophenol
(4) Effect of idoacetate
(5) Effect of low pH.
(6) Effect of high pH.
(7) Respiration of articially activated eggs.

Consult instructor for details and references.

Literature Cited:

Barth, L. G., 1942. Physiol. Zool., 15:30-49

Thompson, T. G. and R. J. Robinson, 1939. J. Marine Research 2: 1-8

Warburg, O., 1908. Hoppe-Seyler's Zeitschr.f.Physiol. Chemie, 57:1-16.

Whitaker, D. M., 1933. J. Gen. Physiol., 16: 475-495

NUCLEIC ACIDS IN EGGS OF MARINE ANIMALS

It is readily apparent that the total amount of nuclear material increases considerably during cleavage and early development. In sea-urchins Godlewski (1908) estimated the increase to be about seventy-fold from the uncleaved egg in which the nucleus occupies 0.2% of the total volume, to the blastula stage, in which the nuclei occupy 14%. However chemical estimations of nucleic acids, based on determinations of nucleic acid phosphorus and of purine nitrogen showed (Masing, 1910, 1914; J. and D. Needham, 1930) no increase during the early development of the sea-urchin. This paradox was largely resolved mainly through the work of J. Brachet (1933-47) and Caspersson and Schultz (1938-40) who accumulated evidence to show that the cytoplasm of animal cells contains principally ribonucleic acid (also called yeast or phytonucleic acid) while the nucleus contains mainly desoxy-ribonucleic acid (also called thymonucleic acid) and that the former is transformed into the latter during development. There is, however, still some dispute concerning the transformation (see, for example, Schmidt et al., 1948).

In the present exercise the student will attempt to demonstrate the two types of nucleic acids by cytochemical methods, making use of an enzyme called ribonuclease (Jones, 1920; Kunitz, 1939) to digest the ribonucleic acid. The eggs and embryos are to be prepared by the flattening technique described in a separate laboratory outline. This method will not give as good results as sectioning, particularly with eggs of Arbacia which are highly pigmented, but will be adequate in most cases to permit satisfactory staining. The present exercise also includes some semi-quantitative tests for the sugar constituents, extractions methods and some properties of nucleoproteins and nucleic acids which may be undertaken if time permits.

Living Material: Eggs and sperm of Arbacia, Asterias, Mactra, Chaetopterus, Nereis or Ostrea.

Equipment (per student): 12+ #1 coverslips (square), 12+ microscope slides, 2+ coverslip staining jars, 12+ syracuse dishes, 1 dissecting needle, 1 forceps for handling coverslips, 6 test tubes (ca. 15 ml), 6 centrifuge tubes (15 ml), 2 small centrifuge tubes (for high speed machine), 1 test tube rack (12 places), 1 beaker (250 ml), 1 bunsen burner, 1 tripod and wire screen, 1 dropper (to reach bottom of 15 ml. centrifuge tubes).

Solutions (per two students): 1 liter of distilled water, 50 ml each of 50, 85, 95, and 100% alcohol and of xylol; 2 ml each of euparal and of Canada balsam in bottles with glass rod; 10 ml of 0.01% ribonuclease in McIlwains buffer at pH 6.6 to 7.0; 10 ml of same buffer; 20 ml of N HCl; 10 ml of Feulgen's fuchsin-sulphurous acid (see Rafalko, 1946):- stir 1 gram of basic fuchsin into 200 ml of boiling distilled water. Allow to cool to 50°C and filter. Under a chemical hood bubble SO_2 gas through this solution for about one hour, from a flask and thistle tube generator containing sodium bisulphite and dilute sulphuric acid; the liquid turns straw-colored; add about 20 grams of activated charcoal (Norit), stir for about two minutes and filter through fast paper. Store colorless filtrate in dark in tightly stoppered bottle; to stand for 24 hours before use; keep bottle well stoppered in dark; 50 ml SO_2 - water (bubble SO_2 through distilled water for one hour; store in tightly stoppered flask; 10 ml of Unna's methyl green-pyronin mixture (0.15 grams methyl green, 0.25 grams pyronin B, 2.5 ml 95% alcohol, 20 ml glycerin, 77.5 ml of 0.5% carbolic acid); 10 ml of saturated aqueous solution of toluidine blue; 20 ml of Bial's reagent (1% orcinol and 0.1% ferric chloride in conc. HCl); 20 ml of Dische's reagent (1 gram diphenylamine + 100 ml glacial acetic acid + 2.75 ml conc., N-free, reagent grade, sulphuric acid); 20 ml of Dische's blank reagent (no diphenylamine); 100 ml of 4 M NaCl; 100 ml of 0.14 M NaCl; 10 ml of Carnoy's fluid (6 vols. 95% alcohol + 3 vols. chloroform + 1 vol. glacial acetic acid); 20 ml of 10% trichloroacetic acid.

General Equipment (for class) 1 water bath at 60°C with rack to hold about 20 coverslip-staining jars; 1 centrifuge for about 3000 g.; 1 centrifuge for about 10,000 g.; 20 ml of 1% solution of ribose, xylose or arabinose; 20 ml of 0.1% solution of desoxyribose; 1 pair of polaroids; 1 water bath for 100°C with wire basket or rack to hold about 20 test tubes (13 x 100 mm).

A. Staining Procedures for the Nucleic Acids. Prepare, as previously described, eggs and embryos at various stages of development of any of the above listed animals, or of others that may be available. Fix three or more sets of eggs and embryos in Carnoy's fluid on coverslips, by the flattening method, for 15 minutes. Separate coverslips, transfer to alcohol and run them down to water. Then stain for the two types of nucleic acids as follows:

1. Feulgen Reaction for Desoxyribonucleic Acid. Place one set of coverslips in N HCl at 60°C for 10 to 12 minutes, leaving another set in water for the same period as a control. Rinse in water, then SO_2 water and stain for one hour in Feulgen's reagent. Wash 1 minute in each of three changes of SO_2 water, then 10 minutes in water; dehydrate in 50, 85, 95 and absolute alcohols and mount in euparal. This procedure stains primarily chromatin material. (See Stefano, 1948, for recent study of the reaction.)

2. Ribonucleic Acid. Place one set of coverslips in a 0.01% solution of ribonuclease (in McIlwain's citric acid buffer at pH 6.6-7.0) at 60°C for one hour and a control set in buffer solution without the enzyme. Staining may be done either with (a) Unna's methyl green-pyronine mixture or (b) toluidine blue.

(a) Stain both sets for 20 minutes in the methyl green-pyronine solution, rinse in water, differentiate in 95% alcohol, run through absolute alcohol and xylol, and mount in balsam. While all basic dyes will stain both types of nucleic acid, the ribonucleic acid tends to stain red and the desoxyribonucleic acid green in this mixture. Digestion with the ribonuclease should remove the red staining material.

(b) Stain both sets for 20 minutes in toluidine blue, wash in several changes of water, differentiate in 95% alcohol (about 5 minutes) and mount as above.

Note and make sketches of the distribution of the stained material in both the enzyme-treated and control sets after one or both of the above methods of staining. Centrifuge a sample of eggs at about 5000 g. for about 10 minutes and subject these to the same procedure as above for the detection of ribonucleic acids. With what material in the egg does the ribonucleic acid appear to be mainly associated.

B. Preparation and Properties of Nucleoproteins and tests for Sugar Constituents of Nucleic Acids. Centrifuge a suspension of eggs to give about 1 ml of packed eggs. Add 5 ml of distilled water and shake vigorously. Centrifuge for about 10 minutes at 3,000 g., and note the separation into pigment, yolk, microsomal (cloudy) and oil layers. Remove most of the microsomal layer and centrifuge this at high speed (ca. 10,000 g.) for 20 minutes. Remove the supernatant and suspend the sediment in distilled water. Add an equal volume of 10% trichloroacetic acid (TCA) to both supernatant and sediment solutions. Heat for 15 minutes 90°C. It has been shown by Schneider (1945, 1946) that this procedure hydrolyzes nucleoproteins, precipitates protein and completely extracts the nucleic acids. Test aliquots of these TCA extracts for pentose and desoxypentose by the Bial orcinol reaction and the Dische diphenylamine reaction described below, and compare the color intensities visually with standards made up from the sugars to give a rough estimate of the quantities present. (Two moles of pentose or desoxypentose represent one mole of the corresponding nucleic acid since only the sugar of the purine nucleosides, not that of the pyrimidine nucleosides, reacts with the reagents).

Prepare a 10% suspension of sperm and add an equal volume of 4 M NaCl. This results in a highly viscous solution of nucleoprotein from which some undissolved material can be removed by high speed centrifugation. Pour this solution into about 6 volumes of water. The nucleo-protein precipitates as a fibrous mass which can be washed in 0.14 M NaCl and redissolved in 2 M NaCl. Note the physical properties of the solution (examine for birefringence of flow if polaroids are available). (See papers of Mirsky, Pollister, and Ris, 1942, 1947, for important contributions to our knowledge of the properties and composition of nucleoproteins and chromosomes.) Extract the nucleoprotein solution with hot TCA as described above and test extracts with the Bial and Dische reagents.

Bial's Reaction. Add 1 ml of Bial's reagent (see Solutions, above) per ml of unknown solution. Heat in boiling water bath for 20 minutes and compare intensity of green color that develops with that of standards prepared from serial dilutions of stock ribose, xylose or arabinose solutions. (See Mejbaum, 1939 for quantitative use of this test.)

Dische's Reaction. To an aliquot of the unknown solution add an equal volume of Dische's reagent (see Solutions, above) and to another aliquot add Dische's blank reagent. Cover with a glass vial and immerse in boiling water bath for 10 minutes. Cool under running tap water and compare intensity of blue color with that of standards of desoxypentose, allowing for blank. The readings should be done shortly after cooling since the color intensity increases on standing.

References

Brachet, J., 1933 Arch. Biol., 44:519

Brachet, J., 1947 Embryologie Chimique, Masson & Cie, Paris.

Caspersson, T. and Schultz, J., 1938 Nature, 142:294; 1939 Nature 143:602

Caspersson, T. and Schultz, J., 1940 Proc. Nat. Acad. Sci., 26:507

Godlewski, E., 1908 Wilhelm Roux' Arch. Entwwicklungsmech., 26:279

Jones, W., 1920 Amer. J. Physiol., 52:203

Kunitz, M., 1939 Science, 90:112

Masing, E., 1910 Hoppe Seyler's Z. Physiol. Chem. 87:161

Masing, E., 1914 Wilhelm Roux' Arch. Entwicklungsmech., 40:666

Mojbaum, W. 1939 Hoppe-Seyler's Z. physiol. Chem., 258:117

Mirsky, A. E., 1947 Cold Spring Harbor Symp. Quant. Biol., 12:143

Mirsky, A. E., and Pollister, A. W. 1942 Proc. Nat. Acad. Sc. 28:344

Mirsky, A. E., and Pollister, A. W. 1946 J. Gen. Physiol. 30:117

Mirsky, A. E. and Ris, H., 1947 J. Gen. Physiol., 31:1

Needham, J. and Needham, D. M., 1930 J. Exper. Biol. 7:317

Pollister A. W. and Ris, H. 1947 Cold Spring Harbor Symp. Quant Biol. 12:147

Rafalko, S. 1946 Stain Technology 21:91

Schmidt, G., Hecht, L., and Thannhauser, S. J. 1948. J. Gen. Physiol 31:203

Schneider, W. C. 1945 Jour. Biol. Chem. 161:293

Schneider, W. C., 1946 Jour. Biol. Chem. 164:747

Stefano, H. W., 1948 Proc. Nat. Acad. Sci., 34:75

RESPIRATORY ENZYMES OF MARINE EGGS

Cellular oxidations are now known to proceed through an elaborate chain of enzymatically catalyzed reactions by which the initial substrate is broken down in stepwise fashion and in which some of the intermediates may undergo cyclical reconstruction (e.g., the Krebs' cycle; see Krebs, 1948). The enzymes involved are, somewhat arbitrarily, termed dehydrogenases and oxidases, based on certain properties such as whether or not they are able anaerobically to reduce certain dyes like methylene blue. But this does not imply any fundamental difference in the mode of action of the two groups of enzymes, since the reversible oxidation and reduction that they both undergo can be expressed similarly on an electronic basis. The differences are due rather to differences in potential at which they operate. The oxidases are capable of reacting with molecular oxygen and form the terminal end of the chain. However, certain dehydrogenases (capable of reducing methylene blue), such as the aldehyde oxidase (Schardinger ensyme) commonly found in milk, are also able to react with molecular oxygen and may be termed aerobic dehydrogenases in contrast to anaerobic ones, such as succinic dehydrogenase.

In the present exercise the student is to perform some simple tests relating to the occurrence of certain of these enzymes in sperm and eggs of marine animals. Relatively little data on marine animals is available in the literature. For references consult Needham (1942), Brachet (1947), Ball and Meyerhof (1940), Ballentine (1940), Runnstrom (1930-'35), Orstrom and Lindberg (1940),1943), and Krahl, Jandorf and Clowes (k942).

Living material: Arbacia, Asterias, Mactra, Nereis, Ostrea or Chaetopterus.

Equipment (per two students): 24+ test tubes (13x100 mm), test tube racks for 24+ tubes, 2+ pipettes (2 ml graduated), 2+ medicine droppers, usual dishes for eggs, sperm and embryos, 1 beaker (250 ml), 1 bunsen burner, 1 tripod with wire gauze, 3 feet of cellophane tubing (ca. 3/4 inch diam.), wooden splints, 2 centrifuge tubes '15 ml).

Solutions (per two students): 100 ml of 0.02% methylene blue in 0.5M NaCl; 20 ml of 0.02% methylene blue in 0.5M NaCl containing 0.01M KCN; 10 ml each of 0.5M NaCl, 0.37M sodium succinate, 0.37M sodium glycerophosphate, 0.37M sodium malate in 1M KCN (prepare hot more than 2 hrs. before use), 0.5M sodium lactate, 0.27M sodium citrate, 1M glucose; 100 ml mineral oil; 2 ml of 0.2% alpha-naphthol in alcohol; 2 ml of 0.2% dimethyl-p-phenylenediamine (prepared just before use); 5 ml of 3% hydrogen peroxide; 20 ml of 10% formaldehyde; 10 ml benzidine solution (2 gm benzidine, 10 ml glacial acetic acid, 10 gm sodium acetate, 100 ml alcohol, 100 ml water); 2 ml of 1% sodium hydrosulfite ($Na_2S_2O_4$, freshly prepared).

General Equipment and Materials for class: 1 centrifuge (for 3000 g), 5 lbs. of dry ice in methyl cellusolve, spectroscope with strong light source.

A. Dehydrogenases.

1. Endogenous Activity: (a) To 1.5 ml of approximately 5% suspensions of sperm, eggs and embryos add 0.5 ml of 0.02% methylene

blue (in 0.5M NaCl) in a 13x100 mm test tube. Cover with about 1 ml of mineral oil (use medicine dropper, forms a layer of about 1 cm). Set up a control with an egg suspension that has been boiled for 1 minute. Note the time for 90% decolorization of the methylene blue (using a ten-fold dilution of the methylene blue in boiled egg suspensions as an end-point standard).

(b) Prepare 20% suspensions of sperm, eggs and embryos. Freeze (in dry ice) and thaw and add methylene blue and oil as above. Compare the rates of decolorization with those obtained with the living material. Do the embryos decolorize methylene blue more rapidly than the uncleaved eggs in living condition and after freezing and thawing?

2. <u>Tests for Dehydrogenation of Various Substrates:</u> To seven 1 ml samples of the frozen and thawed suspensions prepared above (b) add 0.5 ml of the following solutions: - (1) 0.5M NaCl, (2) 0.37M sodium succinate, (3) 0.37M sodium glycerophosphate, (4) 0.37M sodium malate (in 1M KCN-prepared not more than 2 hours before use - use dropper for pipetting and extreme care to avoid cyanide poisoning! Malic dehydrogenase oxidizes malate to oxalacetate which in turn inhibits the oxidation of malate. The cyanide combines with the oxalacetate and prevents this inhibition.), (5) 0.5M sodium lactate, (6) 0.27M sodium citrate and (7) 1M glucose. Reduction of the methylene blue, where it occurs here, will probably take place within 2 hours. Compare the rate of reduction with and without the added substrate. Dialyze samples of the frozen and thawed sperm, eggs and embryos against running sea water for 6 or more hours and test the dialyzed material with the above substrates and the Nacl blank. Has the endogenous activity disappeared as a result of dialysis? Boil a sample of the dialyzed material for 1 minute and test with the substrates that gave reduction of the methylene blue. Set up similar tubes of the dialyzed and non-dialyzed material plus substrate using the cyanide-containing methylene blue solution. Does the cyanide inhibit the reduction of the methylene blue?

The reactions that the above substrates undergo, if the proper dehydrogenase is present, are as follows:-

$COOH \cdot CH_2CH_2COOH \dashrightarrow COOH \cdot CH \cdot CH \cdot COOH + 2H$
succinic acid ---- fumaric acid

$H_2COPO_3K_2 \cdot CHOH \cdot CH_2OH \dashrightarrow H_2COPO_3K_2 \cdot CHOH \cdot CHO + 2H$
glycerophosphate ---- phosphoglyceric aldehyde

$COOH \cdot CH_2 \cdot CHOH \cdot COOH \dashrightarrow COOH \cdot CH_2\ CO \cdot COOH + 2H$
malic acid ---- oxalacetic acid

$CH_3 \cdot CHOH \cdot COOH \dashrightarrow CH_3 \cdot CO{,}COOH + 2H$
lactic acid ---- pyruvic acid

$COH \cdot (CH_2COOH)_2 \cdot COOH \dashrightarrow CO \cdot (CH_2COOH)_2 + 2H + CO_2$
citric acid ---- a-ketoglutaric acid

$CHO \cdot (CHOH)_4 \cdot CH_2OH + H_2O \dashrightarrow COOH \cdot (CHOH)_4 \cdot CH_2OH + 2H$
glucose ---- gluconic acid

and the hydrogen set free, reduces the methylene blue to the leuco (colorless form. If oxygen were admitted to the system the methylene blue would be re-oxidized as fast as it is formed.

B. Oxidases. Tests for these enzymes, in general, involve rather complicated manometric and spectrophometric measurements under various conditions. However, certain of them permit rather simple tests to be performed.

1. Indophenoloxidase: Mix a 0.2% solution of alpha-naphthol in alcohol with an equal volume of freshly prepared 0.2% solution of dimethyl-p-phenylenediamine in water. This mixture is called "nadi" reagent (letters from the words naphthol and diamine). Add about 5 drops of the "nadi" reagent to 1 ml of a 2% suspension of sperm, eggs and embryos. Note the time for development of the blue color. Examine samples of the eggs and embryos microscopically from time to time. Is there an indication of a gradient (see Child, 1944)? The reaction catalyzed by the oxidase here is as follows:-

dimethyl-p-phenylendiamine + a-naphthol--→ dimethyl indophenol blue

$$(CH_3)_2 \cdot N \cdot (C_6H_4) \cdot NH_2 + (C_{10}H_7) \cdot OH + O_2 \dashrightarrow (CH_3)_2 \cdot N \cdot (C_6H_4) \cdot N \cdot (C_6H_4) \cdot N : (C_{10}H_6) : O + 2H_2)$$

Some of the oxidases that catalyze this reaction are also capable of reducing cytochrome and may be termed cytochrome oxidases (also related to, if not identical, with Warburg's "Atmungsferment").

2. Catalase: This is not strictly an oxidase, but its action (liberating oxygen from hydrogen peroxide) is closely connected with physiological oxidation. Add about 1 ml of 3% hydrogen peroxide to about 0.5 ml of a 10% or greater, frozen and thawed, suspension of eggs and of sperm in a test tube. As a control use boiled eggs and sperm. Note the formation of bubbles of gas. Test with a glowing splint.

3. Peroxidase: To test for this enzyme the catalase should first be inactivated by treating the suspensions of eggs or of sperm with 10% formaldehyde for 10 minutes and then washing in distilled water. Place 3 ml of a 1 to 10% suspension of the washed eggs or sperm in a test tube containing 1 ml of benzidine solution. (Add 1 drop of 3% hydrogen peroxide. The development of a blue color indicates the presence of peroxidase. The reaction consists in the oxidation of the benzidine (4,4'-diaminobiphenyl) to a quinone (p-quinone di-imide). Does any color develop before the addition of the peroxide?

4. These important and widely distributed enzymes are detected principally by the absorption spectra of solutions of the substances in reduced form. The reduced cytochromes are readily prepared by removal of oxygen from the system or by addition of cyanide, or of sodium hydrosulfite ($Na_2S_2O_4$). For spectroscopic observation in eggs and sperm, concentrated suspensions and an intense light source must be used. Add 2 drops of the hydrosulfite solution to about 3 ml of a 10% suspension of eggs or of sperm, and examine in the spectroscope. Compare with untreated shaken suspensions. The specific absorption bands for reduced cytochromes are as follows;- 603 millimicrons for the a band (of cytochrome a), 565 for the b band (of cytochrome b), 550 for the c band (of cytochrome c) and about 520 for the d band

(common to all three cytochromes). Which, if any, bands does your material exhibit? Compare with observations of Ball and Meyerhof (1940) and Krahl and Clowes (1939).

Literature Cited

Ball, E. and Meyerhof, B., 1940. J. Biol. Chem., 134:483.

Ballentine, R., 1940. J. Cell. & Comp. Physiol., 15:217 and 16:39.

Brachet, J., 1947. Embryologie Chimique, Masson Cie.

Child, C. M., 1944. Physiol. Zool., 17:129.

Krahl, M. E., Jandorf, B. J., and Clowes, G. H. A., 1942. J. Gen. Physiol., 25:733

Krebs, H., 1947. Enzymologia, 12:88.

Jandorf, B. J. and Krahl, M. E., 1941. J. Gen. Physiol., 25:749.

Lindberg, O., 1943. Ark.f.Kemi., 16:No. 15.

Needham, J., 1942. Biochemistry and Morphogenesis. Cambridge.

Orström, A., and Lindberg, O., 1940. Enzymologia, 8:367.

Runnström, J., 1930. Protoplasma, 10: 106.

Runnström, J. 1935. Biol. Bull., 68:327.

Report Sheet for Part A (Dehydrogenases) of Exercise on
Respiratory Enzymes of Marine Eggs

Name of Student ______________________________ Date performed ______________________________

Species of animal used ______________________________

Material tested	Treatment	Approx. conc. in tubes in per cent	Time (in minutes) for ca. 90% reduction of methylene blue: Without added substrate	Succinate	Glycero-phosphate	Malate	Lactate	Citrate	Glucose
Sperm	living								
	frozen								
	dialyzed								
Unfert. Eggs	living								
	frozen								
	dialyzed								
Fert. Eggs at ____ min.	living								
	frozen								
	dialyzed								
Embryos at ____ hrs.	living								
	frozen								
	dialyzed								