EMBRYOLOGY COURSE, 1963

MARINE BIOLOGICAL LABORATORY

STUDENT ROSTER

NAME	INSTITUTION	LEVEL	FIELD
Hani K. Battikh	U. of Virginia	grad.	developmental biology
Hillard Berger	Vanderbilt U.	grad.	bacteriophage genetics
Morton L. Burdick	Johns Hopkins U.	grad.	developmental biology
Gloria Ann Cassens	Indiana U.	grad.	developmental biology
John R. David	New York U.	M.D.	immunology
Richard L. Davidson	Western Reserve U.	grad.	developmental biology
Michael J. Epstein	U. of Texas	grad.	developmental biology
Daniel Hartline	Harvard U.	grad.	biophysics
C. B. Kimmel	Johns Hopkins U.	grad.	developmental biology
David E. Kohne	Purdue U.	grad.	developmental biology
Dolores J. McWhinnie	Marquette U.	grad.	developmental biology
Dorothy E. Patton	U. of Chicago	grad.	developmental genetics
Robert E. Pollack	Brandeis U.	grad.	developmental biology
Ronald H. Reeder	MIT	grad.	developmental biology
Stanley Shostak	Brown U.	grad.	developmental biology
Elinor J. Spring	Harvard Med. Sch.	grad.	developmental cytology
H. L. Stackhouse	U. of Arizona	grad.	developmental biology
Trygve P. Steen	Yale U.	grad.	developmental biology
Stanley A. Terman	Brown U.	undergrad.	undecided
Shuhei Yuyama	Florida State U.	grad.	cell physiology

Auditor

P. C. Dumonde	New York U.	M.D.	immunology

LECTURE SCHEDULE

Embryology Training Program

Woods Hole, 1963

All lectures in this series will be held at 10:30 A.M., unless otherwise noted, in the Whitman Lecture Hall.

1.	Sunday	June 16	J. D. Ebert	Introduction: The scope of developmental biology; its problems; an appraisal of where we stand.
		Time: 7:30 P.M.		
		After this lecture, students assemble in laboratory.		
2.	Monday	June 17	J. W. Saunders, Jr.	Teleosts I
3.	Tuesday	" 18	J. W. Saunders, Jr.	Teleosts II
4.	Wednesday	" 19	Ch. Devillers	Structure and appearance of marginal zone and bilateral symmetry
5.	Thursday	" 20	Beatrice Mintz	Experimental analysis of differentiation in the mammalian egg
6.	Friday	" 21	N. T. Spratt, Jr.	Morphogenetic movements in the early chick embryo
7.	Saturday	" 22	"Microsymposium" on teleost development	
		Time: 9:00 A.M. - noon		
			J. P. Trinkaus	Cellular basis of Fundulus epiboly
			Ch. Devillers	To be announced
			R. B. Crawford	Energetics of development in Fundulus
			R. Goss	Regeneration and transplantation of scales in Fundulus
			J. Oppenheimer	Contributions, actual and potential of Fundulus to experimental embryology

8.	Monday	"	24	A. Moscona — Sponges I
9.	Tuesday	"	25	A. Moscona — Sponges II
10.	Wednesday	"	26	A. Moscona — Tissue reconstruction from dissociated cells
11.	Thursday	"	27	T. D. Brock — Properties of cell surface in microorganisms
12.	Friday	"	28	~~J. D. Ebert~~ S. W. Bishop — Antibodies, viruses and embryos — Tumorspecific immune responses and Cellular immunochemistry
13.	Saturday	"	29	"Microsymposium" on development of immune mechanisms

Time: 9:00 A.M. - noon

~~J. D. Ebert~~ S. W. Bishop	Introduction
J. F. Albright	Some dynamic aspects of antibody production
R. Auerbach	Spleen and thymus development in relation to immunity

14.	Monday	July	1	A. Burnett — Coelenterates and Ctenophores I
15.	Tuesday	"	2	A. Burnett — Coelenterates and Ctenophores II
16.	Wednesday	"	3	A. Burnett — Experimental approaches to Coelenterate development and regeneration
	Thursday	"	4	NONE
17.	Friday	"	5	J. J. Kollros — Tissue responses to thyroid hormone in regeneration
18.	Saturday	"	6	"Microsymposium" on the single cell

Time: 9:00 A.M. - noon

I. R. Konigsberg	Clonal analysis of myogenesis
F. C. Steward	Development of single plant cells

19.	Monday	July 8	A. C. Clement	The mollusk-annelid pattern of development
20.	Tuesday	" 9	A. C. Clement	Analysis of development in Ilyanassa
21.	Wednesday	" 10	K. R. Porter	Developmental cytology I
22.	Thursday	" 11	K. R. Porter	Developmental cytology II
23.	Friday	" 12	M. Glimcher	Isolation, identification and properties of enamel protein and the ultra-structural organization of mineral and organic components

SPECIAL Friday July 12 D. Mazia

Time: 9:00 A.M. Joint Embryology-Physiology

24. Saturday July 13 "Microsymposium" on keratin and collagen

Time: 9:00 A.M. - noon

J. W. Saunders, Jr.	Introduction - specificity in feather development
E. Bell	Keratin biosynthesis and organization in feather development
J. Gross	Collagen (to be announced)

25.	Monday	July 15	A. Monroy	Echinoderms I
26.	Tuesday	" 16	A. Monroy	Echinoderms II
27.	Wednesday	" 17	NONE	
28.	Thursday	" 18	A. Pardee	Cell regulatory mechanisms I
29.	Friday	" 19	A. Pardee	Cell regulatory mechanisms II

30. Saturday " 20 "Microsymposium" on coding and protein synthesis

Time: 9:00 A.M. - noon

W. S. Vincent	Messengers, nucleoli and the anatomy of a ribosome
F. H. Wilt	To be announced
J. Collier	Fractionation of nucleic acids in Ilyanassa embryos
J. Lash	To be announced

31.	Monday	July	22	J. Lash	Ascidians I
32.	Tuesday	"	23	J. Lash	Ascidians II
	Wednesday	"	24	NONE	
33.	Thursday	"	25	R. S. Edgar	Developmental genetics I
34.	Friday	"	26	R. S. Edgar	Developmental genetics II
35.	Saturday	"	27	D. Bodenstein	To be announced

POST-COURSE RESEARCH

July 30 -- August 31

During this period the class will meet formally on Friday mornings; usually a lecture will be presented by a staff member or visiting speaker.

The speaker at the first of these programs will be Gerald Weissmann of New York University School of Medicine. His topic on Friday, August 2, will be

Vitamin A, Lysosomes, and Metamorphosis

1955

JOINT SESSIONS OF THE GENETICS ... PHYSIOLOGY CLASSES

"DIFFERENTIATION VIEWED AS THE SELECTIVE EXPRESSION OF THE GENOME"

Session I Monday July 15 Chairman: Dr. James Peters

9:00 A.M. An introduction to the problems of differentiation and morphogenesis.

Dr. James Peters

9:45 A.M. The sequential reading of the genome

Dr. Sol Spiegelman

Session II

1:30 P.M. Analysis of development in Echinoderms

Dr. A. Monroy

Session III Chairman: Dr. Sol Spiegelman

8:00 P.M. Sequential protein synthesis during phage infection

Dr. Seymour Cohen

8:45 P.M. Rhythmic physiological events in Convallaria

Dr. J. W. Hastings

9:15 P.M. The timing of enzyme synthesis during synchronous growth in yeast

Dr. Marlyn Halvorson

Session IV Tuesday July 16 Chairman: Dr. Bernard Davis

9:00 A.M. Stability of extrachromosomal characters and their possible role in differentiation

Dr. Martin Pollock

9:45 A.M. Cellular differentiation in the slime molds.

Dr. Maurice Sussman

10:30 A.M. Differentiation of pancreatic cells

Dr. William Rutter

Session V

1:30 P.M. Analysis of development in Echinoderms

Dr. A. Monroy

Session VI Wednesday July 17 Chairman: Dr. Alex Kaynan

9:00 A.M. RNA, messenger, and the problem of differentiation

Dr. Cyrus Levinthal

DISCUSSANTS: Dr. James D. Watson, Dr. William Rutter, Dr. Paul Greene, Dr. A. Monroy, Dr. Martin Pollock

Session VII Thursday July 18

9:00 A.M. The folding of a multichain enzyme and genetic complementation

Dr. Cyrus Levinthal

10:30 A.M. Cell regulatory mechanisms I

Dr. Arthur Pardee

Session VIII Friday July 19

9:00 A.M. The anatomy and physiology of pigmentation

Dr. Thomas B. Fitzpatrick
Dr. George Szabo

10:30 A.M. Cell regulatory mechanisms II

Dr. Arthur Pardee

Session IX Saturday July 20

9:00 A.M. Messengers, nucleoli and the anatomy of a ribosome

Dr. Walter Vincent

9:45 A.M. Characteristics of RNA synthesis in cleaving sea urchin eggs

Dr. Fred H. Wilt

10:30 A.M. Fractionation of nucleic acid in Ilyanassa embryos

Dr. J. Collier

11:15 A.M. Cartilage induction in vitro and sulfate activation enzyme

Dr. James Lash

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2.	Tuesday	June 16	T. J. King	Teleosts I
3.	Wednesday	June 17	T. J. King	Teleosts II
4.	Thursday	June 18	R. L. DeHaan	Cell movements and morphogenesis
5.	Friday	June 19	A. J. Coulombre	The morphogenetic interaction of the tissues of the eye during development
6.	Saturday	June 20	Microsymposium: morphogenesis and tissue interactions	
			E. Zwilling	Morphotypic diversity vs. histiotypic identity
			S. Simpson	Tissue interactions and morphogenesis in lizard tail regenerations
			J. Lash	The induction of chondrogenesis in vitro
7.	Monday	June 22	A. Moscona	Sponges I
8.	Tuesday	June 23	A. Moscona	Sponges II
9.	Wednesday	June 24	A. Moscona	Tissue reconstruction from dissociated cells
10.	Wednesday	June 24 (8:00 P.M.)	H. Rubin	The malignant transformation of
11.	Thursday	June 25	H. Rubin	animal cells by viruses
12.	Friday	June 26	M. Rosenberg	Some applications of surface physics to cell biology
13.	Saturday	June 27	Microsymposium: the cell membrane	
			P. Marcus	Dynamics of plasma membrane modification in virus-infected and normal cells
			A. L. Colwin	Role of the gamete membranes in fertilization
			M. Steinberg	Adhesive selectivity in intercellular reactions

14.	Monday	June 29	A. Burnett	A model of growth for hydroids and tubules of higher organisms
15.	Tuesday	June 30	A. Burnett	The role of neoblasts in the maintenance of form of hydroids
16.	Wednesday	July 1	A. Burnett	Dedifferentiation and redifferentiation of somatic cells in Hydra - an analysis of polymorphism
17.	Thursday	July 2	J. D. Ebert)	Developmental aspects of immunity
18.	Friday	July 3	J. D. Ebert)	

Saturday July 4 NONE

19.	Monday	July 6	J. W. Lash	Ascidians I: General embryology
20.	Tuesday	July 7	J. W. Lash	Ascidians II: Metamorphosis
21.	Wednesday	July 8	G. Reverberi	Ascidians III: Analysis of development
22.	Thursday	July 9	K. R. Porter	Developmental cytology I
23.	Friday	July 10	K. R. Porter	Developmental cytology II
24.	Saturday	July 11	Microsymposium: developmental cytology and genetics	
			E. Hadorn	Developmental aspects of pleiotropic effects of genes
			J. Gall	The nucleic acids of giant chromosomes
			T. J. King	The developmental capacity of nuclei transplanted from advanced embryos
25.	Monday	July 13	R. L. DeHaan	Comparative morphogenesis of annelids, molluscs and
26.	Tuesday	July 14	R. L. DeHaan	echinoderms
27.	Tuesday	July 14 (4:00 P.M.)	E. Hadorn	Problems of differentiation and pattern formation in Drosophila blastemas
28.	Wednesday	July 15	D. D. Brown	(Biochemistry of
29.	Thursday	July 16	D. D. Brown	(oogenesis and
30.	Friday	July 17	D. D. Brown	(early development
31.	Saturday	July 18	Microsymposium: biochemistry of early development	

A principal, introductory lecture by Paul Gross (45 minutes), followed by 30 minute presentations by Eugene Bell, J. R. Collier, Igor Dawid and Alberto Monroy.

32.	Monday	July 20	A. B. Pardee	Cell regulatory mechanisms I
33.	Tuesday	July 21	A. B. Pardee	Cell regulatory mechanisms II
34.	Wednesday	July 22	NONE	
35.	Thursday	July 23	S. Cohen	An epidermal growth-stimulating protein
36.	Friday	July 24	M. Singer	The nervous control of the regeneration of body parts in vertebrates
37.	Saturday	July 25	V. Hamburger	Neurogenesis and the embryology of behavior

July 26 - September 5

Post-Course Research

During this period occasional lectures will be scheduled. The first of these will be given at 9:30 A.M., Friday, July 31 by Leon Weiss, who will discuss his recent studies on the fine structure of spleen and other tissues involved in the graft-versus-host reaction.

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Embryology Course
Marine Biological Laboratory June 17-18, 1963

Teleost Development

I Introduction (on Blackboard: Fundulus, Oryzias, Salmo, Carassius, Etenostomus, Serranus)

A. Defer to the Teleost "experts" who will speak on June 22—

B. Outline lecture plan

1. Survey Normal Development with emphasis on
   a) Fertilization
   b) Cleavage
   c) Gastrulation and Epiboly
   d) Origin of body axis
2. Experimental analysis, with emphasis on
   a) The activation reaction — chiefly as analyzed by Yamamoto
   b) Relationships of cleavage planes to the embryonic axis
   c) Developmental potentialities of the blastoderm
   d) Correlative analysis of morphogenesis
      i Role of yolk
      ii Role of the "dorsal lip"
      iii "Field" properties of the blastoderm
   e) Finally, with apologies to Trinkaus, who speaks later, some words about epiboly.

II Normal Development.

A. "Courtship", Sexual play, etc varies — will not be discussed — Newman, 1907

B. Origin of gametes -- seasonal cycles etc — not discussed -- See Matthews, 1938 on Fundulus

C. Fertilization

1. Appearance of egg when shed — Considerable variation among species — but we distinguish

draw

a) Outer chorion — often fibrous, frequently sticky — which swells somewhat when egg is shed — Micropyle

b) Beneath this, and indistinguishable, ~~except possibly in c.m. (not studied)~~ is, ~~presumably~~ the vitelline membrane, closely applied to it.

c) The egg cortex is a differentiated superficial layer containing characteristic "cortical alveoli. — These (in goby & carp) shown to contain mucoprotein; Wall of alveolus soluble in fat solvents.

→ SLIDE (Yamamoto Fig 9)

d) Yolk and oil -- Oil droplets variously ordered — Sometimes comprise a species specific character.

2. Fertilization reaction —

a) Sperm entry only through micropyle

i colloidal exudate floods out the following sperm — has been seen in medaka & herring (?)

ii Monospermy thus is normal

b) Cortical reaction.

i Breakdown of cortical platelets beginning at animal pole (beneath micropyle) spreads as a wave

→ Slide Yamamoto 5 ii Chorion elevation follows wave

→ Yamamoto 6 iii Detail of release of colloid

iv Cortical reaction in absence of sperm occurs spontaneously - Wessels, 1953, showed pattern of disappearance of cortical alveoli in Fundulus is at random. Barrier to fertilization even if sperm introduced into perivitelline space (but note later analysis).

DRAW v Protoplasmic contraction & elevation of blastodisc.

D. The sequence of morphological changes during development

1. Rapid survey of the emergence of body form

Armstrong 1+2 a) Cleavages lead to high blastula

Armstrong 3 b) Blastula flattens & gastrulation begins i yolk covered by blastoderm

Armstrong 4 c) Embryonic shield gives rise to embryonic axis and "blastopore" closes

Armstrong 5 d) Organogenesis takes place

2. Cleavage to blastula formation

(5) Oppenheimer, 1936; page 409 a) Cleavage planes - meridional or para-meridional through 5th. Alternating at right L's; thereafter diagonal and tangential - Cell Lineage -

Wilson-Seabass-

b) Origin of subgerminal cavity and formation of periblast.
i Make a point of the "surface coat", of which more later.

6
Sumner 1904
Fig 15

c) Appearance of "high blastula"
i Cross section
ii Polar view – note:
A. Germ Ring
B. Embryonic Shield

3. Gastrulation

Slide 7
Oppenheimer
1936 p 412

Also, draw
side view

Slide 8
Sumner - Fig 16

Slide 9
Pasteels, 1936

Slide 10
Sumner, Fig 17
Slide 11
Sumner, Fig 19

Slide 12
Pasteels, 1934
Fig 2

a. Gross movements of epiboly
   i. Movements of germ ring
   ii. Elongation of embryonic shield
   iii. Appearance of embryonic axis
   iv. Identify blastopore closure
b. Formation of endoderm and mesoderm
   i. Turning under of "dorsal lip" to form mesoderm (Sumner, Fig. 16)
   ii. Some question about endoderm
      A. Split off from inturning tongue
      B. By invagination from "Deckschickt" (Devillers and Pasteels)
         1. Detachment of cells from surface coat
   iii. Spreading of endoderm and mesoderm
      A. Beginning gastrula
      B. Later gastrula - beginning of formation of body axis
c. Direction of cell movements during gastrulation -
   i. Pasteels analysis in Salmo
      A. Inturning of mesoderm in shield and germ ring
      B. Expansion of extraembryonic area
      C. Backward movement of cells at margin of epiblast
   ii. Differential proliferation does not account for these movements - (analysis by Richards and students, chiefly Jones)
      A. Will return to question of the source of cells for axial elongation

III Experimental Analysis of Teleost Development.

A. Analysis of the activation reaction.

1. Spontaneous activation

a) Occurs shortly after shedding in most forms —

b) Prevented by immersion in isotonic Ringer's. Fertilization or artificial activation possible after several hours. In some species, activation can not take place in Ringer's in others it can —

2. Effect of Calcium-free sea water

a) Activation occurs neither spontaneously nor as a result of chemical or mechanical treatment in Ca^{++}-free sea water. But — occurs spontaneously upon return to normal sea water.

3. Cortical reaction starts at animal pole after normal fertilization. Where does reaction start if egg activated by pricking?

Slide-Yamamoto '46 Sl 13

a) at point of pricking —

b) (Requires 20u needle to start reaction at veg pole — smaller one elsewhere) —

4. Block to polyspermy.

a) Introduction of sperm beneath chorion after normal fertilization or after spontaneous activation does not (so far) lead to polyspermy.

b) (Recall normal sperm entrance only through micropyle)

c) Denuding egg of chorion after shedding + before fertilization

i Done with hatching enzyme in Oryzias by Sakai, 1961,

ii Polyspermy possible up to 20 minutes, even though cortical granules break down immediately (Yanagamachy, 1957 - up to 30 minutes in the herring)

5) Properties of cortical alveoli

a) Break down only in the cortex - if displaced into yolk by centrifugation, they do not break down. Shows that "activation" transmitted thru cortex.

b) a-granules associated with cortical alveoli break down first -- Suggest release of an enzyme.

c) Yamamoto, 1962, shows that <u>isolated</u> alveoli breakdown in various solvents such as toluene, isoamylalcohol, ether etc, but not in benzene - dissolves only neutral fats. — Also, membrane disorders in hemolysis of cobra venom & bee sting.

d) Content seems to be mucopolysaccharide

a. Correlation between activation site and spread of cortical reaction (Oryzias) — (not shown on slide - if pricked at veg pole by 2nd cell cortical reaction will start there

2. Effects of calcium on activation reactions, Yamamato, 1954, Oryzias.

a. Activation does not occur in calcium-free sea water either spontaneously, by pricking or through use of chemicals.
b. Occurs without further stimulation upon return of eggs to normal sea water.

3. Yamamoto — in isotonic Ringer's spontaneous activation prevented - fertilization and activation pigment gathers after planning .. Ringer's — Also shown by Rappaport for Salmo-

B. What is the relationship of the first cleavage plane to the future embryonic axis? -- No consistent relationship -- many species examined.

Slide 14
Oppenheimer, 1936
Fig 4 - 12

1. Oppenheimer -- Fundulus -- Usually long axis coincides with or is near second furrow.

2. Morgan -- Ctenolabrus -- carmine particles -- no correlation.

3. Argument about plan of symmetry set up in oocyte - Su Wakasa - Fauteg - Clavert + Hibgama

C. Does cleavage segregate cells of qualitatively different developmental potentialities?

1. The omnipotent cells of Fundulus.

a. Morgan, 1895; Lewis, 1912: Excision of one blastomere at 2-cell stage resulting in normal, but smaller embryo.
b. Nicholas and Oppenheimer, 1942
i. 65 of 72 cases gave normal embryos after destruction of one cell at 2-cell stage
ii. Loss of 50% of cells up to 16-cell stage may be regulated (50% normal)
iii. Gastrulation does not occur if defect made at 16-cell stage

2. Qualitative differences between blastomeres in Carassius.

a. Tung and Tung, 1944. Removal of one blastomere in 2 cell stage. 50% of cases, the remaining blastomere made a normal embryo.
b. Eggs bisected meridionally at 2-cell stage - Some cases a normal embryo developed from a half; some formed half-embryos; others formed no embryonic axis at all.

D. What are the prospective fates of the various parts of the blastula?

Slide 15
Oppenheimer
Quart Rev 22

1. Results of vital staining experiments of Oppenheimer (Fundulus) and Pasteels (Salmo).

2. Does the embryonic axis receive contributions from the germ ring?

a. Oppenheimer's dye-marks showing:
i. Elongation of embryonic axis from within shield
ii. Possible contributions from germ ring

Slide 16
Oppenheimer 1936
JEZ 73 p 431

b. Morgan, 1895, Ctenolabrus. Severed connection of germ ring on one side. Embryonic axis usually complete, but cells pile up at edge of cut in germ ring.

Slide 17
Luther 1937
Fig. 1

c. Luther, 1937. Axial structures formed from germ ring after extirpation of embryo.

d. Oppenheimer, 1938. Grafts of germ ring (90° and 180° from shield) into shield area of host gave extensive differentiations, chiefly of tail-like structures.

Slide 18
Brummett 1954
Figure 3

e. But!, Brummett in 1954 showed that with carbon marking, only those placed in the shield, subsequently are found in the body of the embryo.

f. Conclude:

i. Germ ring certainly can form embryonic structures (by induction? -- See later)

ii. A normal component of epibolic movement below the equator would lead marks in germ ring at least to approach the embryo.

iii. Unsure now whether germ ring does or does not contribute.

E. Correlative analysis of morphogenesis

1. Is yolk necessary for development?

a. Morgan (Fundulus, Ctenolabrus) punctured yolk in stages from zygote to germ ring. Found normal development even after loss of 1/2 to 2/3 of yolk.

b. Explantation experiments - Oppenheimer, 1936, separated blastoderm from yolk and periblast up to 128-cell stage and cultured in 2X Holtfreter's solution.

i. All isolates prior to 32-cell stage (and some later isolates) gave only vesicular structures with little differentiation of tissue.

ii. But, after 32-cell stage, gastrulation occurred and good morphological and histological differentiations were achieved -- especially head structures.

c. Does some "morphogenetic factor" move from yolk to blastodisc? Observations of Wilson, Morgan, Roosen-Runge and others on movements from yolk. Experiments of Tung suggest progressive movement of yolk factors.

i. Made latitudinal cuts at various levels. Normal development at different stages depending on an-veg level of cut

1 - cell	--	3/5
2 - cell	--	3/5
4 - cell	--	1/2
8 - cell	--	1/3
16 - cell	--	only blastoderm needed

ii. Possibly this movement is asymmetric:

A. Meridinal cuts at 2-, 4- or 8-cell stages -

1. Sometimes both halves gave normal embryos
2. Sometimes, one normal plus a "hyperblastula"
3. Sometimes defective twins of opposite asymmetry resulted

B. Conclude upward movement of a factor along one side of egg -- compare with location of amphibian gray crescent.

2. The Teleostean Dorsal Lip and Archenteron Roof! Is it comparable to that of the amphibian? Does it show inductive and "field" properties? Etc. -

a. What determines position of "dorsal lip," i.e., the site of mesodermal invagination? Devillers, 1952.
   i. Excise "extraembryonic" half of blastula - obtain normal embryo normally oriented
   ii. Excise "embryonic" half - obtain normal embryo normally oriented
   iii. Rotate blastoderm 180° - Embryonic area correspondingly rotated.
   iv. Replace embryonic half of host with extraembryonic half of donor -- Donor forms the embryo! Normally oriented with respect to host! Suggests the periblast or yolk factors transmitted through the periblast are polarized in original orientation and impose polarity on germ! ~~Controls poor~~! Students read papers of Devillers, 1952 and Luther, 1937.

b. Is the "dorsal lip" inductive?
   i. Oppenheimer, 1936 -- Grafts of posterior edge of shield to extraembryonic area. Obtained secondary embryos.

Slide 19
Oppenheimer
1936, p 419

   ii. Eakin, 1939. Trant. - Separated mesoderm from epiblast and hypoblast, divided it into sectors, packaged it in extraembryonic tissue and grafted to yolk sac of host. Results:
      A. Prechordal plate — no neural induction
      B. Center piece -- brain, notochord, muscles, pronephras, auditory vesicles, etc.
      C. Posterior piece -- neural tube of spinal character, plus other tissues as above.

Slide 20
Eakin 1939
Fig. 1

   iii. Note, however, Oppenheimer frequently obtained brain tissue in absence of notochord in her explants!

Slide 21
Luther 1935
Fig. 7

   iv. Luther (1935) transplantation of tongue of mesoderm to opposite side of blastoderm. -
      A. Result - a secondary embryo induced - facing the primary one head-to-head.

Slide 22
Luther 1936
Fig. 1

   v. Luther (1936) reorientation of a central segment of blastodisc -
      A. Result - normal embryo

4. Does the blastoderm show the classic "field" properties?

a. Progressive restriction -- Luther (1936), ~~Trant~~. Ability of different sectors to form histologically recognizable tissues is progressively restricted to embryonic sector (Tested in grafts to yolk sac).

Slide 23
Luther 1936 (from
Oppenheimer)

b. Regulation -
   i. Excision of entire embryonic sector in early gastrula
      A. Extraembryonic tissue covers yolk, but no embryo forms unless:
      B. Oxygen is restricted; then an embryo forms but does not endergo epiboly.
   ii. Excision of less than entire embryonic sector:
      A. Single embryo or twins, depending on cut. May occur even if gap filled with extraembryonic tissue.
   iii. Reciprocal exchange of right and left halves of embryonic sector. Twins formed - i.e., field split.
   iv. Conclude early germ shows "field" properties.
      A. Gradient of potentialities from high point to center.
      B. Incorporation and organization of other tissue.
      C. Part can form whole.
      D. Fields may be split and fused.

Slide 24
Luther, Fig. 2

Slide 25
Luther 1937
Fig. 9a

Slide 26
Luther 1935
Fig. 1c

I. The analysis of epiboly

1. The character of the surface gel layer -- i.e., surface coat -- Fundulus chiefly (Trinkaus).
   a. Thin layer of gelated protoplasm continuous over blastodisc and yolk.
      i. Non adhesive.
      ii. Thrown into radiating folds when compressed.
   b. Wounds in gel layer -- wound expands and then closes -- carbon marks show that edges of wound contract and rest of gel layer stretches to allow hole to close.
   c. Continuity of gel layer -- wound at edge of blastodisc -- Blastodisc cells stretched out by contraction of wound.

Slide 27

2. Does contraction of the gel layer account for epiboly? Suggestion of W. H. Lewis; tests by Trinkaus.

   a. Note that epiboly concerns both the periblast and blastoderm which rests on it (Draw) -- The two undergo epiboly together.
   b. Evidences for affirmative answer
      i. The contractile power of surface coat as seen in wound-healing.
      ii. Epibolic coverage of yolk hastened by making a wound in surface coat in region of blastopore.
   c. Evidences for negative answer.
      i. Carbon marks on surface coat ahead of advancing germ ring do not move closer together; also, they should not be overtaken by germ ring before closure of blastopore, but they are! (Draw). But [illegible] they are not
      ii. Presumably, a periblast without a blastodisc would offer less resistance to the pull of the yolk-gel layer and would undergo epiboly more rapidly than is normal -- Usually it goes more slowly.
      iii. Epiboly of the blastoderm occurs without attachment to yolk-gel layer -- Detached from periblast, the blastoderm retracts as periblast undergoes epiboly and closes blastopore. Blastoderm then attaches to periblast once more and undergoes epiboly by crawling on the periblast which has no surface coat except at edges.

Tuscona – June 24, 1963

Introductory stuff on aggregation –

I Dramatic demonstration of ease of making cell suspension in Microciona & Cliona

II Movie of aggregation in chick cells

III Association of mouse skin cells – formation of Hair follicles in culture –

IV – Differentiation of Microciona in culture

I Structure of Sponges & their Classification

A. Basic Structure

B. Classification

1 Calcarea – Calcareous spicules

2. Hexactinellida – 6 rayed Siliceous sponges

3. Demospongia

a. Tetractinellida – 4 rayed siliceous spicules.

b. Myxospongia – Slimy sponges – No spicules – no spongin

c Monaxonida – siliceous monaxons – without spongin – Mostly the ones used in course

d – Keratosa – only spongin – bath sponges

C. Cellular Structure

1. Firm Structure of collar cells! – Lining layer – Lice

2. Mesenchymal(?) layer –

a) Collencytes –

b) Amebocytes
i Scleroblasts
A. Calcoblasts
B. Silicoblasts
C Spongioblasts - Produce a species of collagen -
Spongin A is fine, branching fiber -
Spongin B is thicker -

The problem of synthesis of spicules: Founder cell + thickener cell ? -

ii Archeocytes
iii Germ Cells

I Reproductive Methods
Budding - reduction bodies, gemmules, asexual larvae. - Regeneration - Sexual repro.
A. Budding -
1. Sometimes bud drops off as a ball of archeocytes surrounded by dermal cells
2. Continuous budding in colony
B. Reduction Bodies - occur under disadvantageous conditions
1. Cells become detached + aggregate in clusters of archeocytes surrounded by dermal cells.
2 Brien shows archeocytes goes around eating other cells, then clustering as in 1.
C Gemmules - also under adverse conditions.
1. Amebocytes - fed by trophocytes - become loaded with large inclusions - blaster becomes

surrounded by columnar layer of cells -
Special spicules (amphidiscs) formed all over sponge
body - then transported to gemmules
which latch (set in between) on them. - Finally another
layer of cells — to deposited around
cylindrical cell layer, leaving a
micropyle — Then gemmule must
undergo period of cold. — After that,
gemmule cells flow out & repopulate
old skeleton of parent sponge — — If
gemmules are in a glass dish, they come
out individually & form a layer lying
on non-cellular layer — - Cells come
out of gemmule in waves - forming
spicules etc —

see Rasmont

Moscona - Sponges II - June 25, 1963

Gemmules - contd - Hatching (Movie showing the outpouring of cells from gemmule.

Will gemmule cells (Simon-) repopulate old, foreign or treated skeletons -

	Spongilla		Ephydatia		Trophos	
	OM		OM		OM	
Spongilla	+	+	−	+	−	−
Ephydatia	−	−	+	−	−	−
trochospongia	−	+	−	+	+	+

OM = organic matrix - in other box, organic matrix dissolved off (how?)

SEXUAL REPRODUCTION

A. Gametes

1. Sperm

a) from archiocyte which form sperm chambers, which form spermatozoa

2. Ova -

a) Somehow first eat other cells collar cells

b) Nurse cells attach + feed materials in

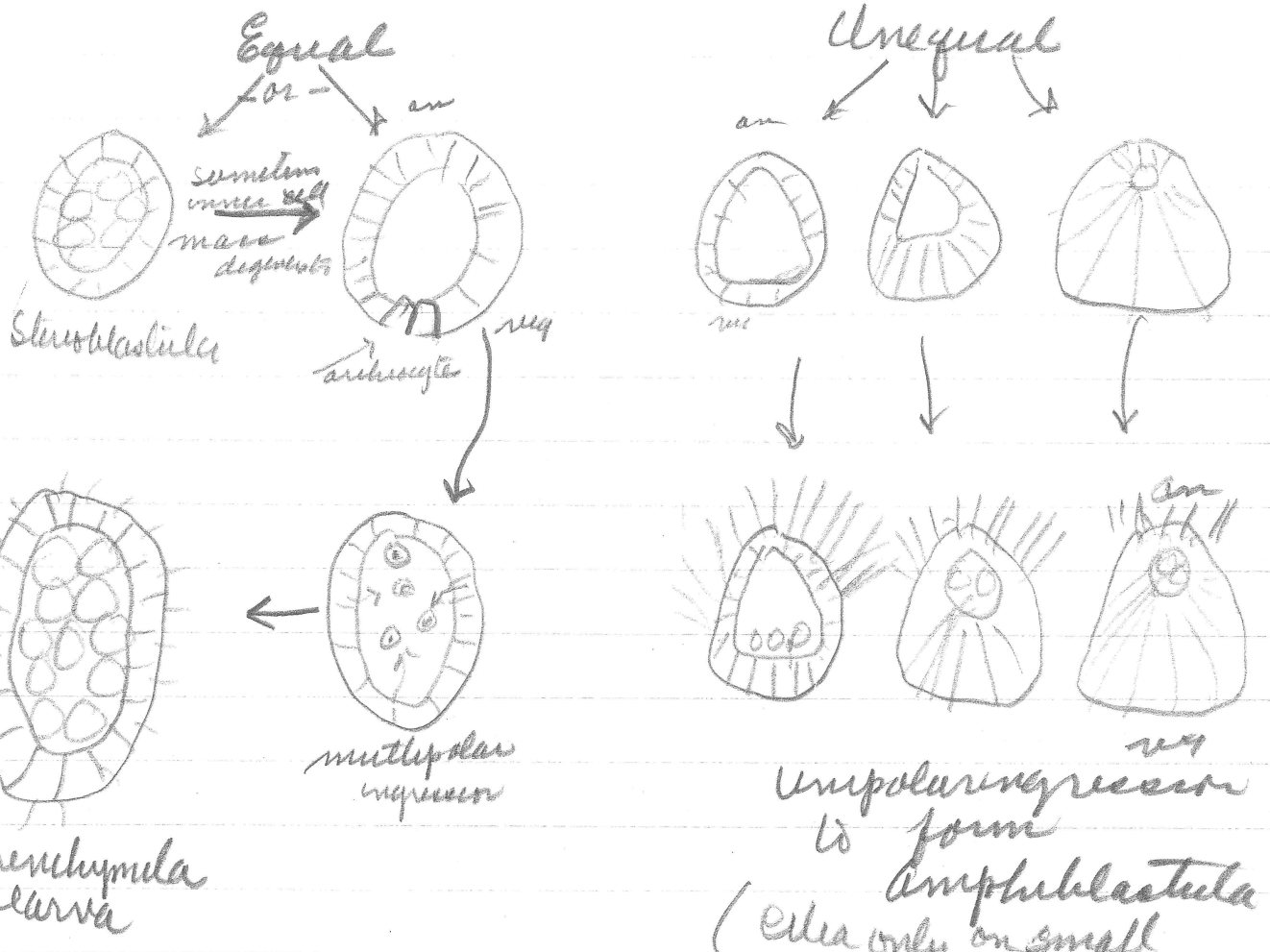
See paper by Dubos + ?

B. Fertilization

1. Sperm, to get at egg, must perforate collar cell. The collar cell pops it into ovum

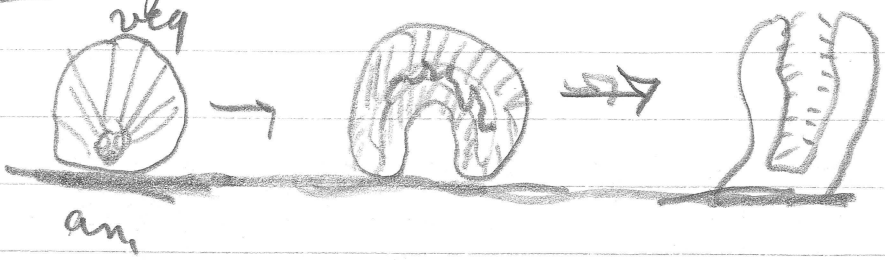
C. Development –

1. A-p axis set up – ant end forms dermal layer. Post end the collar cells.
2. Cleavage.



3 Attachment —

a) Amphiblastula attaches & substrate with animal pole down



b. Parenchymula — reversal of layers — ciliated outside cells move inside & make collar cells — — some cells of inside move to outside & form dermal layer. — Some inside cells remain inside & form archeocytes.

4. Some Exceptions — Cleavage stages

a) In Grantea — ~~amphiblastula~~ connects with maternal cells which penetrate into interior of larva, degenerate & nourish larva. —
After this larva hatches, turns inside out & makes a kind of amphiblastula.

b) In Halisarca (myxospongia) — — goes thru a kind of amphiblastula with no ingression — settles with an side down & invaginates to form collar chamber.

(next page for summary

5. Summary

Amphiblastula by unipolar ingression → inversion → olynthus (as in Grantia or Sycon)

Amphiblastula with no ingression → inversion (Halisarca)

Parenchymula → multipolar ingression → reversal of layers

Parenchymula → multipolar ingression → internal reorganization (Spongillidae.

GRAFTING PROBLEMS - etc Specificity etc

A. Halisarca & Microciona grafts — Some preliminary expts show rejection of foreign grafts -

B. Dissociation - Aggregation - Develpt of aggregate - Metaplasia ?

1. Dissociation procedure -

a) mechanical

b - Survival - Do all kinds survive ? - Does dissoc. procedure select ?

2. Aggregation

a) Interphase upon settling – slow some filamentous projections which meet & may have something to do with aggregation

b) Aggregation pattern depends on density of cells – concept of critical mass.

3. Do restitutions, the selective adherence of cells of appropriate kinds? – Dedifferentiation & primitive types? Survival only of archeocytes

a) Do all cell types survive? Yes, according to Wangula; Galtsoff et

b) Are all cell types incorporated in aggregate? Yes, probably; – Are they in right proportions? Do they all survive? ? ? Don't know

c) Can one separate cell kinds?

i Huxley separated collar cells by picking them out – They do not form sponge

ii Galtsoff separated archeocytes by centrifugation – They did not reaggregate into complete sponge.

iii Fauré-Fremiet selectively

killed collar cells by U.V. Aggregate
2 remaining cells did not make
collar cells.

The preponderance of evidence is
that reaggregation leads to
reassociation of original types

Evidence for plasticity.

Elliot's Mail —

Moscona — June 26 — Cell aggregation etc —

I Intro:
A Necessity for control of cell movement in aggregation
B. Rotation — Gyratory motion — Several cells gently into contact
a at high speeds, shearing component is greater.

II How cell dependent factors affect pattern of aggregation
A. Effect of age of cells — Neural Retina.
1. With age — aggregability declines rapidly — ie. smaller clusters c̄ advancing age.
If cell-binding substance is lost in dissociation process, then it is restored more easily for young cells.
2 Initial rates of aggregation — — Ball's beginning PH Dmp
B. Effect of monolayer cultivation —
1. Dissoc chick long bones at 13 days —
a) Rapid loss of aggregability — almost gone by end of 2nd day.

III Effect of environment on aggregation
A. Temperature effect on Neural Retina.
a) From 38° to 15° aggregability declines almost to nil —
1 Conclude the making of new binding substance is diminished greatly at low temperatures.
b) Preincubate cells at 38° — transfer to 25° & they aggregate very well — contrast to controls without 38° preincubation.

I Sponge – Rotation aggregation.

A. ~~Two~~ Single species mechanically dissociated – reaggregate merely in rotation & if plated out they differentiate, but do not differentiate under constant rotation

B Mix Halisyona & Microciona – In rotation & platina, the species aggregation are distinct –

C Conclude – species cant associate

V Is species distinctiveness retained in vertebrates?

A. Mouse, cartilage, chick kidney dissociates –

1. Initially, in rotation flasks cells are mixed. – Eventually the kidney cells move out & settle on outside of cartilage cells.

B. Mouse skin & chick skin in rotation

1. At 6 hrs aggregates are mixed –

2. Now, graft to chick CA. – cells from the 2 species cooperate functionally in formation of chimaeric tissue – (JUS notes tendency of chick c̄ chick & mouse c̄ mouse – Indeed, however, they do not separate from each other.

C Mouse & chick kidney & cartilage show same phenomenon

VI Effect of monolayer culture on ability of cells to differentiate – Skin

1. ~~Aggre~~ Grow for 5 days, aggregate in rotation apparatus – grow on CAM – Do not differentiate skin – in contrast to dissociated & reaggregated controls.

VI Sponges, again — Rotated at diff temps
An inhibition of adhesiveness brought about by decreasing temp?

Sea water	24°	5°C
Mechanical Dissoc	+	+
Mech Dissoc + rinse in Ca-Mg free SW	+	—
add wash-water		+

→ Contrast c̄ mouse retina
Prebreakment of 5° was followed by aggreg at 24
Note agreement with vertebrate stuff.

Humphries concentrated from washings a factor which causes rapid aggregation into large masses — Effect is dependent on concentration

Effectiveness of factor not destroyed by DNAsc or RNAsc — No peak at 260 mμ.

Is the factor species specific? — Microcione + Halidona factor do not cross react — Mixing factors of the 2 + both kinds of cells will aggregate in same cluster.

VII Block with puromycin at 0.1 γ/ml (blocks protein synthesis at RNA level) and blocks reaggregation — Wash after 6 hrs + return to gyrator — + get aggregation — Thus in Neural Rev tia.

Block c̄ actinomycin (block DNA) — get irreversible block to aggregation.

Burnett – July 1, 1963

I. Lecture Plan

A. Heavy conc on 3 species

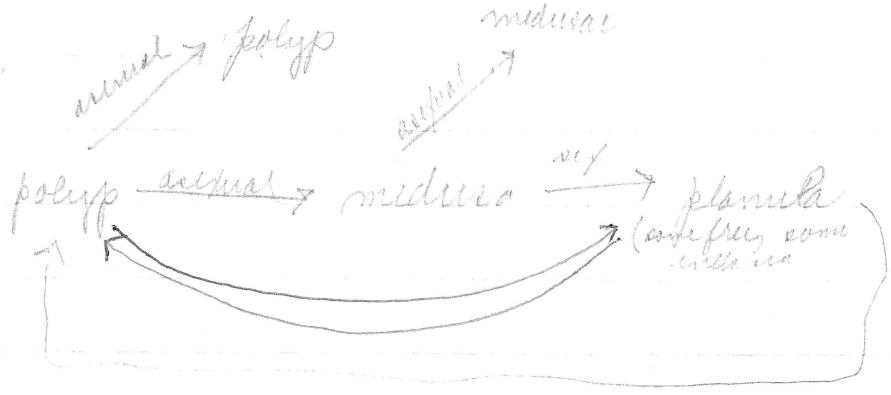
B. List

Tubularia –

Hydractinia –

Hydra –

II. Life Cycles – generalized for Hydrozoa



A. Any selective advantage to this alternation of generations? Gives the advantage of 2 different environments in which the species can propagate

~~B.~~

III. The different cell types in the Coelenterates.

A. Slide of Hydra

1. Gastroderms – Gland cells, mucous cells, digestive cells
and pedunal gland its special

B. Eckhorn

1. Large - mucous producing cells
2. Small, basophilic interstitial cells or "neoblasts" - impt regen & gamete formation
3. Cnidoblasts

C Nerve cells - Burnett did not say what layer?

Burnett - July 2 - growth & Regulation of form in Hydrozoa

I Hydra -

A Intro - Tie simplest in growth pattern & form

B History of Study -

Trembley 1. 1st animal in controlled regen studies

2 1st " grafted

1st " studied with vital dyes

C. Characteristics

1. Rigorously polarized - small s

a) small segment isolated - segments - Retained original polarity in regeneration

(b) Contrast - Tubularia where bipolar regen can occur).

2. Body regions - head - body - foot.

3 Induction

a) Ethyl Browne (Harvey) 1909 grafted small piece of hypostome & applied it to any region - It will induce formation of complete hydranth wherever placed -

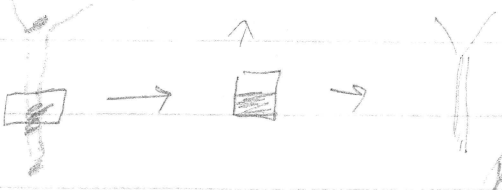
i In combination of green hydra & bleached green hydra it was shown to be a true organizing action.

4. Tripp, 1928 - Growth pattern
a) In animal c̄ a forked tentacle, the fork of the tentacle disappears ⅄ → Y → ∥
b) Grafted base to side of body column - It progressively moved down to basal position.
c) Suggested that just under hypostome is a region of proliferation - forcing cells apically out tentacles & basally down stalk! - When cells reach tip or base they atrophy & slough off - (All of this without direct observation
5. Brien - rediscovered Tripp's work 1948
a) Cells in the "mucous footprint" left behind by hydra.
b) Marked cells c̄ dyes & came out with same conclusion as Tripp!
D. Burnett analysis --
1. "Something" from hypostome induces the cells just below to be "growing region"
2. Interspecific graft of hypostome tissue to foot -
i Cut foot of one & graft head of another to it (sans tentacle) - great movement of neoblasts (usually absent)

into the basal region of the host
A new head begins to form from
host -- Graft falls away &
now the host is a bipolar
animal!! — Contrast with earlier
positive statements about Polarity —

3. Burnett refer to Child's metabolic gradient theory!
   a) Regeneration of head in Planaria
   b) Differential susceptibility to CN
   c) " in redox activities
   d) Conclude anterior end is site of high point in respiratory gradient.

4. Burnett says in Hydra you can dissociate morphogen potential from respiratory polarity
   a) Bud region (even sans bud) is a high point in Hydra as is the hypostome & base.
   b) Cut thru budding region



retain polarity despite the fact that the high point of the gradient is towards base.

effect of op on resp.

5. Burnett theory to account for Hydra polarity
   a) Assume a "head-forming" material produced

in hypostome continually diffuses down the body column becoming attenuated (how?) further from head — poops out by base.

a. Why don't heads form all along stalk? After all - there is h-f material away from hypostome?

i. assume in growing zone an inhibitor, I, blocks action of the growth head forming material (X), but it is rapidly inactivated so, by time you reach bud, the little X material left can now stimulate ~~budden~~ growth of a head of a bud! —

c) Test this idea:

i Put hypostome on agar in the manner of Avena coleoptile test — segment of trunk ~~Block later~~ applied to block & get head formation where block applied.

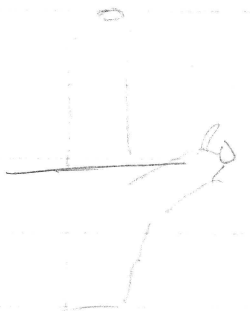
i One of his students puts a segment of Hydra on block & get a mass of Heads. —

Conclude there is some "X"

ii Block flow of X by inserting peduncle below growth zone & get a new head.

iii Reality of I suggested by putting growth region next to budding region by removing intervening column. This inhibits budding altogether until headgrows away from bud region. If bud formation started at time of operation, there is no effect on bud!

iv



Cut parent off just above bud before bud makes own hypostome & tentacles — This inhibits ~~growth~~ regen of parent head until bud's peduncle forms. Latter seems to block flow of inhibitor

II Tubularia

A. Tardent — says he has isolated an inhibitor of growth & regen which seems not to be bacterial.

B Burnett says it is impossible to explain form of Tubularia by postulating a single inhibitor —

a) Remove as little as 1/16 of the oral

cap and graft to stem & get as new hydranth induced — It will induce very close to the original hydranth. — Hydractinia does same.

C. Isolated tentaculozooid of Hydractinia will reorganize into a feeding hydranth. Spiral zooid will do same.

Burnett -- July 3, 1963

I Refer back to yesterday's Hydra discussion -



I = inhibitor - produced in growing region.

- higher areas of respiratory rate -

II Tubules of Dig gland of crayfish (the hepatopancreas)

A has different cell types

- embryonic cells
- absorptive cells
- secretory + fibrillar cells (store iron)
- proximal cells -

Davis, a student of Burnett

B Mitoses occur only at tip, in embryonic area

Inject thymidine - label only at tip at first day

- next day the absorptive cells are labeled

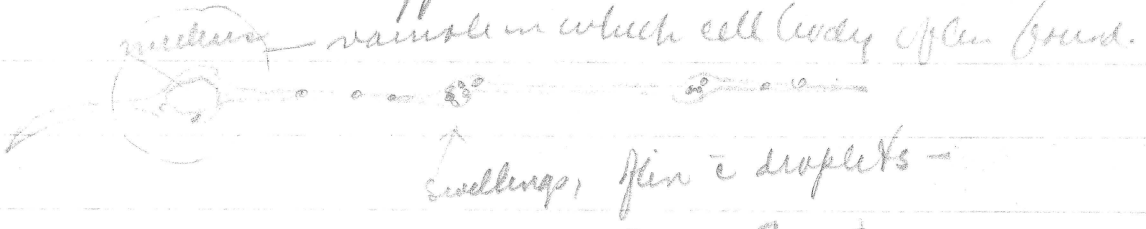
thereafter, moves on down line

C Uridine taken up by all but proximal cells -

D. The proximal cells are pycnotic

III Malpighian tubule of Insect possibly work the same way — — Note also cells of intestinal villus!

IV Expts re Nervous system in Hydra (+ Tubularia)
Possibility that the nerves produce substances that affect growth & differentiation
A. Stain with leucomethylene blue & get specific nerve stain
B Concentrated in hypostome & tentacle bases.



1. Are the droplets secretions? Yes! They can expel the droplet directly out of swellings or axon pulls back, bringing granules into cell body which ruptures releasing granules
C. Are the nerves the source of growth material
1. When bud grows out it has no nerves, but just prior to tentacle formation the N.S. quickly differentiates beginning at hypostome & spreading to base. — reaches latter at time bud separates

2. Stop growth by refrigerating. The nerve cells disappear — or at least only some cells bodies form — Keep at room temp for 2 days & axon sprout again
3. Find the secretion granules in EM's ~~[illegible]~~ thought they come from nucleus. The cells are Schiff positive & show Methylene blue extinction at pH 2 — Stain c̄ alcan blue.
4. Graft hypostome region of growing hydra to base of sexual, non growing animal & reverse sexuality beginning at graft site — gonads grow out on stalks & disappear

V Interstitial Cells or Neoblasts

A. Distrib — All major phyla of invertebrate show them.

B. Characteristics.

1 Highly basophilic
2 Highly motile
3 Considered to be a persistent embryonic reserve which is totipotent.
4 Autoreproductive

C. Can we prove what these cells make?

1. Thymidine label (must injure hydra
   a) ~~Never enters cnidoblasts~~ regardless ~~when pulse~~ given.
   b) Interstitial cells are not the only cells labeled
   c ~~Enter~~ Label first found in cnidoblasts after 24 hrs.
2. Division — Fix in 100% alcohol & treat overnight c̄ RNAse. Stain with M. Thylene Blue & see only nuclei — Find that "I" cells are often in nests ~~other~~ in which all are dividing —
3. Can show all stages in transformation of I cells to cnidoblasts
4. Treat c̄ nitrogen mustard — the I cells become very large — after 3 days they become vacuolated & die — No "I" cells left — Animal lives 10-30 days in cold, then die!
4. After mustard treatment regen ability declines. ; there is division in some cells, but not in cnidoblasts — these ~~pop~~ disappear from entire organism.

5. If a bit of healthy tissue is grafted into irradiated or mustard treated animal, I cells migrate in & repopulate

6. Haynes stuffed epidermis of normal animal down mouth of mustard treated animal. Invasion of host took place, including I cells — But the host dies! Thus I cells not enough!!!!

I cells are never seen in gastrodermis.

7. Can differentiated tissue become "I" cells which can redifferentiate into something else?

a) Dig cells, gland cells, [illegible] cells are only components of gastroderms. Burnett can not confirm that isolated gastroderms will form new hydra.

b) Haynes isolated gastroderms of Green hydra. (Only dig cells have algae) Put at 10°C (crucial) — Dig cells dump their algae & food vacuoles & transform into mucous secreting epidermis. Eventually get a small hydra — die of starvation NO nematocyst or cnidoblast — Cells like

did they have "I cells" & nematocysts?

12(?)

4 cells appear at 8 hrs - Plenty more at 24 hrs - + Nematocysts

2 lectures -

Clement - July 8, 1963

The Mollusk - Annelid Plan of Development

I Introduction

A. Common Plan of Cleavage in Annelids, Molluscs, Nemerteans & Polyclad flatworms.

1. Spiral
2. Similar in cell values

B Exception - Cephalopods

C Determinates - Stereotyped - Presumed by Conklin to show precocious assignment of cell values.

II Development - Ilyanassa chiefly

A. Rearing of eggs of the snail

B Structure of egg



C Meiosis & cleavage

1. Polar lobe with each meiotic division
2. 1st cleavage giving trefoil stage
3. another polar lobe at 2nd cleavage.
   a) In Dentalium, there is no polar lobe, - ditto for quite a few others

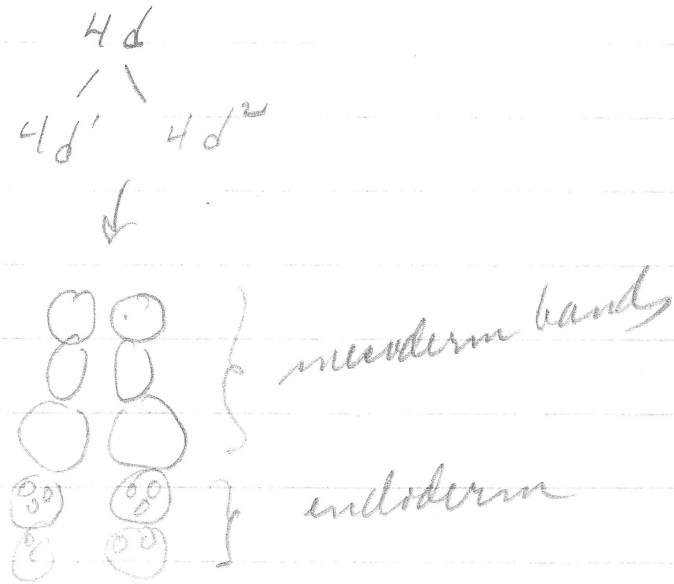
4. Unio - Lithe-no polar lobe, but spindle oscillates about "seeking" an eccentric position leading to asymmetric first cleavage

5. Description of spiral cleavage + cell lineage during early cleavages.

a) Shows 1st 3 quartette

b) Cleavage cutting off yolk free 4d cell - precocious

c) $4d^1$ + $4d^2$ sets up plane of bilaterality.



D. Cell values

1.) Fate

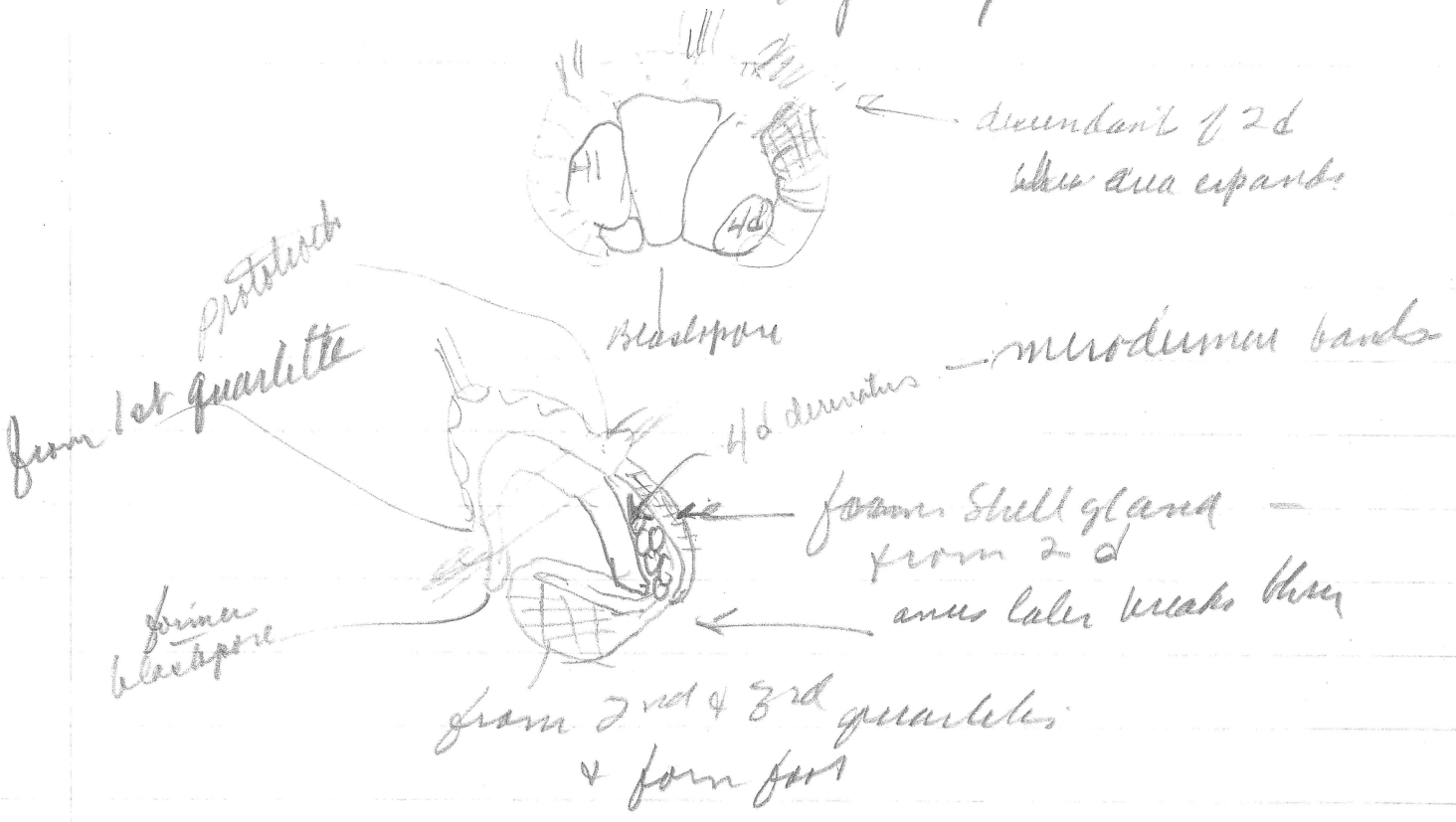
1st, 2nd, 3rd } Ectoderm + mesectoderm

3A, 3B, 3C, 3D } All entoderm

4d some endoderm + Primary mesoderm

2. Special significance of 2d – Often called 1st Somatoblast – 4d called 2nd Somatoblast.

3 Castrulation by Epibaly.



In annelids, trochophore shows pos brev elongation – Merodermal bands elongate

III History of Embryology of Annelids & Molluscs

A. Epigenetic view receives blow from reports of Whitman that of "predetermination" of 4d Clepsine conferens Rubl – Lead to studies of cell lineage.

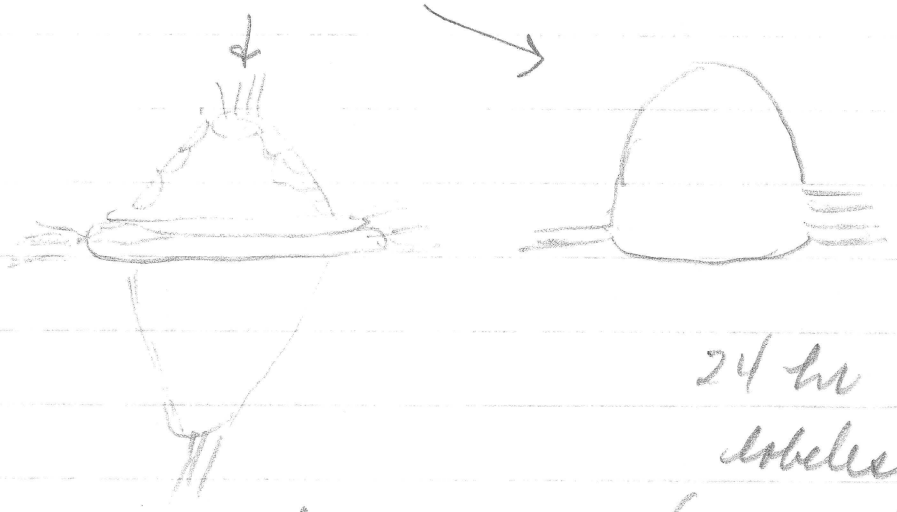
Wilson – Nereis 1892 Lillie – Unio 1895

Conklin Crepidula 1892, 1897

IV Classical Expts on Morphol. Signif. of "Determinate" cleavage. — Mosaic Develop't.

A. Crampton, 1896 (a grad stud of Columbia)
Accidentally knocked off polar lobe of Stynassa
+ isolated other blastomeres
1. No regulation of cleavage pattern
2. In absence of polar lobe — quartette budded off on schedule — but no 4d formed at regular time — No organ formation because of bacterial infection

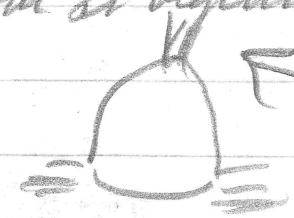
B. Wilson, 1904 Naples.
1. Dentalium + Patella research.



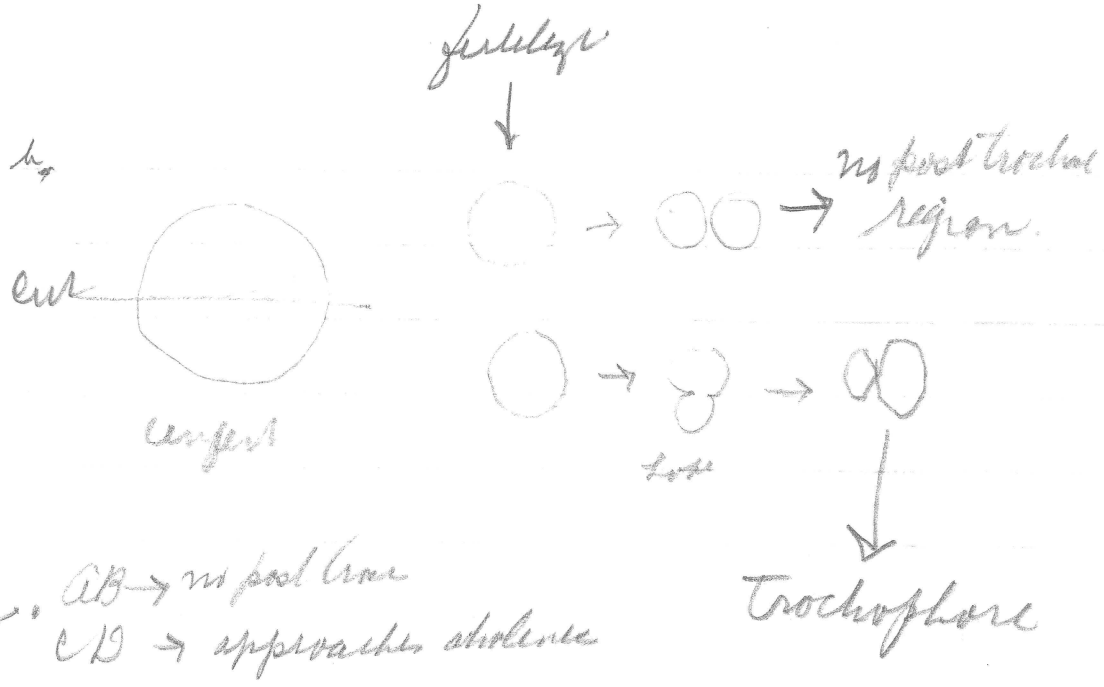
24 hr normal

24 hr lobeless (cut at trefoil

But — cut lobe at beginning of 2nd cleavage →



← Apical Tuft



C. AB → no post troc
CD → approaches cholinea

C. Novikoff - Sabellaria - Polar lobe has same significance

D. Castello on Nereis - Separate blastomere at various stages -- tend to show mosaic nature

E. Expts which tend to show regulation.

1. Albert Tyler, 1930 - Nereis, Chaetopterus, Cumingia - By various means, got polar lobe material into each of 1st 2 blastomeres -- got various types of double embryos.
2. Novikoff - Sabellaria - KCl treatment gave similar results.
3. Penners - Tubifex (a fresh water annelid) à animal & vegetal pole plasms combine & get into 2d & 4d.

b) much of cito from 2d -- also gives some muscles & ventral nerve cord.

c) CD by itself will give a good embryo - so will D.

d Kill 2d with U.V. - knock out normal derivatives

e) Kill 4d -- no meso bands. - 2d does not, under this circumstance form its normal derivatives.

f) But sometimes when 2d is missing, 4d will kind of "regenerate" it.

F. Induction of shell gland in molluscs.

1. Raven - Limnea - Intimate association of endoderm & prosp shell gland.

2 Hi-Bithynia - good evidence for induction in Bithynia of shell gland by endoderm.

Mesectoblast from 2a, 2b 2c -

Similar model of origin of mesectoderm ∃ in polyclads, annelids, molluscs etc

July 15 1965 - Monroy

I How is blastocoele formed in Echinoderm Larvae.

A. Cavity begins as early as 16 - cell stage.

1. K. Dan - During interphase of each cleavage, the hyaline layers of the cleavage cells discharge fluid between blastomeres. - Thus at each cleavage there is a stepwise increase in fluid

2. Gustafson, with time lapse, discover pulsations of entire embryo as blastocoele forms. He finds that volume contracts during interphase & increases during division.

B. Composition -

Blastocoele fluid contains mucopolysaccharide during early development & protein accumulation

II Migration of primary mesenchyma cells.

A. Prop. mesenchyma cells "boil" on inner faces, send out lobopodia - eventually detach.

B. Contrast, independent migration of primary mesenchyma cells with the cohesiveness of cells invaginating in archenteron. - After invagination is half complete, these cells of advancing tip may come loose, forming secondary

mesenchyme —

Invagination ~~is~~ slows down about half way — Lip cells send out long filopodia to make contact c̄ roof & sides of archenteron — ~~[illegible]~~ Now invagination speeds up — Looks like towing.

Analysis of Echinoderm Development. I.

A. Monroy

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[illegible] — Atkinson D

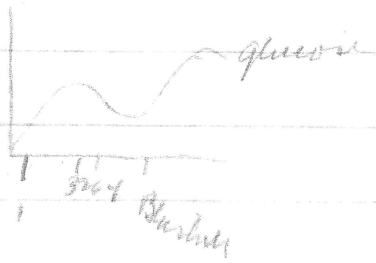
Echinoderms II

Monroy — July 6 1963

In fertilized Psammechinus eggs rate of O_2 consumption increases exponentially until selected blastula forms. Plateau until just before primary mesenchyme formed, whereupon rapid rise again ensues. — New proteins begin to be synthesized as selected serologically.

Examine period fertilization to 1st plateau —

1. Incorp of hot aa's — increases rapidly during 1st few hours — to 64 stage — then declines + rises again at mid blastula
2. Uses hot glucose — rate rapid — plateaus at 32 — 64 cells, declines, then rises at mid blastula.



Disaggregated cells behave as tho they were in the whole ~~re~~ ~~[illegible]~~ re aa incorp

---

Maximum vegitalizing effect of lithium during early stages

Treat with ethionine during 1st 2 or 3 cleavages — when primary mesenchyme cells are formed they degenerate

If you wait until micromeres are formed, then cells of primary mesenchyme do not degenerate.

Suggests that during early cleavage things are going on of fundamental interest to origin of "vegetalizing" influence.

When does utilization of yolk begin? E.M. photos show breakup of yolk platelets beginning right after fertilization.

Phosphoprotein phosphatase activity increases rapidly until 64 cell stage.

---

hatching.

Yasumasu 1960 J Fac Sci Univ Tokyo, Sect 4: 9: 39 –

Hatching enzyme ~~synthesized~~ ~~used~~ during the plateau period of O_2 cons. It is present in homogenates 3 hrs before hatching & disappears thereafter. –

Where do proteins forming cells come from? are they produced anew?

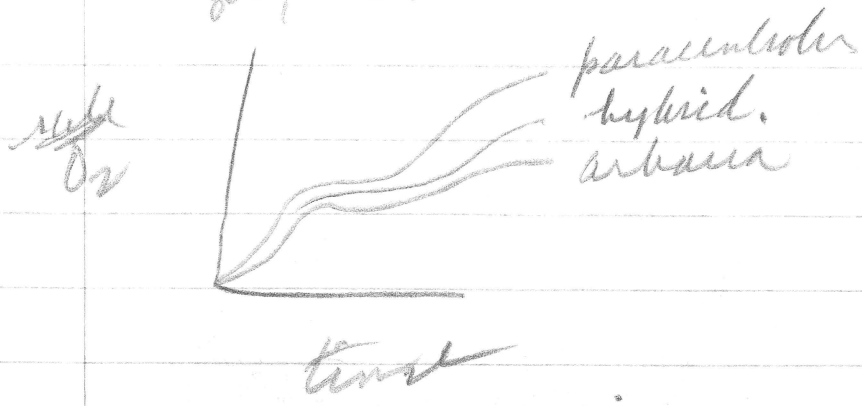
Maggio shows that an activating enzyme increase dramatically at time primary mesenchyme formed.

Alkaline p-ase actively low until the "mesenchyme blastula" stage — the enzyme formed earlier is a faster moving, weaker band on starch gel electrophoresis (I think).

Baltzer — hybrid of Mediterranean Sea urchins — Parental chromosomes often occurred. Elimination of parental chromosomes in many, but all died regardless of loss of a parental set.

Neufach: irradiate nucleus, embryo blastula — embryo dies at blastula — Suggests nucleus is morphogenetically inert to blastula stage.

Baltzer, Chu et al



Die towards end of gastrulation

Fleckinger, BB 112: 21 (1957)

Viable hybrid of strongylocentrotus × Anadrastic shows activity of alk p-ase is intermediate between that of the 2 parent species.

Par ♀ × Psammechinus ♂
Paternal antigen first appears at mesenchyme blastula stage.

Discussion – in unfert Sea urchin egg there are lots of ribosomes but few polysomes – right afterwards, one finds them.

Note that sensitive period for hybrid arrest – as gastrulation begins – is period when nucleoli first appear – this is same for frog hybrids.

Analysis of Echinoderm Development II
A. Monroy
List of References

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Lush - July 22, 1963

I Introduction

Phylum Protochordata

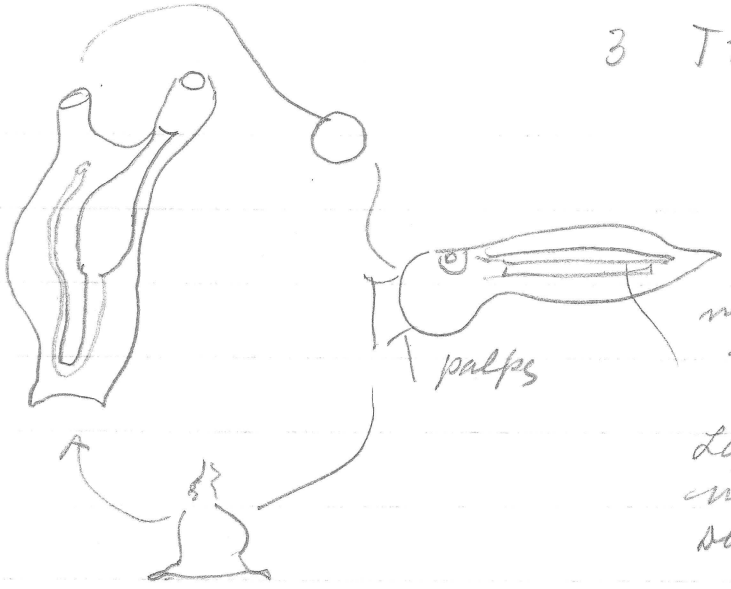
Subphylum 1. Hemichordata (= Balanoglossus

2 Urochordata - Tunicata

Class 1 Larvacea

2 Ascidiacea

3 Thallacea (Salps.



nerve cord notochord

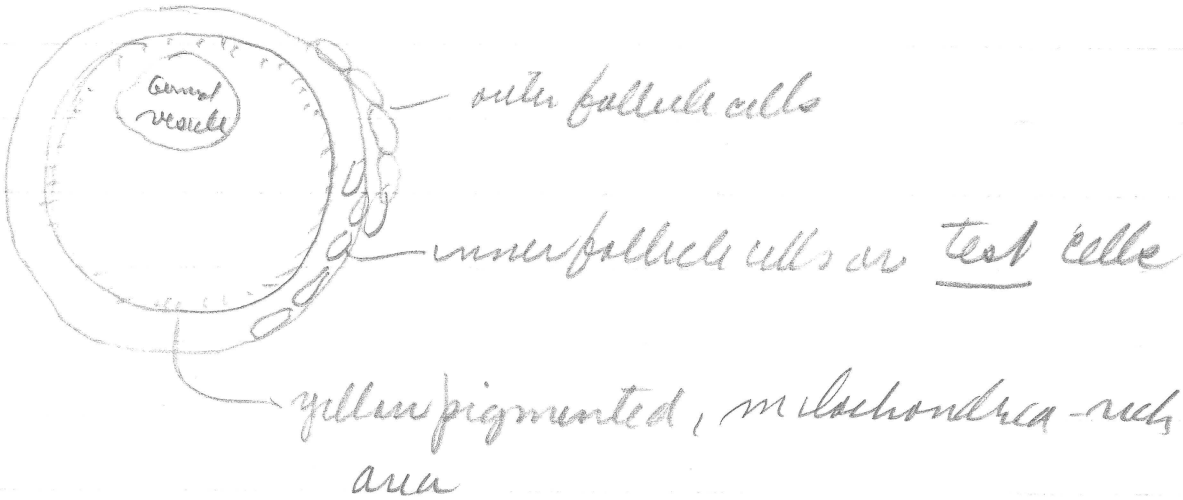
Lumen with gills (branchial basket) notochord - dorsal tubular nerve cord

Some interesting characteristics

- Vanadium carry 10% vanadium and 3% H2SO4
- Cellulose coat
- Extreme "dedifferentiation"
- Reversible heart
- Amazing differentiation of egg -

II The "Mosaic" egg of Ascidians

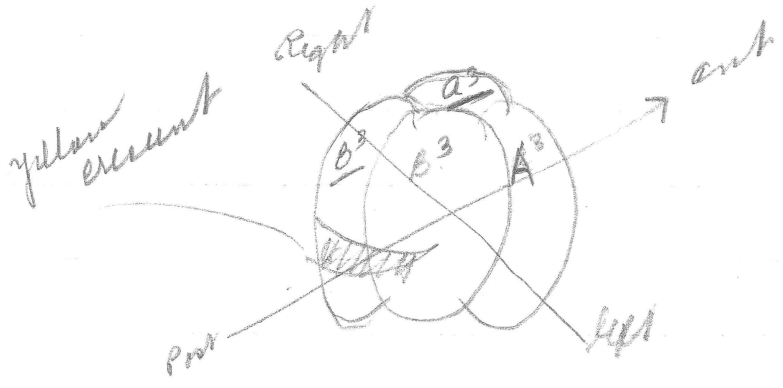
A. Structure of Unfertilized egg



2. Post-fertilization movements
   1. Movement of "yellow" protoplasm to vegetal pole
   2. Clear plasm of germinal vesicle layers over yellow.
   3. Sperm moves in eccentric path and yellow follows it along - Clear material then overlies it.

3. Cleavage
   1. Splits into right & left halves.
   2. Same regulation possible if egg cut at earlier stages -
   3. Cell Lineage -
      a. Chabri - injured blastomeres → defects
      b. Conklin - detailed cell lineage.

(Do I recall correctly that future dorsal side is towards vegetal pole.



Review of cleavage - remarks on dependent differentiation of nervous system on notochord. - See a recent paper by Reverberi.

4. Review normal development to tadpole stage -

a) among other things - note secretion of matrix of notochord - interesting - look it up.

III Remarks of a comparative nature on adult structures mostly - (Skip them much of it)

Bobrytlies - pigments

Black ground pigment - varies thru brown & blue

White - green - yellow - purine

Orange - carotenoid

Sabbadin genetics of peristomal & inter-siphonal bands -

A = orange

a = no orange

B = intersiphonal band

b = no intersiphonal

F = peristomal ring, f = no ring.

These work out in Mendelian ratios.

Orange can be transferred non genetically, — Pigment precursor cells can pass from one colony to one fused with it. — Such "infected" colonies do not give orange genetically.

Showed some nice color photos of colonies of Botrytis.

Lash - July 23

Didemnum - forms 2 buds - one gives rise to a branchial basket & other to viscera. These may fuse with each other to give a whole organism or dig tr of parent may fuse with branchial basket bud & vice versa.

Describes Botrylloides budding
" Perophera "
Amaroecium

Regression or Dedifferentiation - Perophera & Clavellina - regresses to a mass of undifferentiated tissue under unfavorable conditions. - See Huxley

Cell Death? Copy?

Times from fertilization to Tadpole
Morgula - 8-12 hrs
Styela 18 hrs
Ciona 24 hrs
Perophera 40 hrs
Styleopsis 160 hrs

Resorption of tail -
Epidermis
2 Layers of trunc
Nerve Cord
Endodermal strand
Notochord (cells, matrix sheath)
MUSCLE cells, Mesenchyme

All these are drawn into the organism's primary body cavity (around branchial basket). Cells do not fall apart — look healthy.

Larvae of colonial forms are more complex — ready for metamorphosis — attach quickly. Have complex branchial baskets, but simpler eye spots. — These take longer to go from fert. to hatching —

Metamorphosis —

Does metamorphosis "pop" the lysosomes releasing proteolytic enzymes?

Weissman Unpubl — Vit A caused metamorphosis in Xenopus comparable to Thyroxine !!

Vit A accelerates tarnicato tadpole metamorphosis like Janus Green.

This prevented by adding Hydrocortisone

400 tadpoles

Cicona	metamorphosis
Control	62%
Janus Green	100%
Vit A	94%
Vit A + Hydrocortisone	73%
Hydrocortisone	51%
Thyroxin	97%

Thyroxin + A → died immediately

Lack unpubl

Events of Metamorphosis

Cloney

① Epidermis lifts off

2) Depending on species

a) tissues dissociate Styela Bullenia

b) Tissues do not dissociate amaroecium

- Whole tail coils up like a snake in the body cavity. Ectoderm stays out, making an epidermal cap which plugs the hole!

No one seems to have traced disposition of the tail cells. - Look healthy for a long time! this is accomplished in a few seconds in Amaroecium

If tail is severed, the epiderms lifts & contracts but inner mass stays outside

Cut epiderms alone, & it contracts towards body & towards tail tip, but notochord etc is not absorbed into body cavity!

in these, perpendicular by of notochord breaks, and fluid spills into primary body cavity.

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C.O. Whitman, E.G. Conklin, E.B. Wilson, C.E. McClung, Frank and Ralph Lillie, Thomas Hunt Morgan, Gary Calkins, M M Metcalf Jacques Loeb – – Names that you well hear again and again throughout the course

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Don Costello

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J.P. Trinkaus
Chas. Wilde
Clement Markert
Meryl Rose
Anthony Clement
Kalsuma Dan
Jean Dan
CW Metz

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B. But — what is the future going to be

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2. This new outlook is reflected in the composition of our training staff

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Second Wing 1892

1st Embryol Class 1893

Botany Bldg 1894

Embryol Lab – built as

[illegible] hall & res. lab 1896.

1815–1860 – Whaling Center

1836 – Candle House –

1880 – Pacific Guano Works

Lillie F.R. 1942. The Woods Hole Marine Biological Laboratory. U. Chi Press 284 pp.

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→ Insert History Para

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1030 AM Whitman

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time allocated - of the pattern of the past is followed, the lights are now burning in this laboratory, not just for the night, but for the summer.

iv We require no formal records of your work, in the sense of a notebook for us to grade etc - But, as good scientists you will, naturally, have good, neat, organized protocols. These should be available to the instructors on demand and, as essential for certification.

C. Conklin Prize
1. awarded upon selection by staff
2. Award $150.
3. At end of 1st or, perhaps, 2nd period

D. Post-Comm. research
1. Students selected by staff
2. Period for independent work
3. Formal meetings Fridays after July 31.
4. Hopefully you will develop a problem to pursue under guidance (more or less direct) of some staff member.

IV Library

A – Outstanding

B – How organized – Charts show each floor

1. Entry – 2nd floor – – Books
2. Reprints – 1st
3. Journals 4, 3, 2.

C. Regulations

1. Identify yourself to Mrs Harlow
2. Journals do not circulate
3. Sign out books & reprints
   Be scrupulously honest!!!
4. Open day & night!

V Recreation –

A. Outstanding Opportunities.

1. Fishing, boating – trips to Nantucket & Marthas vineyard – Picnic or cruise.

B. Show map of the area

C. Dangers –

1. From currents
2. Sunburn – Caution about reflection from water & sand
3. Poison ivy in woods.

Island Queen – at head of Falmouth Harbor
Capt Bullard
200 each – min of 100 person

VI Simpson talk – Questions –

VII Travel vouchers

PATCH T

Sized for printing unscaled on US-Letter paper

Eastman Kodak Company - 200dpi - TIFF – December 2010 – created with Microsoft Windows 7 MSPaint

Patch Code separator sheet geometry is further defined in the Kodak Publication A-61599 Scanner Patch Code Information
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Narrow black bars and white voids must be exactly 0.08in.
There should be 0.5in of white boarder from edge of page to start of first bar.

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1) **The vertical dimension line must be exactly 8.0 inches (203.2mm).**
2) **The horizontal dimension line must be exactly 5.0 inches (127mm).**
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The paper used should be white or light pastel colored such that at least 65% of the infrared and visible light is reflected in none printer areas.



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EMBRYOLOGY COURSE, 1963

MARINE BIOLOGICAL LABORATORY

STUDENT ROSTER

NAME	INSTITUTION	LEVEL	FIELD
Hani K. Battikh	U. of Virginia	grad.	developmental biology
Hillard Berger	Vanderbilt U.	grad.	bacteriophage genetics
Morton L. Burdick	Johns Hopkins U.	grad.	developmental biology
Gloria Ann Cassens	Indiana U.	grad.	developmental biology
John R. David	New York U.	M.D.	immunology
Richard L. Davidson	Western Reserve U.	grad.	developmental biology
Michael J. Epstein	U. of Texas	grad.	developmental biology
Daniel Hartline	Harvard U.	grad.	biophysics
C. B. Kimmel	Johns Hopkins U.	grad.	developmental biology
David E. Kohne	Purdue U.	grad.	developmental biology
Dolores J. McWhinnie	Marquette U.	grad.	developmental biology
Dorothy E. Patton	U. of Chicago	grad.	developmental genetics
Robert E. Pollack	Brandeis U.	grad.	developmental biology
Ronald H. Reeder	MIT	grad.	developmental biology
Stanley Shostak	Brown U.	grad.	developmental biology
Elinor J. Spring	Harvard Med. Sch.	grad.	developmental cytology
H. L. Stackhouse	U. of Arizona	grad.	developmental biology
Trygve P. Steen	Yale U.	grad.	developmental biology
Stanley A. Terman	Brown U.	undergrad.	undecided
Shuhei Yuyama	Florida State U.	grad.	cell physiology

Auditor

D. C. Dumonde	New York U.	M.D.	immunology

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iii More material will be made available than you can learn in the

time allocated - If the pattern of the past is followed, the lights are now burning in this laboratory, not just for the night, but for the summer.

iv We require no formal records of your work, in the sense of a notebook for us to grade etc - But, as good scientists you will, naturally, have good, neat, informative protocols. These should be available to the instructors on demand and, are essential for certification.

C. Conklin Prize
1. Awarded upon selection by staff
2. Award $150.
3. At end of 1st or, perhaps, 2nd period

D. Post-Course research
1. Students selected by staff
2. Period for independent work
3. Formal meetings Fridays after July 31.
4. Hopefully you will develop a problem to pursue under guidance (more or less direct) of some staff member.

IV Library:

A - Outstanding

B - How organized - Charts show each floor

1. Entry - 2nd floor -- Books
2. Reprints - 1st
3. Journals 4, 3, 2.

C. Regulations

1. Identify yourself to Mrs Harlow
2. Journals do not circulate
3. Sign out books & reprints
   Be scrupulously honest!!!
4. Open day & night!

V Recreation -

A. Outstanding Opportunities.

1. Fishing, boating - trips to Nantucket & Marthas vineyard — Picnic or cruise.

B. Show map of the area

C. Dangers -

1. From currents
2. Sunburn - Caution about reflection from water & sand
3. Poison ivy in woods.

Island Queen - at head of Falmouth Harbor
Capt Bullard
200 each - min of 100 person

VI Simpson talk - Questions -

VII Travel vouchers

Iwanami Productions Presents

THE EGG OF ORYZIAS LATIPES

- Its fertilization and development -

Film Data:
35mm Sound Film, E/K color,
2 Reels, 503m, 19min.
English version

Production Credits:

Producer: Teizo Oguchi
Director: Teruo Atumi
Photographer: Kikuo Namiki
Narrator : William Moore

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Script for Narration:

Life ! The mysterious
The most mysterious of all phenomena:-
giving origin to new individuals ... and transmitting it to their offspring ... through reproduction!!

Indeed, reproduction marks a momentous distinction between the living and the-not-living!

Yet, the process, through which a single-celled egg develops into an individual living creature, is shrouded with the mysteries of life.

For many centuries, scientists have tried to unravel these mysteries by studying numerous forms of life. They have made many valuable discoveries concerning birth of lower animals. But, as for the highly evolved vertebrates, which include mankind, their efforts have met such tremendous obstacles as the opaqueness of eggs, thus it has long been almost impossible to make embryological studies of living beings.

The tiny fresh water fish Oryzias Latipes, quite common in Japan, Korea and China, spawns eggs which are a transparent as glass beads. The eggs of Oryzias Latipes have been ideal subjects for embryological studies. But the great difficulty of artificially fertilizing these eggs has limited their usefulness. Recently, however, Dr. Tokio Yamamoto of Japan succeeded in fertilizing Oryzias Latipes eggs in Ringer's solution. This has made possible detailed observation of the entire process of development.

An Oryzias Latipes egg is about one-point-three milimeters in diameter. Under reflected light, the unfertilized egg looks quite transparent. But transmitted light discloses numerous round speckles all over the surface.
These are the oil globules and the surface vesicles lying between the membrane and the yolk.

The larger dark drops are oil globules, which serve as nutrients during later stages of development, and the smaller drops, lighter in color, are cortical alveoli, which help bring about a vast change in the protoplasm right after fertilization.

There is only one micropyle in the Oryzias Latipes egg through which a sperm cell, or spermatozoon, penetrates.

The spermatoza of Oryzias Latipes look like tadpoles. They vigorously move about by wagging their extremely long tails. The size of the spermatozoon is about three-one-thousandths of a milimeter and of course invisible to the naked eye.

Let's observe the fertilization of the Oryzias Latipes egg in Ringer's solution.

The rapid-motion picture reveals, through the microscope, the movement of the spermatozoa and the changes in the egg following fertilization.

The sperm cells placed near the egg with a syringe briskly move about the egg. Only the first to enter the micropyle will effect union with the egg. And this union, namely fertilization, takes place instantly.

One of the sperm cells makes its way into the micropyle. Another one follows, but the moment the first one penetrates the egg, the micropyle closes, and the sperm cell is shut out.

The rapid closure of the micropyle is due to the quick change brought about in the egg protoplasm by the first spermatozoon.

Following fertilization, the cortical alveoli disappear beginning with those nearest to the micropyle. And such a change in the protoplasm spreads over the egg in a wave-like motion within two or three minutes.

The breakdown of the cortical alveoli creates a space under the chorion, and the chorion hardens as it expands.

Then the oil globules remaining near the surface fuse and move downward while the protoplasm gathers near the micropyle at the upper end nick forms the blastodisc.

As the blastodisc takes shape, a powerful surging motion is created, and the protoplasm bulges out.

The first cleavage, or cell division, now takes place. The life separated into the egg and the spermatozoon for the time being has united, and the egg has now been installed with the vital power.

The blastodisc continues dividing into numerous cells.

The blastodisc shows a violent motion before cleavage.

Probably the higher mammals behave in more or less the same way in the earliest stages of development. The blastodisc gradually spreads over the yolk.

About 17 hours have passed after fertilization.

Twenty-six hours have elapsed. The blastodisc has now covered three quarters of the embryo, and a rhythmic movement has begun. Nothing definite is known as to the purpose of this movement. But it is possible that the movement of the yolk makes it easier for the embryo to absorb its nutrients.

The rhythmic movement is arrested by anaesthetics, the rapid motion camera keeps on showing the development within the egg.

Thirty hours after fertilization. The cells of the blastodisc divide steadily, and they are now surrounding the entire surface of the embryo. Some of these cells have gathered at one part of the blastodisc in two parallel lines.

These lines indicate the border of the neural tube, which eventually develops into the brain and the spinal cord.

The top end of the neural tube bulges into two directions to become the eyes.

The earliest differentiation of the organs in all the vertebrates follow the same pattern as in the Oryzias Latipes--that is, first the nerves and then the eyes.

Before the contour of the body has been perfected down to the tail, a system looking like rows of bricks has appeared along the two sides of the neural tube.

This system, called the "Somite", eventually become the muscles. By this time, the notochord has been laid between the rows of bricks, which serves as a support untill the backbone appears.

Forty hours after fertilization. The contour of the whole body has almost been completed. The circular tissue called "Kupffer's follicle", found at the end of the trunk, is now disappearing.

What Kupffer's follicle does cannot yet be explained.

Although most of the organs have not yet been completed, the eyes are making remarkable progress. The eyes have become rounder and indented.

The lenses of the eyes are not made up of the bulging cells but of the epiderm is which swells and falls into the indented parts.

The various organs do not develop simultaneously. At one period, it's the eyes that grow, at another the brain develops. The space between the eyes is the developing Brain.

Increasing pigment makes the eyes look darker.

Four days have elapsed, and the body has almost been copleted. The blood circulation is active, and the embryo now looks quite like a fish. But, since when, has its little heart been beating?

We will now turn the clock back and examine what was going on fifty hours after fertilization, when the outline of the eyes was more or less taking shape.

The heart at that time was merely a small tube. It was beating very faintly, but no blood was running through it since the blood vessels were not yet there.

Fifty-six hours after fertilization. The cells near the heart underwent a change, and began to form the blood vessels. Blood corpuscles were rapidly increasing in this region, and suddenly the blood began flowing into the heart, and circulation began. In the earlier stages of development, nutrients were directly absorbed through the cells from the yolk. But, now that the blood circulation has begun, the nutrients are carried, chiefly through the blood vessels to the various organs.

The powerful beating of the heart!
The heart pumps the blood through the body, and the development of new organs is accelerated.

Five days have passed. The embryo has grown so large as to coil within the chorion.
The eyes are now quite dark, and the characteristic body color and speckles are conspicuous.

By the seventh or eighth day, the body is almost completed with all the internal organs ready to function.

The tenth day. The embryo is now moving briskly within the chorion.

The eleventh day. The embryo hatches out of the shell, and a new Oryzias Latipes has been born!
The fry still carries a sac containing the yolk and the oil blobules, from which to absorb nutrients.

The young Oryzias Latipes, barely the size of a pin-head, will be an adult fish in three months and begin spawning.

We have observed how a single-celled egg develops into an individual creature through intricate processes. But in each of these processes lie profound mysteries yet to be solved.

The eggs of these tiny fish serve extensively in assisting man to probe these mysteries of life!

- The End -

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Laboratory Unit I

THE DEVELOPMENT OF THE TELEOST

I. INTRODUCTION

A. Purpose

This section has as its purposes: 1) to familiarize you with the facts of Teleost development through direct, personal experience in the handling of living material, 2) to give you new tools for the solution of developmental problems. True, the "facts" and many experimental analyses are available in a plethora of publications; many of these will be covered in lectures on the subject. Reading and listening, however, do not substitute for the insights which derive from working with the embryonic material.

Because of the abundant literature on Teleostean development, this guide provides essentially only an outline of laboratory procedures and experiments. It is important, therefore, that you prepare for work on the Teleosts, first by reading this guide carefully, and then by consulting the reference material which is displayed in the laboratory, listed in the Bibliography, and shelved in the library. Plan your work schedule,based on this preparation, with the view to keeping several observations and experiments proceeding with the greatest economy of time and material.

B. Materials needed in the laboratory

In addition to microscopes and the usual items of laboratory glassware, you will need the following:

1) hair-loops (make in laboratory)
2) watchmakers' forceps (supply department)
3) depression slides (available in laboratory)
4) cover-slips (supply department)

C. Characteristics of the living material

All of the laboratory work will be carried out on the minnow, Fundulus heteroclitus. Adult Fundulus are seined from brackish estuaries and brought to the laboratory in mixed lots of males and females. The sexes are stored separatly in tanks of running sea water until used for stripping eggs and milt.

The mature female 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.

Adult males are usually smaller than females, dull, dark-green, with ill-defined silvery transverse bars or spots; the dorasl fin has black pigment arranged in a mottled pattern.

II. GENERAL DIRECTIONS FOR OBSERVATIONS AND EXPERIMENTS ON THE DEVELOPMENT OF *FUNDULUS* EMBRYOS

A. Records

No particular form is specified for the records that you will keep of your observations. It will, nevertheless, be expected that you keep detailed protocols for all of your laboratory operations. It is particularly important that you record carefully the conditions under which the experiments and observations were carried out and that you keep an accurate time-table of the chief developmental events. As best you can, rear normal and experimental embryos under controlled conditions of temperature.

B. Fertilization and culture of normal embryos

1. Procuring gametes

Both eggs and sperm are procured by stripping. Hold the fish in the left hand, belly-side upwards, anterior and facing your wrist. Gentle pressure is then applied to the abdomen using the thumb and forefinger of the right hand; as the fingers are pushed from thorax to cloaca, the pressure forces out the gametes. When the female is viewed against the light while stripping, the eggs may be seen as they pass through the oviduct which runs along the anal fin.

If the female is ripe, the eggs strip easily, regardless of the size of the fish. Large males are often spent when brought into the laboratory and smaller ones are usually the better source of milt. The milt is expressed as a small issue of milky fluid from the cloaca. Do not confuse milt with whitish fecal material or with the bladder discharge which may accompany stripping.

2. Setting up embryo cultures

Strip eggs into a clean finger bowl containing sea water about 2 mm deep. Immediately strip milt into the egg suspension, immersing the vent of the stripped male in the sea water to assure washing the sperm into the suspension. Alternatively you may remove the milt with a pipette and add it to the egg suspension.

Allow the preparation to stand for 10 to 15 minutes, then wash the eggs in fresh sea water, leaving them in 1/4 to 1/2 inch of water, well separated in several finger bowls. Cover with a glass plate and keep on the sea table or in a constant-temperature device. Label each culture with the time of fertilization. Change sea water daily.

C. Methods of observing development

Gross morphological changes during development are best observed with eggs in Syracuse dishes or Stender dishes of sea water. They should be transferred by pipette from the culture dish to the observation vessel and examined at various magnifications of the dissecting microscope using both transmitted and reflected light, alone or in combination, as necessary to reveal structure.

Coarse fibers of the chorion should be removed by rolling the eggs on filter paper or toweling, leaving the chorion smooth and clean. This permits relatively unobstructed observation of the embryo.

Detailed observations, particularly of the micropyle, cortical granules, periblast nuclei and like features are best made on eggs held in a depression slide (1.7 or 1.8 mm depression) with sea water and cover slip. By manipulating the cover slip against the egg surface the latter may be turned to a suitable position for viewing. Transmitted light is usually adequate, but frequently one finds it useful to use reflected light alone or in combination with transmitted light.

III. HIGH LIGHTS OF NORMAL DEVELOPMENT

A. The timetable

Although there follow a number of specific observations and experimental projects which you are expected to carry out, you should perform the latter, as possible, along with a careful record of the progress of normal development as a function of time and temperature. Familiarize yourself with the normal table of developmental stages in Fundulus, as outlined by Oppenheimer (1936), during the progress of this work.

Strip eggs and milt as described above, noting the time of fertilization. Divide the fertilized eggs among several finger bowls, storing some at 20°C and others at room temperature or on the sea table. Withdraw samples for observations periodically during the period from fertilization to hatching, but give chief attention to events of the first 36 hours of development.

On cross section paper, plot developmental stage on the ordinate against time on the abscissa for normal development at 20°C.

B. Developmental events deserving of special attention

1. The structure of the unfertilized egg

Strip eggs into a Syracuse dish of fresh sea water and observe immediately. Under the dissecting microscope. Note: 1) the fibrous, tightly adherent chorion; 2) the clear yolk; 3) the oil droplets. Can you distinguish animal and vegetal poles? Is there any clear segregation of protoplasm?

Transfer freshly stripped eggs to a depression slide on the stage of the compound microscope. Add a cover slip and, using it, rotate the egg until you locate the funnel-shaped micropyle through the 10 mm objective. This marks the animal pole; it is the only point of sperm entry and the accumulation of the protoplasmic blastodisc occurs in the egg at this point.

Using the 4 mm objective of the compound microscope, and by means of careful focussing and manipulation of the light, distinguish the cortical layer of protoplasm by means of its characteristic cortical granules. These granules, or vesicles, are of a range of sizes, though all are quite small. Rotate the egg and determine whether their distribution in the cortex is uniform with respect to the animal-vegetal axis.

2. The structure of the sperm

Place a drop of diluted milt on a glass slide and add a cover slip. Observe the movement and structure of the sperm using the 4 mm and 2 mm (oil immersion) lenses of the compound microscope. What characteristic structures can you observe? Acrosome? Head? Mid-piece? Tail? Sketch the sperm. If you like, take your preparation to the phase-contrast microscope which will be set up in the laboratory.

3. Fertilization

a. Gross changes

The most obvious events which accompany the fertilization reaction are: 1) elevation of the chorion with consequent formation of a perivitelline space; and 2) the elevation of the blastodisc. These are signs that the egg has been activated, but do not necessarily foreshadow cleavage.

Does the perivitelline space arise simultaneously at all points around the egg surface? What is the limiting membrane of the egg now called? Does a change in the volume of the yolk accompany elevation of the chorion?

After fertilization, clear protoplasm flows to the animal pole from cortical as well as central regions of the yolk forming a hillock, the blastodisc. When is the onset of blastodisc formation first recognized? How long is required for all the protoplasm to be accumulated in it? Is it completely formed prior to first cleavage?

These changes are best observed in time-lapse motion pictures. Hopfeully we shall have available an excellent film by Roosen-Runge depicting the events which follow upon fertilization.

b. More refined observations

According to Huver (1960) the first polar body, very small and transparent, is almost indistinguishable prior to fertilization, but may sometimes be seen projecting into the perivitelline space at the bottom of the micropyle. The second polar body emerges about 5 minutes after fertilization.

Position a freshly stripped egg in a depression slide under the compound microscope with the micropyle in profile. See if you can find a polar body; it probably will not be distinguishable from the cortical granules.

By means of a fine pipette, introduce a small amount of diluted milt near the mouth of the micropyle. Observe the entrance of the sperm into the micropyle and the penetration of the sperm into the egg. The fish egg is not especially favorable material in which to observe sperm penetration, but with persistance and care you will see the process.

Immediately upon sperm entrance you may observe the breakdown of the cortical alveoli, the elevation of the chorion and, with extreme care (and a good deal of luck) the emergence of the second polocyte.

4. Cleavage and blastula formation

After fertilization, the blastodisc or germ disc accumulates at the animal pole and the first cleavage furrow appears within 2 or 3 hours (depending on temperature). Do the cleavage planes divide the whole ovum? The whole blastodisc? Follow carefully the time sequence and geometric relationships of the cleavage furrows through the 32-cell stage. Are there irregularities? Draw and then segregate eggs showing aberrant cleavage patterns to determine effects, if any, on subsequent morphogenesis.

Note the form of the blastula in profile during the cleavage stages. What does one mean by the high blastula? A flat blastula is formed after the period of the high blastula.

During the periods of the high and the low blastula, examine carefully the protoplasm around the margins of the blastodisc. "Circular" cleavages cut off nuclei into this protoplasmic zone, called the periblast, and these nuclei are visible under favorable conditions of illumination.

The periblast nuclei constitute a syncytium which is ultimately continuous beneath the blastodisc (see Wilson, 1891). The syncytial areas at the periphery of the blastodisc is called the marginal periblast; that beneath the disc, the central periblast (the latter is a contradiction in terms!). The marginal periblast precedes the blastoderm as it progressively envelops the yolk during gastrulation.

5. Gastrulation and embryo formation

The details of gastrulation are difficult to observe without the aid of histological sections but, with reasonable care and the exercise of a bit of ingenuity you may observe the essential features in the living material using both the dissecting and compound microscopes. A solution of Nile Blue or Neutral Red (1:20,000 in distilled water; filter it!) will stain the blastoderm and enable you to distinguish thickenings and foldings which otherwise would be seen with great difficulty. Will embryos survive and hatch after treatment with these dyes?

As the flat blastula forms, the edges of the blastoderm thicken and turn under at the margins forming the germ ring. The germ ring is most prominent posteriorly and it is here that the embryonic shield, the source of the embryo body proper originates. The rest of the blastoderm essentially forms extra-embryonic membranes of the yolk sac.

Concomitant with the peripheral inturning of the epiblast is its central expansion and the posterior elongation of the shield. The entire blastoderm thus enlarges and progressively envelops the yolk. Overgrowth of the yolk is called epiboly, and we distinguish epiboly of the germ ring from epiboly of the periblast; the latter preceding the germ ring in advancement over the yolk. The mechanics of epiboly have been considered by Lewis ('49), Devillers (1951) and Trinkaus (1951). Read the papers of these authors for a description and experimental analysis.

Although the precise facts are in some dispute, most investigators indicate that endoderm and mesoderm are formed by invagination of cells at the posterior edge of the embryonic shield commencing with the beginning of gastrulation. These matters will be reviewed in your lectures and should stimulate you to read, especially, the accounts given by Sumner, Wilson and Pasteels.

While the blastoderm is spreading over the yolk, the embryonic axis is established by the posterior elongation of the embryonic shield. By the time the blastoderm has moved half-way over the yolk, the embryonic shield has become a bluntly triangular area extending from the margin to the center of the blastoderm. To see the shield, the germ should be studied carefully from all angles, particularly in profile view. Make a series of drawings showing the shield and embryonic body in profile when the yolk is 1/3, 1/2, 2/3 and 3/4 covered.

With careful observation you will distinguish the solid keel, which is evident initially at the anterior end of the shield and which forms the initially solid neural tube and notochord. As development progresses, the notochord can be distinguished in optical section of the embryo as viewed in profile.

The somites become visible before the yolk is enveloped. When do they first appear? How many of them are there at the time epiboly is completed? What is the rate of formation of somite pairs?

What is the relationship between the rate of formation of the embryonic body proper and the rate of epiboly in Fundulus? Does the end-bud of the embryo form prior to closure of the blastopore? What is morphogenetic significance of the end-bud? Contrast these features in Fundulus and in Salmo (trout).

6. Later development

The analysis of later development is facilitated by the ease with which the embryo may be dechorionated once epiboly is completed (Of course, for those willing and able to develop the technique, dechorionation may be carried out as early as the 1-cell stage, thus making all observations easier!).

a) Nervous system: Trace the development of the central nervous system, sketching the appearance of the optic vesicle, lens, and brain parts. Carefully observe formation of the olfactory and auditory pits. Histological details of the origin of sensory structures will be found in a paper by Jones (1939).

b) Circulatory system: Note the formation of the heart from paired primordia and the onset of its pulsations. How early can you distinguish the pericardium from adjoining areas of the yolk sac? (See Stockard, '05, '15, on the origin and development of mesenchyme and vascular epithelium.)

c) Pigment cells: The origin of the pigment cells will be of especial interest. They usually appear first in beautiful patterns along the walls of blood vessels in the yolk-sac. What kinds of pigment cells can you recognize? Which appear first? What is their origin? Can they be recognized before they begin to synthesize their characteristic pigment?

d) Appendages: Note the appearance of the paired and unpaired fins and follow their morphogenesis.

IV. EXPERIMENTS ALL SHOULD PERFORM

In what follows, a number of questions are asked, for which answers are to be sought by means of simple experiments. Set up the experiments in such a manner that critical evidence and unequivocal answers may be obtained.

A. For what period of time after shedding are eggs of Fundulus capable of being fertilized and undergoing cleavage?

1. Make this determination at 10° and at 20°C using significant numbers of eggs from a single female.

2. Repeat for several females.

3. Plot percent development on the ordinate versus time after shedding when fertilized on the abscissa.

B. Will eggs of Fundulus show spontaneous activation upon exposure to sea water?

1. The criteria of spontaneous activation are: disappearance of cortical granules, membrane elevation, formation of elevated blastodisc.

2. If these events occur without fertilization, in what order do they occur?

3. If activation occurs without fertilization, plot percent activation on the ordinate as a function of time after stripping on the abscissa.

4. Is there a correlation between rate of loss of fertilizability and rate of spontaneous activation?

C. Can eggs of Fundulus be activated artificially by pricking?

1. Prick eggs with a sharp glass needle while observing cortical granules under the compound microscope. Does the chorion elevate? The blastodisc form?

D. What is the effect of calcium-free sea water on the activation reaction?

1. As artificially produced?

2. As spontaneously occurring?

E. What is the effect of dechorionation on the rate of development in Fundulus?

1. Dechorionate eggs at as early a developmental stage as possible, rearing them in sea water along with non-dechorionated eggs from the same clutch.

2. On the same chart, plot average stage of development on the ordinate versus time on the abscissa for each group of eggs.

V. SOME SUGGESTIONS FOR INDIVIDUAL RESEARCH PROJECTS

A. The biosynthesis of melanin pigments

In amphibians, birds and mammals the amino acid, L-tyrosine, has been shown to serve as substrate for the enzymatic synthesis of the insoluble pigment, melanin, which characterizes certain of the pigment cells, the melanocytes. In various members of these major groups, metabolic analogs of tyrosine and phenylalanine have been shown to inhibit the synthesis, and even the differentiation, of melanocytes.

1. Determine the effects of L-tyrosine, L-phenylalanine, L-s-amino-tyrosine and B-2-thienylalanine on the origin of melanocytes in Fundulus and the initiation of their pigment biosynthesis.

2. (You must, necessarily, determine the tolerance of Fundulus embryos to a range of concentrations of the metabolites and antimetabolites and be concerned with the time of onset and duration of the treatment.)

3. Are any effects of the antimetabolites reversible with the normal metabolites?

B. For a number of higher vertebrates, dissociated embryonic cells have been caused to reaggregate by centrifugation and have then undergone rather characteristic organotypic and histiotypic differentiation.

1. Determine whether and at what stages you can obtain visible cells of Fundulus by versene disaggregation.

2. Reassemble the cells by centrifugation and test for histiotypic and organotypic differentiation in vitro using the culture method of Trinkaus.

C. Regeneration of the lens from the dorsal portion of the iris has been extensively studied in the Amphibia and little attention has been directed to this problem in Teleosts.

1. Rear Fundulus embryos through hatching and determine their tolerance to chloretone anaesthesia.

2. With appropriately designed micro-instruments remove the lens of one side, keeping the other as control.

3. Fix the fry at intervals over a period of weeks after operation. Section and stain experimental eyes to observe for signs of lens regeneration.

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Isolation of *Fundulus* Hatching Enzyme

Adapted from his Ph.D. thesis

by

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BACKGROUND

THE HATCHING PROCESS

Throughout its early development the fish embryo is encased within a thick, tough membrane, the chorion, which serves as a protective shell. During this time the embryo uses its large supply of yolk as a source of energy and structural materials. Shortly before hatching, the larva secretes an enzyme from cells lining its oral cavity and gills. This secretion digests the inner layer of the chorion, leaving only the thin fragile outer layer. Finally, the fry escapes from the thin remaining envelope by lashlike motions of its tail.

A. The Teleost Chorion

1. Terminology

The chorion has been variously defined in terms of the cells which are presumed to give rise to it (Needham, 1950; Wilson, 1949). In Fundulus, Kemp (1958) considers that the "chorion internum" is the transformed vitelline membrane or zona radiata of the oocyte while the radial fibrous strands and jelly ("chorion externum") are synthesized by the follicle cells. Although several other terms (capsule, egg casing, zona radiata) have been used in the literature, the term chorion occurs most frequently.

The term chorion will be used in a purely descriptive sense to designate the teleost egg casing without the jelly and is the "chorion internum" of Kemp (1958).

2. Structure of the chorion

Two kinds of structure have been described with minor modifications in different species. The first type, possessing a series of two or more concentric layers has been reported in Fundulus and Oryzias; the other type, in the salmonids (salmon and trout), appears to be composed of one thick layer. It is not known how these morphological characteristics which have been observed microscopically are related to the physical and chemical properties of the intact chorion.

Observations of the egg membranes of fish have been reported as early as 1854 by Lereboullet. Bogucki (1930) published a sketch of the trout chorion which he described as "fibrous". The brown trout chorion was found to be 90 μ thick by Gray (1932). The zebra fish chorion (Brachydanio rerio), studied microscopically by Hisaoka (1958), has been described as a "tough, fibrous membrane" 10 μ thick containing numerous small openings or "pores" 1.5 μ in diameter. Thin sections of Fundulus chorion which had been hardened by exposure to sea water and fixed in osmic acid revealed four somewhat compressed osmiophilic layers (Kemp, 1958). Shanklin (1959) describes it as a tough, transparent, resilient protein membrane 12 μ thick, composed of an outer layer 7 to 8 μ thick containing 10,000 to 20,000 pores per square millimeter which were 1.5 μ in diameter, and an inner layer of densely packed concentric fibers.

Although most of the authors describe chorions of various fish eggs as being penetrated by numerous "pores", the only evidence for this other than microscopic appearance is an observation made by Allen

(1932). He found that droplets of liquid appeared on the previously dried surface of the chorion of the salmon egg when the latter was squeezed.

3. Changes in the chorion upon fertilization

When the egg is extruded from the oviduct the chorion undergoes a change in its chemical and physical properties. The coelomic trout chorion is fragile, flexible, transparent and inelastic. After a period in water that varies with different organisms, the chorion becomes opaque and more elastic. The coelomic chorion will dissolve in 1% NaOH before, but not after, water hardening (Bogucki, 1930).

Although at first it was thought that the breakdown of cortical alveoli, extrusion of colloidal material and elevation of the fertilization membrane were due to fertilization (Kerr, 1900), subsequent studies have shown them to be independent of this event (Yamamoto, 1954). When mature ovarian eggs of the medaka, Oryzias latipes, were isolated in isotonic saline only about 5% underwent spontaneous "cortical activation" as follows: the cortical alveoli (10-40 μ spheres in the cortical cytoplasm) broke down in a wavelike process extending from the animal to the vegetal pole. "Colloidal" material was extruded under the membrane from these alveoli. Elevation of the "fertilization membrane" which becomes the chorion is probably due to imbibition of water by the colloid through the chorion which is permeable to water and small electrolytes but not to colloids. This idea was originally proposed by Bogucki in 1930 who found that swelling of the activated egg could be inhibited by hypertonic saline.

The ovarian eggs can be activated by means other than fertilization, viz., pricking with a glass needle, treatment with surface active agents, e.g., sodium oleate or saponin (Yamamoto, 1954). Calcium appears to be necessary for this process.

The cortical alveoli appear to contain a mucopolysaccharide, although the evidence is far from conclusive. Kusa (1954) reported that cortical alveoli of salmon (Oncorhynchus keta & O. nerka) could be dissected out in Ringer's solution. In Bouin fixed eggs the alveoli gave the periodic acid-Schiff and the ninhydrin-Schiff reactions, stained with basic dyes, showed metachromasia which was not removed by hyaluronidase and reacted with the acid component of Mallory's Triple stain. On the basis of this data, he stated that they were composed of a mucopolysaccharide which possibly contained an amino sugar.

Nakano (1956) described a method for isolating medaka cortical alveoli. They remained intact in isotonic saline but burst and dissolved in water. They were reported to give positive Dische, Molisch and naphthoresorcinol tests for carbohydrates. The material was precipitated by Janus Green and by 50% saturated ammonium sulfate. This precipitate, when dissolved in saline, was said to produce a "contraction" of the chorion. This effect could also be ellicited by gum arabic, gum tragacanth, albumin and gelatin dissolved in Ringer's solution. He states that the chorion is 3.2% "sugar" (method or nature of standard not given). Since no quantitative data are given it is difficult to evaluate this work. No protein analysis was reported. Furthermore, it was not stated whether the changes which take place during "water hardening" of the chorion require the presence of the egg. In other words, can the isolated coelomic chorion become hardened by the action of water alone? If this is not the case then the term

"water hardening" is misleading; if it is the case then a study of the role of the mucopolysaccharide would seem to be superfluous. In any event the chorions of Fundulus and two trout species contain carbohydrate.

In Fundulus, the cortical reaction, i.e., the breakdown of the alveoli, the polar migration of the cytoplasm to form the polar cap and elevation of the chorion to form the perivitelline space, takes place on transfer to sea water. This process requires about an hour and is accompanied by hardening of the chorion. In the trout, complete hardening, as measured by the weight required to break an egg (Hayes, 1949), takes about thirty hours.

In summary, the teleost chorion is composed of fibrous protein, the morphological structure of which varies among different forms. The coelomic chorion undergoes a hardening process on water activation or fertilization which may involve its combination with or "tanning" by a carbohydrate. Carbohydrate containing material is extruded into the perivitelline space at fertilization. The elevation of the chorion is thought to be due to imbibition of water by the alveolar material through the chorion which will pass electrolytes, sugar and water but not protein (Needham, 1950; Prescott, 1955).

4. Chemical studies of the chorion

a. Amino acid analyses

The earliest amino acid analysis of a fish chorion was reported by Pregl (1908) on the "ovokeratin" of the dogfish, Scyllium stellare. He found glycine, alanine, leucine, isoleucine, proline, phenylalanine, aspartic acid, glutamic acid, tyrosine, lysine, arginine, and histidine and commented on the relatively large content of tyrosine (10.6%). Young and Inman (1938) found that salmon (Salmo salar) chorion was partially dissolved after one hour at 100°C in 1% (0.25 N) NaOH or 1% (0.3 N) HCl. Although the authors do not say that this was the result of partial hydrolysis of susceptible peptide bonds, I would like to suggest this possibility. For example, Partridge and Davis (1955) found that aspartic acid was released preferentially from elastin by 0.25 M oxalic acid at 100°C. Similarly, Schulz reported that 0.03 N HCl at 105°C released aspartic acid from ribonuclease (Sanger, 1961). Young and Inman (1938) observed slight digestion with pepsin (pH 2.6, 33°C, 72 hours) but none with trypsin. Arginine, histidine, lysine, tyrosine, tryptophane, cystine, and glucosamine but no hydroxyproline were found in the chorion whose protein component was described as "pseudokeratin". The only study using modern ion exchange methods for the determination of all the common amino acids was carried out on salmon chorion by Young and Smith (1956). These findings will be discussed after presentation of similar data obtained for the Fundulus chorion in this study. These authors also reported that pepsin and papain but not trypsin digested the chorion. Bourdin (1926) concluded that the trout chorion was protein, since it contained 14-15% nitrogen by the Kjeldahl method and gave positive xanthoproteic, Millen and biuret reactions.

b. Studies on the carbohydrate of the chorion

Although it has been claimed that the chorion in salmon (Young and Inman, 1938) and in the medaka (Nakano, 1956) contained a carbohydrate component, the chemical evidence is by no means conclusive. Nakano (1956) did not report the details of the method.

No studies concerning the chemical composition of the Fundulus chorion are available. The report that the cortical alveoli of the trout contain mucopolysaccharide is based solely on histochemical evidence (Kusa, 1954). However, since the alveoli can be isolated a more reliable determination is possible by direct analysis.

Recently, Warren (1960) reported the occurrence of both bound and free sialic acids in homogenized eggs of four common teleosts, brook trout, rainbow trout, shad and perch, but no attempt was made to localize the bound sialic acid in the egg. Sialic acid has also been reported as a component part of structural proteins, e.g., collagen and elastin (Gross, 1957) and in horse erythrocyte stroma it is bound to a mucolipid (ganglioside) (Klenk, 1958). Sialic acids occur in the tissues of a variety of organisms ranging from bacteria to mammals (Gottschalk, 1960; Warren, 1960).

Warren (1960) stated that purified Clostridium perfringens neuraminidase (sialidase) would release the bound sialic acid of trout eggs. One would expect that if neuramidase released sialic acid from the chorion the histochemical method (Spicer and Warren, 1960) could be applied to localize the sialic acid in sections of the chorion.

B. Hatching Enzymes

1. Early studies

Kerr (1900) appears to have been the first to suggest that hatching occurred by an enzymatic mechanism. In anesthesized trout embryos, Wintrebert (1912) observed that the perivitelline fluid became syrupy and could digest the chorion in the absence of any movement by the larva. Bourdin (1926) was the first to demonstrate that the perivitelline fluid of teleosts had proteolytic activity. The fluid was aspirated through the chorion of hatching larva by a micropipette and then mixed with a buffered solution of gelatin. Inhibition of gelation by the fluid was compared with inhibiting action of pancreatin and pepsin. Additional evidence for the role of proteolytic hatching, this time in tunicates, was provided by the interesting study of Berrill (1929). The embryos were allowed to develop normally to tail stages, then transferred to a small volume of water and allowed to hatch. This hatching water, after clarification by filtration and centrifugation, removed the chorion and follicle cell layers from unfertilized eggs within 5 to 50 hours. A heat labile preparation from the frog (Rana pipiens) that could remove the jelly and swell the chorion of early embryonic stages was obtained by allowing a large number of dechorionated embryos to hatch in a small volume of water (Cooper, 1936). After this preparation had been used in an assay, or if it had been obtained from normally hatching larvae, it showed little activity. This may be due to product inhibition which is frequently encountered in the study of enzymes. The hatching process in Fundulus was described by Armstrong (1936) who demonstrated that anesthesized larvae could hatch.

2. Assay of hatching enzymes

Probably the most serious obstacle to the study of the properties and specificities of the hatching enzymes other than the difficulty of obtaining sufficient material for chemical work has been the lack of a reliable, convenient, quantitative assay. Digestion of the natural

substrate, the chorion, liquifaction of gelatin and milk clotting activity have been used.

Hayes (1942) in a study of the salmon enzyme attempted to measure a "rate" by noting the time required for digestion of a hole in 50% of four to six chorion hemispheres isolated from unhatched eggs. The average time required was four hours. The other methods of milk clotting and gelatin liquifaction required even longer periods (five to thirty hours). Obviously, one cannot use these methods to determine a rate. At best they give a rough indication of the relative activities of different preparations. The published methods are summarized in Table I.

3. Properties of hatching enzymes

Studies of hatching enzymes have shown them to be nondialyzable and heat labile (Hayes, 1942; Ishida, 1943; Berrill, 1949). Although some of these authors reported temperature and pH "optima" it must be realized that these values are only approximate. These optima vary considerably with the degree of purity of the enzyme and the substrate, the presence of inhibitors or activators and with the method of assay employed (Neilands and Stumpf, 1955).

4. The nature of hatching enzyme activity

The hatching enzymes which have been studied so far have all been shown to have proteolytic activity, although some workers have contended otherwise (Hayes, 1949). No other type of enzymatic activity has been found in these preparations. In addition to the activity toward gelatin already discussed, several other lines of evidence support the hypothesis that hatching enzymes are proteases. Hatching enzymes from the tunicates, Phallusia mammillata and Ascidiella aspersa clotted milk at pH 8, 30°C in 20 hours (Berrill, 1949). Both the milk clotting and biological activities were destroyed by heating. Diastase and lipase activities were not detected. The enzyme liquified gelatin. The action on gelatin of the hatching enzyme was duplicated by 1% crude trypsin at pH 8, 12°C. Hayes (1949) found that crude salmon and trout hatching enzymes required three times as long to liquify gelatin as to digest a hole in the chorion. Trypsin (1%), on the other hand, liquified gelatin in ten minutes but even after four days showed no activity on the chorion. He questions whether the hatching enzyme is hydrolytic because he could only detect a slight increase in amino nitrogen in the perivitelline fluid prior to hatching and using isolated chorions in vitro he found that only six percent of the total nitrogen was released as free amino groups after incubation with the enzyme. In support of his idea he offers no evidence but invokes as a possible mechanism something analogous to the keratinase system in the clothes moth (Linderstrom-Lang & Duspira, 1935). In this case, wool keratin is digested by the combined action of a reducing substance (possibly cysteine) which reduces disulfide cross links in the keratin, and an ordinary protease. An alternative explanation of his observations would be only a small number of peptide bonds would have to be split in order to dissolve the protein. Furthermore, Smith (1947) reports a marked increase in amino nitrogen in the perivitelline fluid before hatching. Milkman (1955) claims that the Fundulus enzyme is proteolytic but presents no evidence in support of this. He could demonstrate no milk clotting activity.

It has been claimed recently that in the hatching glands of Kuban fish embryo two types of granule are formed containing hatching enzyme and hyaluronidase respectively (Baburina & Buznikov, 1957). The hatching enzyme was assayed by the Hayes method; the hyaluronidase was assayed viscometrically. Hyaluronidase could not be detected in loach (Misgurnus fossilis) perivitelline fluid. The loach enzyme is inhibited by a 0.25% ovomucoid trypsin inhibitor and not affected by sulfhydryl poisons (Buznikov, 1959). In the carp he found that hyaluronidase activity could be inhibited by 0.001% trypan blue. When hatching took place in the presence of trypan blue 0.0025%, it began earlier and took longer than in the controls. Since this experiment was performed in vivo the role of hyaluronidase in digesting the chorion was not clarified.

A substance with anti-proteolytic activity which appears shortly before hatching and disappears sometime thereafter in fish and frog embryos was isolated and partially purified by ethanol fractionation (Wu and Wang, 1948). The inhibitor was assayed by its ability to prevent release of red color by the action of pancreatin on congo-red dyed fibrin. The authors felt that the substance was formed in the cutaneous mucous glands and acted to protect the embryo from the action of its hatching enzyme.

In summary, then, the following lines of evidence support the hypothesis that hatching enzymes are proteases:

1. Digestion of gelatin
2. Milk clotting activity
3. Release of amino nitrogen during hatching
4. Inhibition by ovomucoid trypsin inhibitor
5. The appearance of a protease inhibitor just before hatching

There is no evidence that any other type of enzymatic activity is involved.

C. Secretion of the Teleost Hatching Enzymes

Armstrong (1936) reported that if Fundulus embryos were treated with ethanol their mouths could not open. These embryos could not hatch. Histological studies showed cells in the mouth and pharynx which contained eosinophilic granules. These disappeared after hatching. In the medaka (Ishida, 1943), granular structures are found in the mouth and gills which break down before hatching. When these were isolated and placed in water they burst giving a preparation which digested chorions. Ignativa (1960) dissected hatching glands of the sturgeon in Holtfreter's solution. These glands, cultured in vitro, released active enzyme.

A method for inhibiting hatching in Fundulus was described by Milkman (1954; 1955). Three day embryos were sealed in a flask of oxygen-saturated sea water and allowed to develop for three days after the controls had hatched. Transfer of the embryos to fresh sea water initiated hatching within a few minutes. He described the following sequence of events. First the mouth began to open and close rapidly and a granular, milky secretion appeared in the perivitelline fluid.

The yolk sac and the larva began to somersault or spin around in the chorion. After about eight minutes hatching began. Cultures of embryos treated in this way hatch at approximately the same time. My procedure for collection of enzyme is based on Milkman's method. Since work reported by Ishida (1943, abstract in Japanese) considered the question of inhibition of hatching on which the Milkman method depends, Ishida's work will be summarized here. Development of the hatching glands of the medaka was studied by histological methods. On about the third day of development, cells appear in the future gill region which contain small granules. These cells move forward with the formation of the intestine. The granules have no enzymatic activity until the mouth has completely formed. From the seventh to the tenth day these cells with their granules increase in size. Just before hatching, the nuclei of the secretory cells disappear. Rapid gill activity precedes hatching (this is true in Fundulus) and it is thought that the collapse of the gland cells is triggered by the increased pressure of water across the gills. Respiration increases greatly just before hatching as a result of gill activity. Stimulation of hatching is observed in 0.001 M HCN. Ishida feels this is due to a deficiency of oxygen caused by decreasing the heart rate. This would, in turn, result in an increase in carbon dioxide tension in the blood stimulating gill movement which then would initiate hatching. If this is so, a high oxygen tension should tend to suppress gill movement and inhibit hatching. Milkman's procedure seems to be based on this principle although he does not mention it. In addition to Ishida's abstract, a Russian worker (Buznikov, 1959) reported that reduction in oxygen tension stimulated secretion of the loach hatching enzyme.

TABLE I

Hatching Enzyme Assay Methods

Organism	Assay Method	Reference
trout, perch	Inhibition of gelation Digestion of chorion fragments	Bourdin, 1926
tunicates (Phallusia & Ascidiella sp.)	Gelatin liquifaction Milk clotting activity Removal of chorion from unfertilized eggs	Berrill, 1929
frog (R. pipiens)	Removal of jelly layer and swelling of chorion of early stages	Cooper, 1936
salmon	Time to make a hole in isolated chorion	Hayes, 1942
medaka	Digestion of chorion fragments	Ishida, 1943
Kuban fish	Time to make a hole in isolated chorion	Barburina & Buznikov, 1957
Fundulus	Digestion of unhatched chorions after abrasion of outer layer	Milkman, 1955

SUMMARY OF KAIGHN'S FINDINGS

1. Most of the proteolytic enzymes tested did not attack the chorion. Although pepsin and papain digested the chorion, they did so slowly and under non-physiological conditions.

2. Microscopic studies revealed that the Fundulus chorion is about 7 μ thick and is composed of 4 or 5 alternating birefringent and non-birefringent layers. The trout chorions are non-layered and about 20 μ thick.

3. Ninety five percent of the nitrogen of the chorion was accounted for in the amino acids of an acid hydrolysate. The content of proline, amide nitrogen and aliphatic amino acids was found to be relatively high while that of cystine, aromatic amino acids and those with ionic side chains was low. The overall amino acid composition of the Fundulus chorion resembles that of the salmon chorion more than any other structural protein. Only traces of N-terminal amino acids could be detected by the FDNB method.

4. Three carbohydrate components were found in the chorion: a non-amino sugar (1.4%), hexosamine (1%) and a sialic acid (1.5%).

5. Preliminary fractionation of papain digests of the chorion by ethanol precipitation, changes in pH and gel filtration suggested that the carbohydrate moiety was firmly bound to the protein.

6. From their solubility in cuprammonium hydroxide, it was inferred that Fundulus chorions were stabilized for the most part by strong hydrogen bonding. Disulfide bonding was ruled out.

7. The hatching enzyme (chorionase) of Fundulus was partially purified by dialysis, gel-filtration and sucrose density gradient ultracentrifugation. Its molecular weight, on the basis of sedimentation behavior, was found to lie between 15,000 and 40,000.

8. Assay methods for the chorionase were investigated. The chorionase hydrolyzed powdered chorions releasing soluble ninhydrin-positive and OD_{280} material. It also hydrolyzed congo red-dyed elastin and B-chain of oxidized insulin. These methods were unsatisfactory because of the slow rate of hydrolysis and the large amount of substrate required. A sensitive, reliable, semiquantitative assay was described using tiny chorion fragments.

9. Chorionase appeared to act within the ionic strength range $\mu = 0.4 - 0.40$. Lower activity was observed above and below this range. Further evidence ruled out a requirement for any particular cation or anion. However, the enzyme tolerated greater variations of ionic strength in sea water than in buffers.

10. The B-chain of oxidized insulin was partially hydrolyzed by the chorionase. Two of the points of cleavage were the peptide bonds of Tyr-Thr and Thr-Pro.

11. Evidence was presented which showed that chorionase binds DFP^{32}. The pattern of radioactive spots obtained by partial hydrolysis and ionophoresis of the DIP^{32} enzyme differed from that of chymotrypsin and elastase. The active site probably contains threonine in contrast to the serine found in other proteolytic enzymes. The fact that DFP inhibited the activities of chorionase against the chorion, elastin and B-chain and the fact that heated chorionase did not bind DFP strongly suggest that the active site was involved in DFP binding and that a valid comparison with elastase and chymotrypsin fingerprints can be made.

12. The evidence presented proves that chorionase has proteolytic activity; however, other actions, e.g., lipolytic or mucolytic, were not ruled out.

13. Fundulus chorionase digested Fundulus chorions and also chorions from two trout species, but rainbow trout enzyme could only digest trout chorions.

Isolation Of Fundulus Hatching Enzyme

1. Fundulus eggs

Eggs are stripped into finger bowls moistened with sea water (SW). As soon as possible, milt (sperm) is added and thoroughly mixed with the eggs. After five minutes sufficient SW to cover the eggs is added. The SW is poured off after 15 minutes, the eggs are washed with SW to remove excess milt and the bowl is half filled with SW and covered with a glass plate. The jelly coat is removed by rolling the eggs on paper towels after the chorions have hardened. Undeveloped eggs are removed and the viable eggs transferred to fresh SW (50 to 100 per bowl) which is replaced daily and allowed to develop at room temperature.

2. Collection and purification of chorions

The eggs are washed with SW and forced through a 5 cc hypodermic syringe. The shearing force developed during passage through the opening, which should be just slightly larger than the egg is sufficient to rupture the chorion and at the same time homogenize the embryo. The hollow chorions are washed five times by filtration and decantation with SW until no trace of tissue can be detected on them. They are then extracted with 1% NaCl at pH 8 for several days in the cold with mechanical stirring and daily solvent changes. Washing is repeated with distilled water for several days. Finally, they are rinsed in 95% EtOH, absolute EtOH, ether to remove unbound lipid, and dried *in vacuo* over P_2O_5 and KOH. They are stored in the dessicator.

3. Collection of crude enzyme

The hatching of cultures of three-day embryos is synchronized by the procedure described by Milkman (1954). Apparently, the process of sealing up the embryos in oxygen saturated SW, as described below, in some manner inhibits hatching in the more rapidly developing embryos in a given culture and allows the slower ones to catch up. This inhibition is released upon transfer to fresh SW and hatching begins almost immediately.

There are two advantages to this method of enzyme collection. First, since all eggs hatch within a short time interval, the enzyme does not remain at room temperature for more than 30 minutes. This minimizes autolysis and denaturation. Second, the number of embryos that can hatch by this method in a given volume of 10% SW is far greater than the number which can develop in the same volume otherwise. This permits a much more concentrated crude enzyme to be obtained. Another advantage is the lower salt concentration (for 10% SW $\mu = 0.07$). The method has a disadvantage in that crude hatching water contains a high concentration of dissolved chorions.

The embryos (25 to 100) are rinsed in filtered SW and transferred to 250 ml Erlenmeyer screw flasks containing 200 ml SW. Pure oxygen is bubbled through the SW for two or three minutes and the flask tightly capped. One to three days after the control culture maintained with daily SW changes in finger bowls begins to hatch, the crude enzyme is harvested (normally hatching occurs in about 7 days). The contents of the flasks are filtered through plastic screen with 1.5 mm pores which retains unhatched embryos but through which hatched fry can pass.

(obtainable from Supply Dept.) The unhatched embryos are washed with 10% SW, transferred gently to a Syracuse dish with the aid of a rubber policeman and 2 ml of 10% SW added for each 100 embryos. Hatching usually begins immediately, sometimes even during rinsing and transfer. Within a few minutes, the characteristic rotation of the embryos within their chorions, which always precedes hatching, is observed in from 10% to 50% of the embryos. This "somersaulting" is soon followed by hatching. Within thirty minutes the hatching water is filtered through Whatman no. 1 filter paper into a tube cooled in an ice bath. The clear filtrate constitutes the crude enzyme and is stored frozen. Several cycles of freezing and thawing denatures the enzyme, therefore it is desirable to freeze small aliquots (about 0.5 to 1 ml).

4. Assay for "chorionase" activity

The term "chorionase" will be used to denote the action of the hatching enzyme system on small fragments of isolated chorions from the same species under the conditions described below.

Either purified, dried chorions or freshly isolated chorions washed in 10% SW and distilled water are suitable. If dried chorions are used these are first hydrated by immersion in distilled water. They are digested in the same way as fresh chorions by the enzyme. Under the dissecting microscope the chorions are torn into small fragments with forceps about 1 mm^2 and placed inner surface upward in a watch glass. One drop of buffer (0.04M tris, pH 7.5) and one drop of the enzyme are then added and the watch glass is covered with a cover slip and incubated at room temperature. Sequential microscopic observations are made. As a control, a fragment of chorion is incubated with one drop of buffer and one drop of 10% SW.

Observe changes in the inner surface of the chorion fragments. Look closely at the layered structure. Record the time required for complete digestion when only the flimsy outer layer will remain undigested.

SUGGESTED EXPERIMENTAL APPROACHES

1. Synchronization of hatching

The factors which inhibit hatching could be investigated e.g., partial pressure of CO_2, O_2 and hydrogen ion concentration.

2. Study of properties of chorionase

Although no quantitative assay is available, one can test the effect of changes in pH, ionic strength, enzyme concentration or inhibitors on activity of the enzyme by observing the time required to digest chorion fragments. The crude enzyme can be dialyzed against 10% SW or 0.1 M tris or phosphate buffer (pH 7.1) in the cold (4°C) for 2 or 3 hours without loss of activity.

3. Specificity of chorionase

It would be of interest to see whether the enzyme would attack chorions of other teleosts (it does digest the chorions of 2 trout species) or other structural proteins.

4. Studies on the chorion

This material could be collected while the eggs are available and stored for later use. All of the common amino acids have been found except tryptophan in acid hydrolysates. In addition, sialic acid, hexosamine and another non-amino sugar are present (about 1% of dry weight each). These have not been isolated but simply identified colorimetrically.

5. Isolation of hatching enzymes of other species.

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Embryology Course
1963

DISSOCIATION AND AGGREGATION OF MARINE SPONGE CELLS

Outlines of laboratory exercises

I. Dissociation and Self-Aggregation

Materials: Microciona prolifera; Haliclona permollis (other species optional) Bolting cloth; Large forceps; Petri dishes (glass, plastic; small large; Beakers; Calcium and magnesium-free sea water (CMF-SW); Hemocytometer; Graduated pipettes.

Protocols: Make detailed protocols of your work describing the purpose of each experiment; preparation; observations; conclusions; significance of results; other or better ways of doing the experiment to answer further questions; references, etc.

A. Cell density, temperature and self-aggregation.

1. Prepare a sponge cell suspension (start with Microciona) by pressing 1 gm of sponge fragments through bolting cloth into cold (approx. 4°C) sea water (100 ml) in a beaker. Count the cells and prepare several dilutions of the suspension. Dispense aliquots of each dilution into petri dishes, calculate cell density per 1 cm^2. Cover and leave undisturbed for 24 hours at room temp. and at 4°C. Determine number and size distribution of aggregates in the different dishes and relate to original cell density and temperature. Compare Microciona with Haliclona.

2. Put fresh suspension in two small petri dishes and observe the process of cell aggregation under low magnification (dissecting microscope) and higher power.

3. Place drops of cell suspension on glass slides and stain the cells with toluidine blue, neutral red, giemsa. Compare Microciona and Haliclona cells. Try to identify cell types. Do vitally stained cells survive long enough to aggregate? (There are practically no histochemical studies on sponge cells).

B. Cations and self-aggregation.

Prepare a cell suspension of Microciona in cold CMF-SW. Rinse the cells twice in CMF-SW (cold) by centrifugation (1000 rpm 2 min). Dispense aliquots of this suspension into petri dishes with (a) CMF-SW; (b) CMF-SW with $CaCl_2$ (at sea water concentration) (c) CMF-SW with $MgCl_2$ (at sea water concentration); (d) sea water. Compare after 24 hours at room temperature aggregation of the cells in these solutions. Devise additional tests to determine the effect of cations and pH on aggregation of sponge cells. Is it possible to distinguish between effects on cell mobility and cell adhesiveness? You might want to repeat some of these tests using aggregation by rotation.

C. Substrate and self-aggregation.

1. Coat coverslips with materials which will give you surfaces with different properties (for instance: paraffin, paraffin oil, agar, celloidin, silicone, lanolin, etc). Determine to what extent is aggregation of sponge cells

affected by different substrates. Compare with what is known in this respect about vertebrate cells (see: L. Weiss, Intern. Rev. Cytol. 9, 187, 1960).

2. With a diamond pencil scratch plastic or glass cover-slips to make grooves of different width, depth and density. Find out whether aggregation of sponge cells is effected by such surface heterogeneities. Any evidence of "contact guidance"? (ref: P. Weiss, Intern. Rev. Cytol. 2: 391, 1958).

3. By squeezing out all the cells from sponge fragments prepare cell-free "skeletons" of Microciona and Halichona. "Seed" such skeletons with cells of its own species, of the other species and with mixtures of cells from both species and find out how the cells behave in each situation. Is it possible to "modify" the reaction of the cells to the skeleton by washing, boiling, extracting, etc., the skeleton? (ref. L. Wiens - see ref. list).

II. Aggregation by agitation

1. Prepare a cell suspension of Microciona (about 10×10^6 cells/ml in sea water. Distribute 3 ml aliquots of suspension into 25 ml Erlenmeyer flasks and seal them with parafilm. Place two flasks on gyratory shaker at room temperature (22°C) and rotate at 80 rpm. Place another set of similarly prepared flask cultures on a shaker in the refrigerator. Sample flasks hourly to determine "growth" of aggregates. (ref. D. A. Moscona, P. Nat. Acad. Sci., 49: 742, 1963).

2. Place aggregates in plastic petri dishes in sea water and follow their development. The dishes should be kept on the water table and their sea water changed daily.

3. Prepare a mixed suspension of Microciona and Halichona cells and study the aggregation of such interspecific cell populations in rotating flask cultures at 22°C. Observe further development of such aggregates by transferring them to plastic petri dishes, as above. How do sponge cells compare with embryonic vertebrate cells (ref. Townes and Holtfreter, J. Exp. Zool., 128: 53, 1955; Moscona, D., Cell. Comp. Physiol. 60 (suppl. 1): 65, 1962.

III. Aggregation of chemically dissociated cells.

1. Dissociation

Use Microciona. One gm of sponge is blotted free of excess sea water and foreign material, placed in 80 ml. CMF-SW at 0°C, cut into small fragments, and soaked for 30 minutes. Dissociate the pieces by pressing through bolting cloth (#25 standard quality) into another 80 ml. of cold CMF-SW. The resulting suspension of cells and clumps is sedimented by centrifugation, using 5 ml aliquots and spinning for 2 min. at 2,000 in 15 ml centrifuge tubes. The cells are then resuspended in fresh cold CMF-SW at a concentration of 20×10^6 cells per ml. Pour the suspension into a beaker to a fluid depth of about ½ inch and place on a shaker at 80 rpm for 6 hours, at 4°C. After this time, dissociate remaining loose cell clusters by flushing through a fine pipette; spin cells by centrifugation and redisperse in cold sea water or test medium. Adjust concentration of cells (to about 10×10^6 cells per ml.) dispense 3 ml of suspension into 25 ml Erlenmeyer flasks and place on shaker.

2. Compare aggregation of mechanically and chemically dissociated cells, dispersed in sea water and rotated at 5°C, and at room temperature.

3. Do chemically dissociated cells aggregate in CMF-SW.

4. Do chemically dissociated cells self-aggregate in sea water?

SPONGES

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DEVELOPMENT OF ECHINODERMS

GENERAL PRECAUTIONS

1. Cleanliness: Use only dishes that are biologically clean. Avoid using bichromate cleaning solutions unless followed by prolonged rinsing. Detergents are good fertilization inhibitors; rinse dishes carefully and lay them upside down on towels. Pipettes used for eggs and sperm should be rinsed immediately in sea water to avoid encrustation of dried gametes.

2. Avoid overinsemination: Remember that very few spermatozoa are required to bring about fertilization. If the egg suspension becomes noticeably more milky after insemination, you have added too much sperm. Either use a toothpick to introduce sperm, or measure amount as described later. Label sperm pipette and use only for that purpose.

3. Keep temperature low: Remember that marine animals are accustomed to temperatures 5 to 15 degrees C. below room temperature. Never leave stock cultures at room temperature; they may not develop normally. Remember that eggs which normally develop, for example, at 8°C. may be heat killed at 15°!

4. Overcrowding: Remember again that eggs spawned under natural conditions probably never have to face overcrowded conditions. Avoid this very common source of abnormal development by placing so few eggs in a single culture that they can hardly be seen. If they form anything like a continuous layer on the bottom of the dish, they are too numerous. It is far better to discard half a culture than lose the whole culture through overcrowding.

5. Equipment: The climate at Woods Hole is especially hard on equipment and optics. Be sure to rinse off objectives or any other parts of microscopes that may come into contact with sea water or the sea fog that carries salt. Use stopcock grease on the mechanical parts of your microscope.

The student should spend three days studying thoroughly the development of Arbacia punctulata (sea urchin).

Later in the week, Echinarachnius parma (Sand dollar) and Asterias forbesii or (A. vulgaris -- starfish) will also be available.

Fertilize a batch of eggs at the beginning of the laboratory period. These may be studied comparatively by keeping a table of "cleavage times" and are to be used for the study of gastrulation and larval formation on the second and third days of observation. Detailed study of fertilization and early cleavage stages should be done with a second batch of eggs for each species separately and consecutively.

Fertilize a new batch of eggs each night to cover the developmental stages you would otherwise miss overnight.

It is wise to take special precautions to avoid overcrowding and overheating of eggs in the sea water dishes. Have no more than a single layer of eggs on the bottom of a finger bowl and keep the temperature of the sea water below 25°C. for best results. Temperatures above 30-32° are lethal for Arbacia eggs.

ARBACIA PUNCTULATA

The eggs of various sea urchins have been possibly the most used egg material for embryological and physiological studies. The egg of Arbacia is rather different from the eggs of most other sea urchins in being smaller and containing red echonochrom granules. It is interesting to note that Hyman has classified Arbacia in a different order from the other sea urchins so often studied.

In the early years at Woods Hole, investigators placed a heavy drain on the supply of urchins; whether this was the reason for a depletion in their supply is not known. However, for the last five or ten years, the general supply of urchins has been jealously guarded. Recently there have been signs that urchins were becoming more common locally; it is not known how much of this is due to "seeding" operations carried out by the supply staff in the area of former urchin beds, and how much is due to other factors. Sea urchins normally shed several times during a season, often at rough weather.

To preserve the supply of urchins, it is highly desirable to "sex" them, to avoid opening several males in a row, for example, while searching for a female. Mrs. E. B. Harvey has described a simple electrical method for sexing Arbacia; this method is preferable to the KCl injection method, which can also be used. The apparatus department provides a simple electrical set-up with which to stimulate the surface of the sea urchin (usually with a lead electrode). If the animal is ripe, gametes will ooze out of the gonopores (aboral surface) in sufficient quantity to determine the sex of the specimen. Eggs are red; sperm are white. Shedding usually ceases if the current is stopped; the animals should be rinsed with sea water and placed in separate, appropriately labeled aquaria. For experiments the desired amount of eggs can be obtained by electrical stimulation, or the Aristotle's lantern can be removed and the animal inverted over a container filled with clean sea water. If this does not induce spawning, a few drops of 0.5M KCl should be pipetted into the body cavity. Sperm should be collected "dry" by the same treatment, in a Syracuse watch glass.

Spermatozoa

Undiluted Arbacia seminal fluid contains approx. 2×10^{10} spermatozoa/ml. One drop of this "dry sperm" in 10 ml. of sea water brings the concentration down by a factor of 200, to 10^8/ml. One drop of this stock sperm suspension in 10 ml. of sea water containing eggs dilutes the sperm concentration to 10^6 sperm/ml. Densities between 10^7 and 10^8 sperm/ml. result in varying percentages of polyspermy. Undiluted sperm will keep in a refrigerator for at least two days; dilution, however, rapidly destroys fertilizing capacity. Consequently, a new stock sperm suspension should be made up for each insemination.

Observe some living spermatozoa under oil immersion, or preferably phase contrast. The acrosome, nucleus and middle piece are roughly 0.3, 1.0 and 1.0 microns in length, respectively. The tail is about 45 microns long and 0.1 micron wide; its axial filament protrudes a short distance beyond the end of the sheath. Sketch a spermatozoon and describe its movement. Place side by side on a slide a drop of stock sperm suspension and a drop of "egg water" (supernatant from eggs which have stood for an hour or more). Place a cover-glass over the two drops so that they mix at the middle of the cover-glass. Note the agglutination; do the spermatozoa stick together by their heads or tails? Can you detect any changes in the sperm acrosome?

Add one ml. of 10^8/ml stock sperm suspension to A, 9 ml. sea water; B, 9 ml. egg water. Observe at 10-minute intervals. What effect does egg water have on the sperm besides agglutination? Will these sperm activate eggs?

Unfertilized ova

Arbacia punctulata eggs complete both meiotic divisions while in the ovary; only seldom are eggs observed with maturation spindles or polar bodies. Unripe gonads often do exhibit large numbers of eggs in the germinal vesicle stage. These will often respond to insemination by forming hyaline blisters at the points of sperm entrance. At this stage, the mechanisms for membrane elevation and for preventing polyspermy have not developed. No development results from insemination at this stage.

The mature Arbacia egg has a transparent jelly layer that swells to about 30 microns in thickness. Most of the jelly can be removed if desired by swirling the eggs for 20-40 seconds in sea water pH 4.5. Longer treatment removes the jelly entirely, but may injure the eggs. Beneath the jelly layer is the vitelline membrane. It is too thin to see on the egg surface, but is visible as it elevates from the surface at fertilization. Beneath the vitelline membrane is the cortex, containing characteristic granules from 0.5 to 0.8 microns in diameter. The cytoplasm of Arbacia contains oil sperules, mitochondria, yolk granules and the slightly larger red echinochrome granules. (As Hartmann (1939) and Tyler (1939) showed, the red echinochrome is a substituted naphthoquinone related to the K vitamins.) The formed cytoplasmic components and the cortical granules are best seen in centrifuged eggs.

Centrifuge a sample of unfertilized eggs at 3,000 g for 10 min in 20% sucrose and sketch one in "side view," noting the five layers of stratified material: oil cap, hyaline zone, mitochondria, yolk zone, and pigment zone. Where is the nucleus? Have the cortical granules been displaced? Are they thicker in the centrifugal hemisphere? If time allows, centrifuge some eggs at higher speeds in the air-turbine centrifuge (consult papers of E. B. Harvey) to separate them into halves and quarters. Inseminate these and watch development. The mitochondrial layer is stained brilliantly by either Janus Green B or by Cresyl Violet.

Cortical reaction

Place a drop of dilute eggs on a slide and cover with cover-glass supported on the four corners with plasticene "feet." Withdraw a little water so that the water interphase shrinks away from the outline of the cover-glass. Add a drop of stock sperm suspension to one corner of the preparation and select the sperm density that is most convenient for the subject under study. (For the details of sperm penetration, select an egg far from the point of insemination to get a low sperm-to-egg ratio; for observing the time of membrane elevation, select an egg with plenty of sperm around it.) The penetrating sperm is usually one of the first to arrive at the egg surface (see Hultin's "first orbit theory"), Exptl. Cell Res., 1956, and it is safe to assume that the sperm has entered by the time the membrane begins to elevate. Watch for a characteristic wrinkling on the egg surface; this is caused by explosion of the cortical granules. These can be observed best in the clear zone of centrifuged eggs (focus on upper surface; compare fertilized with unfertilized egg). Note also distribution of red pigment

granules before and 10 min. after fertilization. Are these granules also shifted by centrifugation after fertilization? Observe unfertilized eggs under dark-field with the most brilliant light source possible (direct sunlight is best). Note the orange ring around the egg surface. Inseminate and watch the orange ring fade, either gradually and regularly if the sperm enters directly above or below the egg, or as a wave if the sperm enters anywhere near the equator. For the kinetics of the cortical change, see papers by Lord Rothschild, and by Allen and Hagstrom.

- Observe what happens if the eggs are washed and fertilized in Calcium-Magnesium-free sea water. Are divalent cations necessary for fertilization?

Lifespan of the sperm: Take a small amount of dry sperm and dilute it 50 times. Inseminate a batch of eggs with a portion of the dilute sperm suspension, and save the remainder. After one hour, use it to inseminate another batch of eggs. Compare the percentages of fertilization.

By varying the time, find the lifespan of the sperm at several temperatures and dilutions.

Induction of Polyspermy: Remove the fertilization membrane with Calcium-Magnesium-free sea water. Before each reinsemination, wash the eggs in CMF-sea water; but keep them in normal sea water. Inseminate several times, rather heavily, and observe cleavage.

Polyspermy may also be induced by suspending the eggs in M/3 $MgCl_2$ or 0.2% chloral hydrate (in sea water), or in sea water with a few drops of nicotine added. Is it necessary to inseminate the eggs within these solutions?

Cleavage

The rate of cleavage and development in general are dependent upon the temperature of the sea water. The following figures give the average time for the first three cleavages (Fry, '36):

Minutes after fertilization	First	Second	Third
	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%. These differences may still be apparent during larval stages.

The plane of the first cleavage is meridional (in the polar axis) and divides the egg into two equal-sized blastomeres. The egg then passes into a resting stage (lasting ca. 25 min. at 24°) before the onset of the second cleavage.

The plane of the second cleavage is also meridional but at right angles to the first, resulting in the formation of four equal-sized blastomeres. A resting stage then follows.

The plane of the third cleavage is equatorial or horizontal (at right angles to the polar axis). Each of the four blastomeres divides into an upper and lower blastomere of equal size.

The student should follow these early cleavages carefully. Note the relationship of the hyaline plasma layer to the cleavage furrows (see Dan and Ono, '52). It will also be interesting to attempt to follow the movements of the orange-red, cortical granules in relation to the early cleavages.

At the fourth cleavage the upper four cells divide meridionally, forming equal-sized cells called mesomeres. The lower four cells divide unequally and horizontally, forming four larger cells called macromeres and below them, at the vegetal pole, four small clear cells called micromeres.

At the fifth cleavage the eight mesomeres divide equally and horizontally forming two tiers of cells in the animal hemisphere (an_1) and (an_2). The four macromeres divide meridionally, forming a tier of eight cells. The four micromeres also divide. Thus a 32-cell stage is attained.

At the sixth cleavage the an_1 and an_2 cells divide more or less rapidly. The macromeres divide horizontally, forming two tiers termed (veg_1) and (veg_2). The eight macromeres fail to divide. In the 56-cell stage five layers or tiers of cells may be distinguished. From the animal to vegetal pole these are: 16 an_1, 16 an_2, 8 veg_2 and 8 micromeres. These layers are difficult to distinguish in Arbacia.

On the basis of staining with vital dyes (e.g., Nile blue sulfate) Horstadius ('39) has shown for Paracentrotus that an_1, an_2, and veg_1 cells form the ectoderm of the pluteus-larva (see below), veg_2 cells form the gut endoderm and part of the mesoderm (coelomic pouches, secondary mesenchyme), the micromeres form the remainder of the mesoderm (primary mesenchyme) which produces the skeleton of the larva. Sketch the various stages through the sixth cleavage.

Dissociation of cells: Remove fertilization membranes with CMF-sea water. If the eggs remain in CMF-sea water, what happens to the hyaline layer? The cells should reaggregate if replaced in normal sea water.

Try varying the times of dissociation and reaggregation. How do reaggregated cells develop? Will cells of different species sort out?

Blastula:

At the eight-cell stage there is a very small central cavity which enlarges, as cleavage continues, to form the blastocoel. This is filled with a fluid which, presumably, is mostly sea water plus colloid (see Dan, '52). About 6 hours after fertilization a smooth-surfaced, spherical young blastula is formed, the wall of which is one cell thick. Cilia develop on the surface and the blastula is rotated by their action within the fertilization membrane. At about 10 hours after fertilization the blastula, composed of about 1000 cells, hatches out of the fertilization membrane. It has been shown (Ishida, '36; Kopac, '41) that the blastula releases a "hatching enzyme" at this time that weakens and dissolves the membrane sufficiently for the blastula to break through.

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 vegetal plate. Meanwhile, the micromeres located in the approximate center of the vegetal plate region have begun to

migrate as individual cells into the blastocoel, forming the primary mesenchyme which later forms the skeleton. There is evidence that the micromeres continue to divide during this "ingression" process. Sketch early and late blastula stages.

Gastrula

At about 20 hours after fertilization the cells at the vegetal pole invaginate to form a blind tube, the archenteron. Invagination is apparently brought about by a fairly uniform stretching in an animal-vegetal direction of the whole veg$_2$ disk, i.e., the vegetal plate (Horstadius, '39). Note the gradual lifting or wrinkling of the hyaline layer at the vegetal pole of early gastrulae (See Dan, '52).

The outer wall of the gastrula, as well as the wall of the archenteron, consists of a single layer of cells. The primary mesenchyme cells form a ring around the archenteron and form a pair of triradiate spicules. Draw beginning and completed gastrulae.

Prism:

At the completion of gastrulation the tip of the archenteron bends to one side of the gastrula which becomes flattened, representing the ventral side of the embryo. The blastoporal side represents the posterior or anal surface of the embryo.

Where the tip of the archenteron touches the ectoderm the latter bends inwards to form the stomodeum. An opening, the larval mouth, later develops between the stomodeum and the adjacent tip of the archenteron (Gustafson). The archenteron becomes divided by two constrictions into oesophagus, stomach, and intestine. The apical tuft changes into a ciliated band surrounding the oral area, the embryo begins to elongate in the dorso-ventral axis and the direction of swimming changes so that the ventral (oral) 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 traverse rods). The rods are each made up of three or four parallel parts joined by cross bars. 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 through oesophagus, stomach, and intestine 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 the oesophagus and intestine. Two coelomic sacs extend posterolaterally 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 studied here. It should be mentioned, however, that the adult organs are built up in and around a structure termed the Echinus rudiment which is formed by the fusion of an invagination (amlotic invagination) of the ectoderm on the left side with the mid-portion (hydrocoel) of the coelom. The left side of the pluteus becomes the future oral face of the adult. Draw a 3-day-old pluteus in postero-ventral and side view.

ASTERIAS FORBESII (or A. VULGARIS)

Specimens of the common starfish, Asterias, are dredged in the neighborhood of Woods Hole. Fresh material seems to yield the best eggs. The largest and fattest specimens are really the only ones worth opening. Sexes are separate in Asterias, but it is not possible to distinguish them by external characteristics. To obtain eggs, remove an arm of a female and split it along the middorsal line to expose the pair of bulging ovaries, which are a typical pale salmon color. Then, with a pair of clean forceps, carefully detach each plume-like ovary with as little injury as possible and transfer it to a finger bowl of sea water. Do not cut up the ovary, but rather let it shed normally for about 5 minutes; these first shed eggs are best. Discard the rest of the ovary and save the specimen for use later in the day. Injured animals should be kept in a separate aquarium with rapidly running sea water and not kept for more than two days. The eggs should be washed by siphoning off the supernatant fluid and replacing it with sea water at least twice. During this time, the eggs should be examined occasionally to observe meiotic stages. About the best that can be expected of Asterias eggs is 85-90% germinal vesicle breakdown.

Spermatozoa

Remove testes from one arm of a male to a dry Syracuse watch glass and cover. Small amounts of the "dry sperm" should be freshly diluted with sea water for each insemination.

The sperm of the starfish are perhaps the best material in which to detect acrosome filaments. These can be seen with phase contrast, and perhaps even with bright field. It used to be thought that the filament which Fol (1879) and later Chambers (1930) saw joining the sperm and egg was a product of the egg; now, however, this has been shown to be the acrosomal filament of the sperm (J. Dan, '54). As the Colwins have shown in Japan and Woods Hole, the egg reception cone climbs up the acrosome filament after the latter attaches to the egg surface.

Eggs

Observe some freshly shed Asterias eggs. Note the large clear germinal vesicle and the yolky, yet surprisingly transparent and unpigmented cytoplasm. These eggs contain so much yolk that it is difficult to stratify them by centrifugation. Note the nucleolus. If the oocyte is placed on the stage of a horizongal microscope, the nucleolus will fall through the nucleus (Gray, Harding).

Note the cortical layer of the immature Asterias egg. The number of cortical granules in this layer seems to increase during maturation. (The origin of these granules has been studied in the eggs of the sea urchin by Monne and Hørde and by McCullough).

Fertilization

Watch the equator of an egg being attacked by only a few sperm. Remember that the chances of sperm penetration in the equatorial band that you can see are about one in three. Accordingly, make several observations. Can you see a reception cone? A filament between the egg and sperm? It must be stressed that for extremely fine observations of this sort, clean optics and slides are required; also good illumination and patience! Watch for details of the cortical reaction (cortical granule breakdown, membrane elevation, dark-field color change as in the sea urchin).

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 horizongal, 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 a spherical shape, resulting in a rather loose arrangement of cells. Note that no micromeres are formed. Draw the early cleavage stages.

Blastula

Eventually (10-12 hours after fertilization) the cells arrange themselves as an epithelial wall enclosing the blastocoel. The surface cells acquire cilia, and the blastula rotates within the fertilization 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. Note that the cells at the vegetal pole are thicker than those at the animal pole. Note also that there is no "ingression" of cells at the vegetal pole, there being no primary mesenchyme comparable to that found in Arbacia. Mesoderm formation in starfishes occurs after gastrulation, i.e., after formation of the archenteron.

Gastrula

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. Note the large space between the archenteron and the outer wall of the gastrula.

Middle and late gastrula: The gastrula becomes pear-shaped ca. 20 hours after fertilization. The blind inner end of the archenteron becomes thin-walled and expands. From this end mesenchyme cells wander out into the blastocoel. 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, and that the archenteron extends only about halfway from the vegetal toward the animal pole.

Dipleurula Larva:

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.

Fully formed Dipleurula (early Bipinnaria) larva: This larva represents an early larval type common to Asteroidea, Echinoidea, Ophiuroidea, and Holothuroidea (see Korschelt, vol. 1, p. 499, or Bell, 1948). Study carefully a ventral, dorsal, and lateral (preferably left) view.

Observe the following:

Shape of larva. Notice convexity of ventral side and mouth openkng underneath the overhanging oral lobe. Compare with the flattened oral lobe of Arbacia.

Locomotion: What part of the larva is directed forward in swimming?

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 mid-line. Eventually the loops meet in the mid-line, 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.

Alimentary tract. This consists of the three parts characteristic of Echinoderm larvae: oesophagus (with constriction near entrance into stomach) stomach and intestine. Study ciliation in oral field and different parts of the digestive tract.
s
Coelom. Study the 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 blastocoel. Note that these cells are connected to one another by filamentous pseudopodia.

Vital staining with neutral red is helpful; but study unstained specimens first. Study also stained whole amounts 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 must be used, if available.

Fully formed Bipinnaria Larva:

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 antero-dorsal, postero-dorsal, postero-lateral, postoral and preoral arms. See Gemmill, plate 18, fig. 7, and

Intestinal Tract.

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

Brachiolaria and Metamorphosis:

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 Note the following:

Intestine

Coelom, in different stages of subdivision.

Disk, or developing starfish, on left side.

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

ECHINARACHNIUS PARMA

The supply of Echinarachnius comes from the cold water on the north coast of the Cape; consequently, even the temperature of the sea water table is likely to put quite a strain on eggs of this species. They are best used on cold days or in an air-conditioned room. If a specimen turns green, either all over or in spots, it is probably ready to be discarded.

Gametes can be obtained by cutting the oral surface completely around, about 1/4 inch inside the margin. The oral part of the testes and the Aristotle's lantern can be removed. Specimens cut open in this way will usually shed if placed aboral surface down over a stender dish. A second method of obtaining gametes is to inject isotonic (0.53M) KCl into the visceral cavity. Sperm should be obtained dry by either method, or the testes can be kept dry in small dishes in the refrigerator. It is important that the eggs should be washed a few times before insemination. Sperm should not be diluted until ready for use. The electrical method may also be used with reasonable success.

Fertilization

Sand dollar eggs are larger than those of Arbacia (135 microns instead of 74) and are surrounded by a much thicker jelly layer in which very beautiful red pigment granules are suspended. The egg itself, freed from jelly, is pale yellow and resembles very closely the eggs of the Camarodent urchins (Psammechinus, Paracentrotus, etc.). Echinarachnius eggs are fragile compared to those of Arbacia, but they can be stratified by centrifugation in much the same way. The cortical granules of Echinarachnius are larger than those of Arbacia and other sea urchins, and, consequently, this form is the best one at Woods Hole in which to observe cortical reaction phenomena. If you can locate the cortical granules at the top of an egg under high power or water immersion, inseminate and watch these granules break down in a wave which sweeps the cell from end to end. Watch the membrane elevate and try to determine the time relationships between dissolution of the cortical granules and membrane elevation. Could there be a causal relationship between these two events? Do you see a hyaline layer?

Early development of the sand dollar is not markedly different from that of Arbacia; compare the corresponding stages: early cleavage, blastula, gastrula, and pluteus. Can you distinguish animal, vegetal, and micromere tiers of cells? Is primary mesenchyme formed prior to archenteron formation? Is there a folding or wrinkling of the hyaline layer at the vegetal pole during gastrulation? Does the archenteron proliferate secondary mesenchyme from its tip? Is the volume of the gastrula larger or smaller than that of the blastula? Compare the plutei of the sand dollar with corresponding plutei of Arbacia. What obvious differences do you see?

Experimental Procedures with Echinoderm Embryos

Since the most thorough and extensive experimental studies have been done using various species of sea urchins, the student may be interested in repeating some of the same experimental procedures with Asterias and Echinarachnius eggs. A few types of experiments are listed below.

1. Experimental analysis by operation: Development of isolated blastomeres (easiest); development of isolated animal and vegetal halves (more difficult); development of recombinations of animal and vegetal layers (extremely difficult); combination of operation and chemical agents.

2. Experimental analysis through the effect of agents: Physical: Centrifugation; development of light and heavy halves and quarters; development of whole centrifuged eggs; combination of centrifugation and chemical treatments. Chemical: Effect of development of: LiCl, NaSCN, iodosobenzoic acid, proteolytic enzymes (trypsin, chymotrypsin, ribonuclease), sodium azide, mercaptoethanol, etc.).

3. Gradient studies: redox gradients as demonstrated with methylene blue, Janus green, Nile blue sulfate or Neotetrazolium chloride.

4. Artificial parthenogenesis.

5. Partial suppression of activation by heat.

6. Permeability properties of the blastular wall.

7. Isolation of mitotic apparatus

For the above, or any other experimental procedures which members of the class might wish to try, special directions, reagents, and equipment will be made available by the instructors.

EMBRYOLOGY OF MOLLUSCS AND ANNELIDS

Representative molluscs and annelids commonly used for experimental and descriptive embryological studies will be supplied. Our laboratory work will be concerned with descriptive development of type forms--or forms which are readily available at this time. It may be well to remind students that cleavage in these forms is characterized by partial rotation of the micromeres, so called spiral cleavage. The spiral pattern is not observed with equal ease in all of the forms, thus it is obscure in the eggs of Spisula, but very clear in those of Crepidula, etc. In addition to their precise pattern of cleavage, the eggs and early embryos of molluscs and annelids are noted for their lack of ability to regulate. When blastomeres are isolated, under most circumstances they continue to develope as though still part of the whole embryo. They exhibit a mosaicism (or determinative cleavage) which has frequently been contrasted with the regulative (tendency of parts to form a whole) behavior of the eggs of other forms. Finally a structure which is unique to these eggs (and a few related forms) is encountered. This is the polar lobe, a large mass of nonnucleated ooplasm which frequently resembles a blastomere. The polar lobe forms and retracts in synchrony with the first few cleavages.

Some basic information concerning the handling of eggs, and development of embryos, of a few of the more common annelids and molluscs, is given below. This should be supplemented by references to Costello et al., Methods for Obtaining and Handling Marine Eggs and Embryos, MBL, 1957, and to the bibliography supplied at the end of this section. These eggs, like those of other marine forms, require a reasonable degree of care in their handling (see Just, 1939, Basic Methods for Experiments on Eggs of Marine Animals). The eggs are sensitive to adverse conditions. In order to avoid high temperatures, evaporation, etc. , a stock batch of eggs or embryos should be kept in a covered finger bowl or stendor dish on the seatable or in the B.O.D. units. Samples of the embryos may be taken out at intervals for microscopic observation.

Glassware should be thoroughly washed to avoid contamination.

For observing rapidly moving larval stages it is necessary to immobilize them without causing excessive harm. Polyvinyl alcohol may be used for this purpose (1 part polyvinyl alcohol to 1 part sea water containing the larvae). This solution is very viscous and slows down larval movement without apparent damage. A 1:2 solution of 0.1% chlorobutanol or MS 222 may also be used.

Embryology of Mollusca

Two forms will be available for study------Ilyanassa and Spisula (Mactra). Primary emphasis will be placed on Ilyanassa. Spisula is offered as an example of a spirally cleaving egg in which polar lobes do not form and the spiral cleavage pattern is not so obvious. Due to the opacity of most of these eggs, the details of maturation, association of germ nuclei, and cleavage can best be studied from prepared slides. For this purpose, sets of Conklin's original slides of Crepidula development will be provided.

Spisula (Mactra) solidissima (Pelecypod)

1. Obtaining eggs. Remove hinge of the clam, breaking the ovary and forcing the eggs to extrude into the beaker of filtered sea water. Decant 4 times in the first half hour, leave the heavier more mature eggs on the bottom of the beaker. Add two drops of a sperm suspension (one drop dry sperm in 10 ml. of filtered sea water) to eggs contained in 40 to 50 ml. of filtered sea water. When observing the eggs, limit the egg concentration to 20 - 26 per low power field since they are very susceptible to overcrowding. Dry ovaries and testes may be kept overnight in a refrigerator. Washed eggs may be kept for 4 to 6 hours.

2. The unfertilized egg: Obtain a sample of unfertilized eggs in a stendor dish and transfer to a depression slide for study. When shed, the eggs are irregular in shape due to pressure within the ovary, but they become spherical on standing. They are small (56 microns) and the center is almost completely filled by the enormous germinal vesicle with its prominent nucleolus. Unless eggs are inseminated they will maintain 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.

3. 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 to 20 minutes there is only a lighter area in the center of the egg, marking its former position. A thin fertilimation 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 within 30 - 35 minutes after insemination. Note the position of the polar bodies, for they mark the plane of the coming cleavage.

4. 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 ablou about half an hour. In the case of the larger cell, cleavage is again unequal, resulting in one larger cell and three smaller ones. The following cleavages are rapid, perhaps only ten minutes intervening between the 4 and 8 cell stages. The cleavage is undoubtedly of a spiral type, but this characteristic is more dificult to detect than in Crepidula because of the size differences of the blastomeres.

5. Timetable of development: These are times recorded from insemination at 25°C. as reported by Schechter, 1941.

10 minutes	Germinal vesicle reaction
30 "	Polar bodies formed
50 "	Pronuclei visible
1 hour	First cleavage
1.5 hour	Second cleavage
5 Hours	Swimming blastula

6. Later development: (The figures of the development of Dreissensia (Meisenheimer, 1900 will prove helpful)

Gastrulation and early Trochophore stages: Remove samples 4 - 9 hours after insemination. If forms are moving too rapidly, add a drop of polyvinyl alchhol. 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 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 visimble . By 9 hours the embryos have lost their somewhat barrel-shape 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 shell gland is visible as a conspicuous concavity on the dorsal surface. By 14 hours the shell gland will have evaginated and this concavity will no longer be visible at this stage.

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 vellum.
d. The stomadeal invagination on the ventral side, just below the velum. The proctodeal invagination appears later (23 hours).
e. The internal structures are difficult to recognize at this time, for a large dark mass of undifferentiated endoderm and mesoderm cells fills most of the post-velar area.

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Crepidula fornicata (Gastropod) - prepared slides

Due to the opacity of the living eggs, the details of maturation, association of the germ nuclei, and cleavage can best be studied from prepared slides. The eggs are not sectioned, but are whole mounts. Number one or zero cover glasses have been used, ma king 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, and the males locomotive, and at the breeding season or perhaps once for all, the females are visited and inseminated by these motile males. All of 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.

4. Crepidula - Derivatives

1'st 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 connectives
f. cerebro-pedal connectives
g. possibly the pedal ganglia
h. an apical sense organ
i. paired eyes

2'nd quartette of micromeres:

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

3'rd quartette of micromeres:

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

The Veliger Larva of Crepidula

Study the typical Veliger larva of Crepidula formicata. 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 of 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 text books of Parker and Haswell volume 1 (1928) fig. 657 on p. 734, or MacBride figs. 263 ff. on pages 301 ff. or Korschelt volume 2 (1936) 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. Ciliated velum
2. Head vesicle (dorsal)
3. Eyes with lens (dorsal)
4. Foot (ventral); study it in lateral view.
5. Statocysts (ventral) at the sides of the foot
6. Ciliated mouth (ventral) above foot
7. Oesophagus (dorsal), stomach and 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

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Ilyanassa obsoleta (Gastropoda)

The Obtaining of Eggs and Early Stages: The best method of obtaining very early stages consists of removing egg capsules which have been deposited on glass plates by snails in aquaria. Eggs are visible at the oviducal opening (anterior median part of foot). After the egg is fastened to glass, the snail may be removed gently and the capsule transferred to a watch glass of filtered sea water with a pair of forceps. Since snails seem to prefer to deposit eggs on the wooden sides of the tank, in preference to the glass, glasss plates have been placed over the wooden portions. These plates can be removed for inspection purposes without disturbing the animals.

Opening of Capsules: When the proper stage is found, remove the capsule from the dish of filtered sea water and gently roll it over a piece of paper toweling to remove contaminating debreis.

Next, place the clean capsule in a Syracuse watch glass or small petri dish half full of either pasteurized sea water or autoclaved sea water. Pasteurized sea water can be prepared by heating filtered sea water to 70 - 80°C for 15 minutes, cooling it and shaking to aerate.

The capsule can be opened in a variety of ways. Clement (1952) has suggested the following technique: Using a dissecting scope, hold the capsule against the bottom of the dish, with a sharpened dissecting needle. With a pair of sharp scissors, snap off the end of the capsule at a point where the eggs are not clustered/ The eggs are held in the capsule by a thick jelly which is soluble in sea water and which will ultimately dissolve. The eggs may be more rapidly freed, however, by exerting a very slow pressure on the capsule with a second needle. With appropriate care, the capsules can also be opened using a pair of fine watch makers forceps.

Preparation of Cultures: Once the jelly has dissolved, individual eggs may be transferred to stendor dishes (half filled with sterile filtered sea water). No more than 10 to 20 eggs should be placed in a given dish. Using this method, perfectly normal veliger larvae can be reared.

Time table of development 23 - 24°C (Costello, 1957)
The time is recorded from the initial appearance of the first anti-polar lobe.

Stage	time
Egg spherical, first lobe withdrawn	11 minutes
Begining of second polar lobe	21 minutes
Egg spherical, second lobe withdrawn	1 hour, 21 minutes
Appearance of third polar lobe	1 hour, 59 minutes
First cleavage	2 hours, 59 minutes
Second cleavage	4 hours, 9 minutes
Third cleavage	5 hours, 9 minutes
Ciliated, rotating embryo	2 days
Veliger	4 - 5 days

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

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Embryology of Annelida

Two forms are available for study---Nereis and Hydroides. Cultures of advanced stages will be prepared by the instructors. Students should prepare their own cultures of early stages. It is suggested that both forms be carefully studied.

Nereis limbata

Nereis can be made to swarm in the Eel Pond about an hour after dark at certain phases of the lunar cycle (see Lillie and Just, 1913) by shining an artificial light on the water from the dock. This procedure will yield a few animals almost any evening. The females (fat and greenish bodies) should be isolated in separate finger bowls. A number of males (reddish anteriors and cream-white posteriors) may be placed in the same dish.

Females may burst while in captivity and shed their eggs; if not, they can be cut or pinched and the eggs will then be shed into the dish. Place a few eggs in a Syracuse dish or depression slide and observe with a low power of the microscope. Nereis eggs are anisodiametric: 140 microns as seen from above, and 100 microns as seen from the side. Note the large germinal vesicle (typical of eggs before meiotic divisions), the oil droplets (note what happens to them in the first few minutes after fertilization), the thick, finely granular cortex, and the vitelline membrane. Is there an external jelly layer?

In your observations of fertilization and early development, never rely on preparations that have been kept on the stage of the microscope, as temperature and possibly other factors may be operating to make these cultures abnormal. A convenient way to keep constant observations on narmal material is to remove a drop of eggs, place it on a slide under a cover glass supported at the four corners by scraping each corner ofer soft plasticen. Observations should be made on the eggs in the center of the preparation; an occasional look at eggs near the edge of the cover-glass will tell when the preparation should be renewed. All powers of the microscope can be used with such a preparation, and manipulations of various kinds (sorting of eggs, isolation of blastomeres, etc.) can be carried out under such plasticene-supported cover glasses.

After you have become familiar with the morphology of the unfertilized egg, look closely at sperm under the oil immersion objective. You will see the most detail if you partially dark-adapt your eyes and use low light intensities. Advantage should also be taken of the phase contrast microscope. Note the head, middle-piece and tail regions of the sperm and their characteristic movements. Draw a sperm cell to scale, noting especially the acrosome in front. Next observe some of the "rejected sperm" attached to the extruded jelly of fertilized eggs. Is their shape the same?

Find two eggs that are touching one another and inseminate them. Note how they are pushed apart by jelly extruded from their cortices. The margin of the jelly layer can be seen by the ring of rejected sperm and their characteristic movement. By 5 or 6 minutes after fertilization, the "fertilization membrane" has raised slightly from the surface. Find an egg showing a tangential view of sperm attachment; by 8 to 10 minutes an entrance cone begins to form. The sperm head becomes hidden from view within the next ten minutes, but does not enter for some time. When it finally penetrates (or is drawn in), the middle piece and tail are left outside. Observe eggs during cleavage to determine the relationship of the entrance point to cleavage plane.

Exaggerated entrance cones

Place some Nereis eggs inseminated 5 to 8 minutes earlier in a Syracuse dish containing alkaline NaCl (pH 10.3 to 10.5). Observe immediately. The vitelline membrane will elevate due to the sudden inhibition of jelly release through the membrane and a subsequent accumulation of jelly in the perivitelline space. The vitelline membrane remains permeable to water; swelling of the perivitelline space is due to colloid osmotic pressure. Elevation of the membrane stretches out the sperm entrance cone between the membrane and the egg surface, forming a long filament which frequently causes marked indentation of the membrane.

Development

The first developmental changes to follow insemination are those involved in the meiotic divisions. The dissolution of the germinal vesicle is followed by the extrusion of the first and second polar bodies. These establish the animal pole of the egg. What relation has the polar axis to the plane of cleavage?
At 24 °C. half of the eggs in a developing culture will form the first polar body at 45 minutes, and the second polar body at 60 minutes.

Observe the cytoplasmic segregation which begins at the time of fertilization (Costello, 1945). This is a segregation of cytoplasmic inclusions along the polar axis so that by the time of the 3rd cleavage the micromere region is oil free and nearly yolk free. In the unfertilized egg the large oil droplets form a ring around the germinal vesicle and the other inclusions (yolk spheres, mitochondria, granules, etc.) are uniformly distributed. After germinal vesicle breakdown there is a gradual movement of oil droplets and yolk to the vegetal hemisphere. By the four cell stage this segregation is completed and at the 3rd cleavage the micromeres are free of yolk and oil droplets.

The first cleavage (90-100 min.) is unequal and forms a smaller AB and a larger CD blastomere. These are divided unequally at the second cleavage forming blastomeres A, B, C, and D in order of increasing size. At the third cleavage four smaller cells (1st quartet of micromeres, 1a-1d) at the animal pole are separated from four larger vegetal cells (1st generation of macromeres, 1A-1D).

Time Table of Nereis Development (22-24 °C.) (from Costello et al 1957)

Stage	Time
Disappearance of membrane of germinal vesicle	10-15 minutes
First polar body	42 "
Sperm penetration	48 "
Second Polar body	58 "
First cleavage	81 "
Second cleavage	108 "
Third cleavage	132 "
Fourth cleavage	162 "
Ciliated trochophore	8-10 hours
Pigmentation in trochophore	24-38 hours

Later development

Keep a sparse culture of Nereis eggs going as long as possible to have an occasional look at the development of the trochophore larva.

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 appears very early. Study larvae from all sides. Consult the figures in E. B. Wilson, 892.

Observe:

A. Trochophore

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 stomadaeum; the latter is a short ectodermal invagination.
5- The large macromeres have ont yet differentiated into the endodermal parts.
6- Several "frontal bodies" near the upper end. Circular disc-like structures of unknown function.
7- Two pairs of seta sacs (chetae) or setae will differentiate inside of them. The sacs appear as spherical structures in the post-trochal hemisphere. These sacs are the first indication of the first two segments of the worm.
8- Observe the trochophore in locomotion.

B. Advanced Trochophore: two days old (Wilson, figs. 85-89) Observe all structures described in (A) above. 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 three segments. Setae can be seen in the process of formation, inside these sacs; in slightly older stages they will be seen projecting from the sacs.
3- Pigment appears in the prototroch cell.

C. Metamorphosis: 2.5 to 3 days (Wilson, figs. 90-91) Notice the change of shape and the gradual demarcation of the first three segments. Observe the following new features:

1- Sense hairs at the apical pole.
2- Additional eye spots; number variable.
3- Mesotrochal ciliary bands at the boundary of the segments.
4- Parapodia with parapodial cirrhi appearing on segments 2 and 3.
5- Tentacular cirrhi on the head.
6- Stomadaeum, stomach, intestine becomes distinct.

This stage combines larval (Trochophore) and adult structures in a peculiar way.

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Hydroides hexagonus

Hydroides, both male and female, 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). Clouds of white sperm will flow from the male, while the female will release large numbers of pastel-orange eggs.

Remove the animals after they have spawned. Let the sperm stand for about one-half hour. Add a few drops of sperm suspension to a dish of eggs. Extrusion of the polar bodies and cleavage may be studied readily. The blastula stage is reached after 5 - 6 hours, gastrulation after 8 - 12 hours, the trochophore stage lasts from 20 hours to two weeks. The trochophores are best for study when 2 - 5 days old.

Trochophore of Hydroides: The trochophore is a typical Annelid trochophore. Consult the excellent figures in Hatschek ('86) and Shearer ('11). The larvae show positive phototaxis and gather at the light-side of the dish. They are transparent, and proper adjustment of the illumination by moving the condenser will bring out all structures. The student should also make use of the phase contrast microscope. The apical tuft and the anal vesicle are landmarks for the poles, the mouth is on the ventral side, the eye is on the right side. Study the animals in lateral and in polar views (both from animal and from vegetal pole).

Observe:

1. Shape of the trochophore
2. Apical tuft. (several long cilia, probably function as sense organ)
3. Apical organ. a thickening of the ectoderm at the animal pole; a nerve center and probably the primordium of the cerebral ganglion.
4. 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. It consists of a few large prototroch cells which become pigmented in older stages.
5. Metatroch (paratroch), a circular band of cilia in the middle of the postrochal 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, stomadaeum (oesophagus-ectodermal) enlarged stomach (endodermal), narrow intestine (endodermal) except for the end portion which is invaginated ectoderm = proctodaeum, and anus, an opening behind the vegetal pole. Alll parts are ciliated. 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 the intestine and the outer body wall is not a true coelom but a primary body cavity, the persisting blastocoele.
12. The larval kidneys (paired) are typical protonephridia with flame cells; they open near the anus. They appear as slender cords near the statocysts, extending between the esophagus and anus. They are best identified in animals with vegetal pole up (consult figures in Hatschek 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 apicall plate, the other inserts at the esophagus. These longitudinal muscles. Other longitudinal muscles extend from the stomach to points of the upper hemisphere. A strong circular muscle is found near the metatroch; the constriction of the larva caused by its contraction will be 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 4a 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.

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ASCIDIANS

The subphylum Tunicata includes the sessile ascidians, the pelagic thaliaceans and the appendicularians with a permanent chordate tail. Each group has a unique organization and their interrelations are obscure. An excellent survey is available in Berrill's monograph ('50).

Only forms representative of the sessile tunicates (the Ascidiacea) will be studied here. Their adult structure and life history may be reviewed in a standard text or Berrill's monograph (see Bibliography).

All Ascidian larvae have the following chordate characters:

1. Hollow dorsal nerve cord.
2. Notochord ventral to nerve cord.
3. Pharyngeal gill slits, patent or incipient.

The post larval and adult stages of the life cycle lack items 1 and 2, but have well developed pharyngeal gill slits. The following summary of the life cycle of compound and solitary Ascidians will serve to demonstrate some of the major differences between these groups.

Solitary Ascidians (e.g. Styela, Ciona)

SMALL EGGS → EXTERNAL FERTILIZATION → ZYGOTE → DEVELOPMENT IN PLANKTON → SMALL TADPOLE LARVAE

↓

DISPERSAL: larval period few hours to 5 days

↓

ATTACHMENT AND METAMORPHOSIS

↓

POST-LARVAL DEVELOPMENT

↓

GAMETES (no budding)

GAMETES → EGGS

Compound Ascidians (e.g. Amaroucium)

LARGE YOLKY EGGS → INTERNAL FERTILIZATION → ZYGOTE → DEVELOPMENT IN ATRIUM OR ATRIAL BROOD CHAMBER → LARGE COMPLEX TADPOLE LARVAE

↓

DISPERSAL: few minutes to few hours

↓

ATTACHMENT AND METAMORPHOSIS

↓

POST-LARVAL DEVELOPMENT: reorientation of tissues

↓

OOZOOID (no gonads in some spp)

↓ budding

COLONIES OF BLASTOZOIDS WITH GONADS HERMAPHRODITIC

All Ascidians are hermaphroditic. Some species are self-fertile, others self-sterile. Styela, Molgula, and Ciona are most self-sterile. The best material for study is obtained from naturally shed gametes.

OBTAINING GAMETES: (1) Gametes can be obtained from solitary Ascidians by bisecting two or more adults in the sagittal plane with a clean razor blade, removing the gonads and gonoducts, and mincing them on bolting silk spread over a beaker of filtered sea water. Excess sperm should be washed away by changing the water several times after the eggs settle. Development can be obtained from a small to moderate number of eggs by this method at any time when the animals are "ripe", but this method is wasteful of specimens and should be used only if method 2 cannot be used.
(2) Styela normally sheds gametes between 4 and 7 pm. If adults are kept in the dark in oxygenated sea water until 10-12 hours before they are needed and then illuminated with a 40 watt bulb, eggs and sperm can be obtained (Rose, 1939). In Molgula and Ciona spawning usually occurs at daybreak. Animals kept in the dark can be brought into the light just before eggs are needed. Molgula sheds in about 15 minutes and Ciona sheds immediately after exposure to daylight.

EMBRYOLOGY OF STYELA: The egg of Styela was made famous by Conklin ('05). His 1905 monograph is the best reference on the normal development of simple Ascidians and should be consulted for details not outlined below. The following description holds for the eggs of Styela and, in some respects, for Molgula and Ciona as well. There are certain important differences which the student should work out for himself(e.g. the occurrence of yellow pigment in Styela, the large follicle cells in Ciona).

1. Structure of the fully formed egg.

The eggs of Styela are about 150μ in diameter exclusive of the chorion (Ciona 150μ; Molgula 100μ). A large germinal vesicle can be seen in gonadal oocytes but the GV breaks down soon after spawning. The spindle of the first maturation division forms near the animal pole and remains arrested in metaphase until fertilization. As soon as a spermatozoon enters the egg, movements of the cytoplasm begin. This results in the redistribution of ooplasm. The anaphase of the first maturation division begins, the first polar body is subsequently formed followed by the second maturation division and the formation of a second polar body. The polocytes mark the animal pole.

Post-fertilization changes in the egg can be followed in Styela especially well because the cortex contains lipid yolk granules (droplets). Critical microscopy is essential.

2. Post-fertilization rearrangements.

Within 2-3 minutes after fertilization, the peripheral pigmented ooplasm begins to stream toward the vegetal pole (near which the sperm has entered). The inner follicle cells also move similarly through the perivitelline space. While the cortical pigment becomes concentrated in a vegetal cap, gray yolk granules rise toward the animal pole. The sperm nucleus migrates up the

periphery of the egg to a point just below the equator, then moves inward toward the center of the egg where it joins the female pronucleus. The yellow pigment tends to follow the sperm toward the equator and soon assumes the form of a broad crescent. As soon as this stage is reached, the three major axes (A-P, D-V, R-L) of the future larva can be determined. The center of the yellow crescent marks the posterior pole, and the arms of the crescent wrap around the right and left sides. The vegetal pole is directed dorso-posteriorly, and the animal pole ventro-anteriorly.

Another remarkable feature of the egg at this stage is the fact that several areas are already visibly different from one another. Each of these areas is destined to form specific larval tissues. For example, the yellow crescent goes exclusively into muscle and mesenchyme, the gray cytoplasmic crescent on the opposite side of the egg constitutes neural and chordal materials (cf. Conklin's figures).

3. Cleavage. The following approximate time schedule is for normally spawned eggs; eggs from "minced" cultures are delayed apparently because they ripen at variable intervals after striking sea water.

From	To	Time required	Total time since fertilization
Fertilization	2 cells	40 min	40 min
2 cells	4 cells	30 min	1 hr 10 min
4 cells	8 cells	30 min	1 hr 40 min
8 cells	16 cells	20 min	2 hrs
16 cells	32 cells	20 min	2 hrs 20 min
32 cells	64 cells	20 min	2 hrs 40 min
64 cells	112 cells	20 min	3 hrs
112 cells	218 cells	20 min	3 hrs 20 min
218 cells	Neural plate	2 hrs	5 hrs 20 min
Neural plate	Tadpole	6 hrs 40 min	12 hrs

First cleavage: Meridional and equal; cuts yellow crescent in half, hence separates future right and left halves. Is the plane of reference for all cleavages after the third; hence cleavage is bilateral. According to the system of nomenclature proposed by Conklin ('05), the 2 cells are designated AB2 (right) and AB2 (left).

Second cleavage: Meridional and nearly equal, at right angles to the first. Separates 2 anterior blastomeres (A3 on right, A3 on left) from 2 posterior blastomeres (B3 and B3). The two B cells contain little yolk and practically all of the yellow crescent. The clear cytoplasm goes equally to all four cells.

Third cleavage: Equatorial and unequal, producing 4 animal cells (a4.2, a4.2, b4.2, and b4.2) somewhat smaller than the four vegetal cells (A4.1, A4.1, B4.1 and B4.1). Not strictly in the frontal plane but roughly separates future dorsal and ventral halves.

Fourth cleavage: Oblique, varies in different blastomeres. In the two vegetal A4.1 and the two animal b4.2 cells, the furrows cut in an ant-post direction to meet the 2nd cleavage plane. In the two vegetal B4.1 and the two animal a4.2 cells, the furrows run from the lateral border of the egg obliquely inward to reach the 1st cleavage plane (future mid-sagittal plane). Thus, the animal and vegetal hemispheres (as well as the right and left halves) become mirror images.

Sixteen cells are produced and they and their subsequent derivatives are labelled as follows:

Cell lineage in Styela (Conklin, 1905). Left half of egg only.

4 cells	8 cells	16 cells	32 cells	64 cells	Gives rise to
				A7.1	Entoderm
			A6.1	A7.2	"
		A5.1		A7.3	Chorda
			A6.2	A7.4	Spinal cord
				A7.5	Entoderm
	A4.1		A6.3	A7.6	Mesenchyme+notochord
		A5.2		A7.7	Notochord
A3			A6.4	A7.8	Spinal cord
				a7.9	Cerebral vesicle
			a6.5	a7.10	" "
		a5.3		a7.11	Epidermis
			a6.6	a7.12	Cerebral vesicle
	a4.2			a7.13	Epidermis
		a5.4	a6.7	a7.14	"
				a7.15	"
			a6.8	a7.16	"
				B7.1	Entoderm
			B6.1	B7.2	"
		B5.1		B7.3	Mesenchyme
			B6.2	B7.4	Muscle
	B4.1			B7.5	Mesenchyme (muscle)
			B6.3	B7.6	" "
		B5.2		B7.7	"
B3			B6.4	B7.8	Muscle
				b7.9	Epidermis
			b6.5	b7.10	"
		b5.3		b7.11	"
			b6.6	b7.12	"
	b4.2			b7.13	"
			b6.7	b7.14	"
		b5.4		b7.15	"
			b6.8	b7.16	"

In using the above plan of cell lineage, note that: (1) after 3rd cleavage, animal cells receive small letters (a and b), vegetal cells capital letters (A and B); (2) cells of right half have underlined letters (a, b, A, and B); (3) first exponent after a letter gives the generation to which a cell belongs; (4) second exponent indicates position of cell relative to vegetal pole. To determine the mother cell of a given blastomere: (1) leave letter unchanged; (2) subtract 1 from first exponent; (3) if second exponent is even, divide it by 2; if second exponent is odd, add 1 to it and then divide it by 2. To determine daughter cells reverse the process. It is also possible to pick a different type of problem to work on.

Fifth cleavage: The fifth and subsequent cleavages are more difficult to follow. Some details may be observed with the help of Conklin's

figures. The scheme of cell lineage can be mastered. In favorable material, the student may confirm Conklin's observations that different cells can be recognized by their natural color, as follows: endoderm-slate gray; chorda-lighter gray, less yolk; ectoderm and neural plate-clear and free of yolk; mesoderm-yellow.

Gastrulation and neurulation: Gastrulation begins during the 7th cleavage and is substantially over by the close of the 8th; some 40 minutes are required. The gastrula passes successively through disc-shaped, cup-shaped, and egg-shaped stages. Observe the gradual tremendous transition to the neurula stage and, finally, the formation of the definitive tadpole.

Metamorphosis and post-metamorphic development: Prepare cultures of developing Styela, Molgula and Ciona and study them at 1 day intervals. The small size of the larvae makes observations difficult, but many external changes can be followed. Older cultures which have been developing for a week or more will also be available for study.

EMBRYOLOGY OF AMAROUCIUM: Fertilization is internal in Amaroucium and the embryos develop within "brood spaces". The eggs are large (250u) and yolky. Eggs and embryos can be obtained by tearing colonies apart with forceps or by squeezing them. The early embryology of this species has been described by Scott, '45. Our main concern will be with the tadpole larva and metamorphosis.

Tadpoles are usually released from colonies after sunrise but if the colonies are kept in the dark, shedding can be postponed. Some larvae are usually released within 15 minutes after bringing the colonies into the light. The behavior of the larvae should be studied, observe their sensitivity to gravity and variations in light intensity.

Study the morphology of the larva with a dissecting microscope and then with a compound microscope. Note the size and relative complexity of this species as compared to the solitary Ascidian tadpoles. The siphons are well developed, but they are occluded by the test which covers the entire larva. The sensory or cerebral vesicle is located between the siphons. A large solid pigment granule is located within a single cell attached to the wall of the vesicle. This is the statocyst. Pigment associated with the ocellus is made up of many minute granules (posterior). Look for evidence of the pharyngeal stigmata and the endostyle. Notice the prominent adhesive papillae and the test vesicles. With good resolution the muscle cells, notochordal cells, and epidermal cells can be seen in the tail. Observe the dorsal, ventral, and caudal fins.

Metamorphosis: Place several larvae in a small amount of water and observe the first stages of metamorphosis. Scott ('52) lists some of the early events of metamorphosis in this species.

Place a dozen or so tadpoles in a drop of seawater on a dry watchglass. When (within an hour) the tadpoles have attached to the glass, add more sea water to the dish. After attachment the tissues of the tail withdraw into the body, the test swells, and metamorphosis is under way. Within a few hours, movements of the body may be observed.

Observe the rotation of the body and organs of the post larval stages. This rotation is effected by strong contractions of the larval body. The intestine, endostyle, yolk mass and heart rotate through an arc of about 90° within one hour after attachment (Scott, '52).

Observe metamorphosed specimens 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 off debri in the seawater tank, and avoid tipping off their cover of water. After making your records, return the specimens 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 by gently lowering a coverglass on them. At one end of the animal are the atrial and oral siphons; at the other end 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 indented 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.

Epicardial budding: Asexual reproduction of new individuals may be seen in laboratory cultures about 17 days after attachment of the tadpole. Or swarms of buds in all stages of growth and migration can usually be found at the bases of the tiniest transparent finger-like lobes of a large healthy colony.

Asexual reproduction is accomplished by Strobilization, i.e. segmentation of the post-abdomen which contains the epicardial strand. The buds consist 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. The epicardial buds while differentiating into new zooids move up and take their place around the parent. During the strobilization of the parent's post-abdomen, the old heart is isolated and degenerates, and a new heart is regenerated in the parent.

Permanent preparations of larvae and metamorphosed individuals can be made by the following method, kindly supplied by Sister Fordnee Scott.

Fix about a dozen specimens of desired age in a small Stender dish in Schaudinn's fluid (sat. sltn. $HgCl_2$:80 pts; abs. alc.: 20 pts; HAc gl.: 5 pts added before using). Heat mixture 60°C and pour over animals in a small amount of sea water. After 10 minutes change to pure fixative for ½ to 2 hrs. In this and subsequent changes, use pipette to remove and replace fluids, keeping specimens always in the same dish. From fixative, change to 50% alc (20 min), 70% alc plus iodine (overnight), 70% alc to rinse out iodine, 50% alc (15 min), 35% alc (15 min), dist. water.

Change to 1 N HCl (60°C) for 7 min, rinse in water, Feulgen stain for 6 min, SO_2 rinse for 5 min (1 change). Upgrade slowly through alcohols.

35%(10 min); 70% (15 min); 80% (20 min); 95% (½ to 1 hr); 100% (½ to 1 hr); 100% + toluene (½ hr); toluene (1 hr). Change to a small quantity of fresh toluene and add balsam a drop at a time over a period of 30 minutes. Permit toluene to evaporate. Mount in balsam, supporting coverslip with paper or chips of coverslip.

EMBRYOLOGY OF BOTRYLLUS: 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.

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 eight ampullae which are diverticulae of the body wall, destined to be parts of the as yet non-functional and incomplete circulatory system (Groves and Woodbridge, '24). 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.

Two-day old form atrial budding: Allow tadpoles 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 the three rows of stigmata which let water pass out into the atrial cavities of the two sides. The endostyle lies on the underside 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 oozoid) 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 (Berril, '41).

Week-old form: Will be provided. In these animals, general structure already studied may easily be seen under low power. The pharynx of the oozoid 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 symmetrical pairs. The same organ structures may be seen in all the individuals, not withstanding 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 seperate 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 (Berril, '41).

//Excelent accounts of the asexual reproduction in Tunicates, both descriptive and theoretical, can be found in Berril ('45, '51) and Trion ('48).//

The experiments and observations called for in the above outline are basic for an appreciation of the Ascidians as an experimental animal. Additional experiments will be assigned during the class interval devoted to the Ascidians.

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SUGGESTED EXPERIMENTS

1. Add Vitamin A, Hydrocortisone, Thyroxin to flourishing cultures xx of tadpoles and observe effect upon time of metamorphosis. Also use dilute solution of Janus Green (1:10,000 in sea water). Do at various times after hatching of tadpole.

2. Are proteolytic enzymes released during metamorphosis? Appropriate solutions will be available for doing B-glucuronidase assays.

3. Radioactive sulfate will be available for autoradiography of notochordal matrix. Nothing is known of its composition, but a fair guess would be that it is a sulfated polysaccharide.

4. Radioactive sulfate and appropriate solutions will be available to assay for sulfate-activating enzymes during the secretion of the notochordal matrix.

5. Melanogenesis in the eye-spot of the tadpoles can be inhibited with tyrosinase inhibitors. There appears to be a correlation between eye pigmentation and normal structure and function of the tail.

6. Regeneration studmies on: Botryllus, Perophora, Amaroucium, Ciona.

7. Colony fusion: Botryllus. What determines whether colonies will fuse?

8. Budding experiments with Botryllus, Perophora, Didemnum. Do inhibitors of DNA or RNA synthesis affect budding?

9. Mechanics xof tail resorption in Ciona. Does it differ from Styela or Amaroucium? Do the tails of induced tadpoles (e.g. Janus Green) undergoe normal metamorphosis?

10. For unknown reasons, eggs of Botryllus have not been fertilized out of the parents body.

11. Neurula and tadpole stages may be dissociated and grown in cell culture. To assist in freeing tissues from chorionic investment, various proteolytic enzymes will be available. Vertebrate serum, embryo extract, and plasma may be used to supplement the medium.

12. Study cleavage stages - Styela.

13. Solutions for nerve stain (Protargol) will be available. The xx connections between the eye-spot and musculature of the larva have not yet been determined.

WHOLE MOUNT PREPARATIONS: For tadpoles and metamorphosing animals

Fixation in Bouin's fixative, 15-30 min; wash in 70% alcohol until picric acid is removed (several changes over a period of 4-8 hrs.).

Stains:

Mayer's Carmalum

carmalum stock.....5.0 ml.
glacial acetic acid.....0.4 ml.
distilled water.....100.0 ml.

Stain 15-30 min, wash in two changes 70% alcohol and dehydrate through alcohols. Clear in one change abs. alc-xylene 50/50 and one change xylene.

Delafield's Hematoxylin

Stain used directly from bottle 15-30 min.
Wash in distilled water until water clear.
Destain in acidified distilled water (HCl-few drops 4N in 50 ml. H_2O).
Transfer directly to alkaline distilled water (NaOH-few drops 4N in 50ml H_2O). Animals will turn from red to blue.
Examine under microscope-if animals appear too dark they may be returned to acid water.
Dehydrate through alcohols to xylene.

Mounting:

A

Animals may be pipetted from xylene to slide and the excess xylene can be allowed to run off without washing off the material. Add several drops of HSR or Permount and some fragments of coverslip to the periphery of the mounting medium to support the coverslip.

Solution changes are best carried out in centrifuge tubes. Cleared animals should be transferred to watch glasses for inspection before mounting.

<u>Examples of prepared whole mounts are available for study</u>.

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Problems in Lab Embryology

Devillers, Charles - June 19, 1963

Class concerned i. embr of Vetrat

Structure and appearance of marginal zone and bilateral symmetry -

Professor at the Sorbonne, Paris

Has been in residence at Yale University since last September and is presently the Lillie fellow at The Marine Biological Laboratory.

I Introduction

A. Contrast teleost & Amphibian egg structure

1. Amphib - Bilateral symmetry determined.

2. Teleost - only an-veg (cephalocaudal axis) determined

B Structure of trout egg at spawning & early cleavage

1. Disc

2. Periblast - emphasizes contribution of periblast cytoplasm to embryonic disc - goes on through to blastula

3. No obvious plane of bilateral symmetry until blastula is formed.

C. Earlier evidences of symmetry

1. Oppenheimer - differentiation of good embryo of Fundulus from blastoderm isolated in Holtfr only after 32nd cell stage.

2. Tung - Goldfish - 6 cell stage is critical

3. Devillers - late blastula is critical.

He ~~suggests~~ says that Oppenheimer suggested migration of special substance or plasm from periblast to blastoderm - could setup symmetry

9. Tung's expts bisecting entire egg – Concludes plane of bilateral symmetry is established prior to first cleavage. – The germ itself has no plane of bilateral symmetry, (is isotropic), but the periblast cytoplasm has asymmetric distribution of material – This moves into the germ by 6 (?) cell stage.

II Denellen's expts on trout egg

A General remarks about trout egg

1 Large 4-5 mm yolk granule

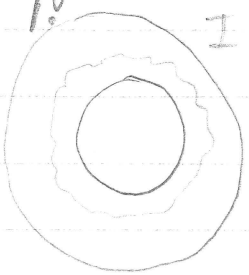
2 Small embryonic disc

3 Hard to keep alive after operation, but easy to operate on –

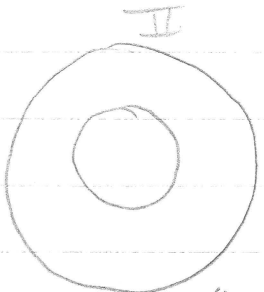
" In some (rabbit) trout eggs, eccentric position of blastocoele shows plan of bilateral symmetry at time of blastula.

B. Experiments

1.



morula



beginning gastrula

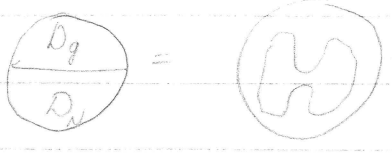
Attach germ I to yolk II. – it undergoes epibolic but forms no embryo or a poor one.

Conclude – Tung's substance has not reached the graafian germ & not present in host yolk

2. Epen embryonic half – the ventral half regulates and makes a perfect disc – at gastrulation, it gives a perfect embryo normally oriented. — Considers that the central peridast is controlling orientation.

3. Replace embryonic half with ventral half (embryos same age) — Gives one embryo in normal orientation — Competition between 2 parts of same prospective value – the winner is the one in the portion of the normal gem.

4. Remove ventral half & replace by a dorsal half –



– 2 embryos opposite each other —

Central peridast not able to change the fate of a dorsal half — –

5 Rotate blastoderm 180° – rotate embryo accordingly –

Microsymposium on Teleost Development

Trunkau – Saturday June 22, 1963

I Introduction

A. Germ at time of gastrulation begins
   1 Blastula
   2 Periblast

B. Spreading movements both – Blastoderm spreads <u>on</u> periblast
   1 Periblast runs & precede blastoderm in epiboly initially
   2 Blastoderm catches up
   3 By time blastopore is ½ closed, periblast once more gets ahead of blastoderm & periblast closes blastopore first.

II Questions –

A. Does periblast require blastoderm for epiboly –
   1. Detach – periblast goes alone

B. Will blastoderm reattach.
   1. Detach – wait while periblast epiboly continues
   2 Reattach – Blastoderm catches up but never goes beyond periblast.

C. Does spreading capacity require <u>intact</u> blastoderm?
   1. Remove shield → rest of embryo undergoes epiboly – (No embryo – melanophores appear)
   2 Remove part of central germ ring – Likewise remainder of blastoderm spreads.

D. Do the cells tell us something about epiboly?

1. Dissociate cells in versene - observe behavior on glass.
   a) Blastula cells chiefly show blunt lobopodia
   b) Gastrula cells largely flatten on glass
2. Comparisons by stage - shows progressive flattening behavior (all within 6 hrs) correlated with beginning of gastrulation - Stages 8 → 11 2/3
3. Flattening is much more frequent when cells re-aggregate!

III Conclude -

A. Gastrula cells have greater adhesivity. Change in surface property such that they stick to each other & to ~~the~~ glass

B - NB - the change stable thru dissociation process

Discussion - -

Inhibit mitosis c̄ colchicine - - epiboly goes on!!

Reaggregation occurs more rapidly in gastrula cells than gastrula.

NB - Cells crawl over each other in epiboly

Devillers – Saturday – June 22, 1963

I Problems concerning yolk

A. Formation of yolk in oöcyte –

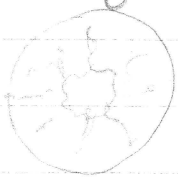
1. Vacuoles in which yolk droplets appear
2. These fuse into homogeneous mass. –
3. – Note difference from organization of frog egg.

B. Relationship of yolk & cytoplasm.

1. Note sturgeon egg as an exception – more like an amphibian egg
2. Note Amia – studied only by Whitman and Eckeyshymer. – Yolk in form of platelets as in amphibian – Holoblastic cleavage! even tho lots of yolk.

II Bipolar Differentiation

A. At spawning – Stain egg c̄ vital dye – get appearance of streaming of protoplasm towards disc.



← seen from animal pole –

1. Mechanism of streaming?
2. What activates this streaming in spawning?
   a) In trout – activated by contact with H_2O
   b) In anoxia or in with KCN (M/1000) the movement stops – reversible
3. Note difference in degree of mixing of yolk & cytoplasm in trout & in zebra fish

B. Proposes that some interaction of cortex & yolk leads to motility

Discussion — Hauser points out that in primitive fish the yolk droplets remain separated -- suggests that complete separation of yolk & cytoplasm does not occur --

Trinkaus suggests that fine structure studies must be done.

Devillers - points out you can't change position of blastodisc — Hauser says he concurs for majority of cases, but in pricked eggs he sometimes gets disc formed at point of pricking & sometimes 2 discs; one below micropyle & other at point of pricking.

Crawford – Energetics of Development June 22, 1962

I. Intro –

A. Purpose of such investigation

1. Control of Development
2. Clue to fast the differentiation has occurred might be found in change in energetic pathway – indication of new protein synthesis

B. Specific

1. Are there changes in source of ATP during
2. development
3. ~~Are~~ development affected by the kind of ATP source?

C. Note that in anaerobic metabolism, ATP comes directly from oxidative phosphorylation of ADP from high energy substrate – – But in aerobic metabolism, it comes from electron transport

II Inhibitors – But with Cyto C_5 (in microcomes) (new) one can bypass the antimycin block ~~to some extent~~ – this only for DPNH reduction – still no ATP formed however.

A. Intro –

Antimycin A inhibits between cyto b & cyto c,
Dinitrophenol prevents coupling of energy of oxidation to form ATP
Cyanide blocks cytochrome oxidase

B. Using Antimycin – DPNH – cytoc reductase system not inhibited in young embryos, but 100 % in adult muscle & little inhib in adult liver.

C. Cyanide did not kill Fundulus but did stop development or differentiate — remove from Cyanide & get development (.02 M is a high dose, but doesn't kill). Dolimpt accelerated!!

D. ~~DNP~~ Dinitrophenol in Salamander T.C.'s knock out

Conclude ATP from glycolysis is enough for maintenance, but for differentiation we need Oxidative part of cycle — Maybe small change in local source or small change in quantity determines whether differentiation can occur.

June 22, 1963

Goss — Triplett & Barrymore

Transplant scales → Pigment cells disintegrate in homograft reaction — a very abrupt reaction — rate at which it is achieved is temperature dependent. — In Cymatogaster adults 1st transplant breaks down in 7 days at 20° C — with repeated grafts, get it down to 3 days

In 1 to 3 day fry, reaction time is 13 days, but by 15 day embryos rejection reaction is 10 days

When grafts made to pregnant host, her offspring can be grafted from same donor! — Will rejection time of offspring be accelerated? Yes — 1 to 3 day embryos from mother sensitized once rejected in 8 days instead of 13

In Cymatogaster, the embryos develop in the ovarian follicle — maybe transfer thru ovarian

Goss in Fundulus — graft pigmented scales to white tiltys — Pigment cells disintegrate in 3 days at 28° — — 4 to 6 days at 20° 14 to 16 days at 14° — at 7°, no disintegration in 26 days —

Treat fish with antimitotic agents after homografting to see if mitosis is a necessary concomitant of protein synthesis

Purine & pyrimidine analogs fed — the treated fish showed prolonged survival of homografts —

Chloramphenicol & Puromycin also prolonged homogr survival

Some amino acid analogs were slightly effective
- Possibly wrong kind of aa does not render protein greatly less effective as antibody

Mitomycin C (very lethal - $1000 gm) supposed to specifically interfere with DNA synthesis - didn't get any thing yet

With Colchicine, the fish reject homograft on schedule.

Gross suggests maybe mitoses is the result of rather than an antecedent of antibody production

Op 65 Madison 233 2929

Brock - June 27 - Properties of Cell Surface in Microorganisms

I Intro -

4 types of specific associations of microorganisms

1. Non-sexual - (a) flocculation of yeast cells in "bottom fermented" beers - When cells (population) reach a certain size they specifically adhere & flocculate - 3 genes involved in this flocculation process - a protein & a polysaccharide are involved.

(b) In certain bacilli, cells adhere side by side prior to spore formation.

c. Specific ones - bacterial attachment at infection.

2. Sexual associations -

a) Sperm & egg

b) found in yeasts, bacteria, algae etc

B. Advantage of many sexual associations - Evolved at and the expression of adhesivity -

C. Hydrogen bonding mediating specific association -

II Yeast - Kansenula - has a sexual association

A. Life cycle -

Kansenula diploid mate - agglutinate - form diploid ~~haploid cells~~ which, under right conditions reproduces vegetatively - occasionally sporulates, producing 4 haploid spores, 2 of each mating type.

B. Effect of varying #'s of cells of each mating type —

1. Best ppt when mating types are in 50-50 ratio. — It's not 2 & 2, but all cells adhere & as many of opposite mating type as there is room —

2. Even isolated cell walls agglutinate

C. Effects of heat — — Agglutination enhanced by treatment at 100°C —

D. Ph Optimum — from 3 to 9 — — Specific substance of one mating type destroyed by high ph — Don't know why it does not agglutinate prior to Ph 3. —

Isoelectric ph is 1.5 — thus all agglutinates at ph 3 to ph 10 is with cells negatively charged

E. Agglutination will not occur at in washed cells suspended in dist H_2O. — Various non-specific co-factors — NaCl, $CaCl_2$ mucin etc — also protamine sulfate, gelatin — will permit agglutination when added.

F. Effects of extracting cells on their agglutinability —

Extraction	Str 5	Str 21
80% phenol	++++	0
8M urea		
Et OH	++++	++++
Acetone	++++	++++
1N NaOH	0	0
1N HCl	++++	++++

+ = retains agglutinability.

trypsin knocks out specific agglutinates components of Strain 21 but not Strain 5.

Quantitatively, trypsin removes as much protein from one strain as the other. But —

Penicillin, binding polysaccharides, knocks out aggl for Str 5. — Thinks they adhere by the portion of 21 adhering with polysaccharide of Str 5

Alcohol ppt of simple growth medium — from Strain 5 agglutinates cells of strain 21. It is a polysaccharide, has been crystallized — has no amino acids. — Multivalent polysaccharide

Protein extracted from medium of Str 21 prevents agglutination of Str 5. — It must be monovalent, can adhere by only 1 combining site, thus can't stick cells together.

Genetics. — Genes controlling mating specificities are alleles in most microorganisms. — Must determine for Neurospora whether this is so for its mating types.

Cellulose inhibits prot synthesis in yeast & mammalian cells — Same for chloramphenicol does in bacteria.

Proteins synthesis required for conjugation —

Why do not diploids agglutinate with either parent strains ? — Antigens formed in diploid — Ag products mutually inhibitory & don't get to surface.

Bishop - June 28, 1963

Tissue Specific immune Responses and
- Testicular Immunochemistry -

I. Background -

A. Freund & collaborators -- homogenize testis, - inject with adjuvant - - Intra ~~Sub~~ cutaneous !!! Inject into (single injection) another guinea pig -- Germ cells completely destroyed, but interstitial & Sertoli cells remain - Secondary sex characteristics

B. Bishop now has highly purified antigen which produces effect, even injected without adjuvant & intramuscularly

C. Antigen comes from the acrosome, apparently - First knocked off by the antigen - Testis without differentiated sperm because of immaturity or damage will not produce effect -- ie does not contain antigen

D. Testis reacts to anti-brain antibodies in same manner - only deviation from "spissus specificity found.

E. No regular pattern of recovery - not sure, therefore, if reaction is reversible.

ad Dow

~~Does reaction work with other species~~
How about isologous

F. Do not get this reaction cross-species —
G Best response is with autologous injection
— even better than homologous. ~~—~~ — ~~How~~

II Can we block differentiation of germinal epithelium?
A Inject neonatal guinea pig — Find spermato-
genesis goes on for 1 month — sometime
within 2 weeks more, ~~[illegible]~~, the germ
cells are all gone! — Necrosis evident
even ~~when~~ in early stages when spermato-
genesis is still in process.
B. Antibody production begins before birth in
guinea pig — (sheep also) — thus no tolerance.

III ~~Various autoimmune diseases can be duplicated~~
~~A. Rabbit tra~~

III What if adjuvant + antigen not mixed?
A. Repeated inj of antigen sans adjuvant will
produce damage — Contrast c̄ single
shot with adjuvant
B. Put adjuvant injection near antigen inj
on same side → reaction
C If ~~separated~~ ~~by~~ injected alone, one either side
of midline, → no reaction !!!
D. Why? Don't know!!

Good cytol studies not made of the breakdown process in testis — cells come off in layers —

polygalactose Fb

IV Purification — turns out the galactoside moiety is antigenic

A Ammonium sulfate purified fraction } went to sleep & missed —

B Chloroform purified material —

V Serum of injected animal transferred to non injected animal — never gave an aspermatogenic response.

VI Questions

A. Why is this phenomenon limited to testis??

B " " " " " " Guinea pigs?

Variations with genetic factors

a. Strain = 4/3 are more responsive than other strains.

C. Where does antigen go when injected? — does it induce a new synthetic pathway? Modify an old? — Is it a template

Ask Dave about necrosis — in hypersensitive skin in testis —

Question says — who? * Selective destruction of testis by Cadmium

Searles says dithiol enzymes inhibited by Cadmium — uncouples!

All acrosomes stain c̄ PAS

Microsymposium June 29, 1963

Albright,

I Propose to show that proliferation is an integral part of the production of soluble antibody

A. Antibody production curve is like a growth curve

B. Antibody production from plasma cells

These increase after antigenic stimulation

Organizational change in spleen & lymph nodes — support idea of proliferation

II Test system — Irradiated Recipient or (host) receives spleen cells from "primed" (preimmunized) donors —

A. Amt of ab. produced by host is proportional to # of cells injected

The donor cells are put in diffusion chamber

they produce antibody one can detect it with fluorescence techniques

Mitoses in one of 7 cells during big phase

— only 1 in 35 in controls

Small cells are counted the "Reticulum blast & immature plasma cells show increase

Other cell types — lymphocytes, histiocytes

granulocytes normoblasts — no change.

Plot of later res. antibody containing cells

with K

+ ab. cont. cells

Auerbach [illegible]

I Embryonic origin

A. Mesenchymal capsule around epithelium - which rudiment forms the lymphoid cell.

B Isolation in culture

1 - Epithelium alone

2 - Epithelium + mesenchyme gives ~~morphogenes~~ growth & budding of epithelium -- morphogenetic pattern different c̄ different mesenchymes.

3 Separate epithelium from mesenchyme by filter

a) epithelium proliferates & makes buds only opposite the mesenchyme brass filter

C Species Chimaeras -

1. Chick-mouse inductions work.

All lymphocytes come from the epithelium

D Single cell suspension reaggregate & make thymus.

II Differentiation -

A In mouse, from 16 days to birth the maturation from large lymphocytes to small ones (coulter-counter studies).

B - This goes on in vitro almost identically with what goes on in vivo

II Thymus lymphoid differentiation in anterior chamber only of isologous hosts > ~~Ab~~ homografts. Only an epithelium formed — Makes thymus look interesting immunologically — When one looks at the + previous.

Is thymus the primordium for cells involved in immune response

A. Corollaries —

1. Immune

Miller — Neonatally thymectomized mouse accepts homografts — Others showed it for rabbits + rats — plasma cells decreased, gamma globulin decreased

Presumably, a spleen should not get lymphocytes in absence of a thymus — In culture it does not.

Someone? —

Diamond -

Classifies antigens

Species specific - what do they mean? are they better than taxonomic criteria

Group-specific for ex blood groups

Individual specific - revealed by transpl immunity - is this true of lower phyla? - can we isolate - problem of gene expression.

Tissue specific antigens - whence differences trivial

Heterogenetic antigens -

Problems -

Biochemical

How statistics

The immune response - how do above antigens correspond to classical antigens.

Sensitization -

Damage following immune response

Tolerance -

Are tissue specif antigens likely to be not readily diffusible & thus other tissues do not get in contact -- infections

Should we consider possibility that "Group" specifications are characteristic [illegible]

Jane Kattenbach

Kollros, Jerry J. July 5, 1963

Tissue responses to thyroid hormone in regeneration -

I Introduction

A. Reduced powers of regen after metamorphosis in amphib

B. Metamorphosis & Requirements thereof -

1. Stages of metamorphosis - Taylor & Kollros for R. pipiens
NB. These stages are closely graded -- St 11 is latest stage at which regen can occur in any cases after amputation thru thigh.

2. Control of Metamorphosis

a) Acquisition of sensitivity to thyroid hormone - Insensitive until Shumway st 23 whereupon one tissue after another begins to show sensitivity

b) Each metamorphosing tissue has its own threshold and, in act of responding it then changes its threshold.
Hind limb responds to .0002 g/ml but threshold goes up to .04 g/ml.

c) Each tissue has a rate-concentration characteristic - which enables one to put metamorphosing events out of synchrony - Also there is a maximum dose beyond which no further effect

d) Temperature dependence -- Lower temperature & increase threshold - 3½ fold increase when temp lowered from 25° to 15°

e) Analogs of thyroxine — differential effects on different tissues

f) Pituitary gland → TSH → Thyroid gland → thyroid hormone.

g) "Readiness" to respond ~~is differ~~ among a list of tissues & events is in different sequence from order in which they respond.

3. Regeneration of amputated ~~Tadpole hind~~ limb (showed slides of newt from Singer).

a) Migration of cells to form cap epidermis

b) Clean-up period - cell breakdown and phagocytosis

c) Cell proliferation in stump just back of area of breakdown

d) "Dedifferentiation" of stump tissues to form blastema.

C. Requirements for regeneration

Amurogenic regenerate

1. Singer & Nerves -- threshold factors

2. Appropriate hormonal milieu.

a Hypophysectomized → no regen unless done 10 days or later after amputation (adult newt)

II Frog regeneration

A. Progressive loss of regen. power — from proximal to distal — thigh loses by St XI — shank by XVI.

Van Stone suggests this parallels diminution of cross sec area of nerve in proportion to x-sec area of limb — Reduction from stages 9-11 thru St 18 in # of cells of lateral motor column supplying limb — No reduction in hypophysectomized — give thyroid & # of cells reduced — Hughes & Bretton a year or two ago!!

First effect of thyroid is stimulate of nerve growth

N B for cell death!!!

Look up reference cf. Chuck-Rita-Lever Mortalities

B. Restoration of capacity of adult frog limb to regenerate

1. Rose — salt treatment
2. Polejaev — trauma — pricking
3. Singer — deviate sciatic nerve to ipsilateral forelimb — cut — get regeneration.
4. Schotte & Wilber — Implant adrenal into lymph space under jaw — get ~~beginning of~~ regeneration (Correlate c̄ restoration of regen power in hypophysectomized newt).

In hypox frog, adrenal gives only the beginning of regeneration — at best a fair blastema.

C. Can we get thyroid conc such as not to cause degen of lateral motor columns but sufficient to give increased regen activity of limb tissues.

1. Kollros lab -- use hypophysectomized animals; bring them slowly along c̄ low thyroid hormone treatment. — Ordinarily limb cut thru thigh does not regen ~~ap~~ cut after St XI. K & students have brought limb to stage XIV & get regen of digits after cut thru thigh.

D. Xenopus Lens

1. Lens regenerates lens ~~fr~~ in tadpole, but not ~~an~~ adult.
2. Lens regenerates from cornea
3. Adult cornea implanted in Tadpole regenerates.
4. Local thyroid treatment of tadpole cornea prevent its regeneration of a lens.

D. Skin - Local effects of thyroxine implants

E. Speidel -- Thyroid ~~regen~~ treatment of regenerating tails — old tissue responds earlier than young —

In regenerate, thyroid hastened the

completion of each regenerative phase &
more rapid entrance into metamorphosis
Pretreatment with thyroid inhibits
regen of tail.

F. - Mesencephalic V^th Nucleus — Large cells
Responds to thyroid — Brought along by
very low doses of thyroid, they are very
small as compared with control 5th nucleus
cells.

In Xenopus larva size of mesencephalic 5th nuc cells dependent
on continued presence of thyroid - without
it, they shrink.

Dr. K. C

Can Dr Stewart have his room an extra night?

[illegible] fired? any other differentiation? any collagen?

Can you continue the cells in bipolar state

Cell Death in monolayers? Has anyone really studied it?

Stewart –

I Intro — R

A. Review angiosperm fertilization & embryo formation

B. Haberland – 1902 suggested that isolated cells could make a whole embryo

C. Van Overbeek v — Coconut milk to culture embryos

D. 1 growth is unorganized when plugs of carrot tissue planted

2 Growth curve –

DNA synthesis in Lag period?

D. Corn "milk" – Horse chestnut "bladder" & & this all have growth promoting fluids –

1. Not due to auxin

2. Separable into 2 factors, neither of which is active alone, but active when recombined –

a) the "neutral fraction hexitols" (sorbitol, myo inositol sylo inositol are identified –

b) the other fraction uncharacterized

Can you maintain cultures of free cells.

III

E. Synergistic substances required for other plants – eg. Potato needs 2,4,D (dichlorphenoxyacetic acid)

II Free cells in the medium of revolving cultures.

A. These float free & divide –

1. In a manner quite different from that shown in confines of plant body.

B. Multinucleate giant cells form – septation occurs – clump formation

C. Some show bipolar organization c̄ "cleavage" planes as in an embryo.

D. Frequently, when single cells divide, the progeny are not alike – i.e. one may make anthocyanin & the other not.

E. Roots forming spontaneously in liquid culture from free cells.

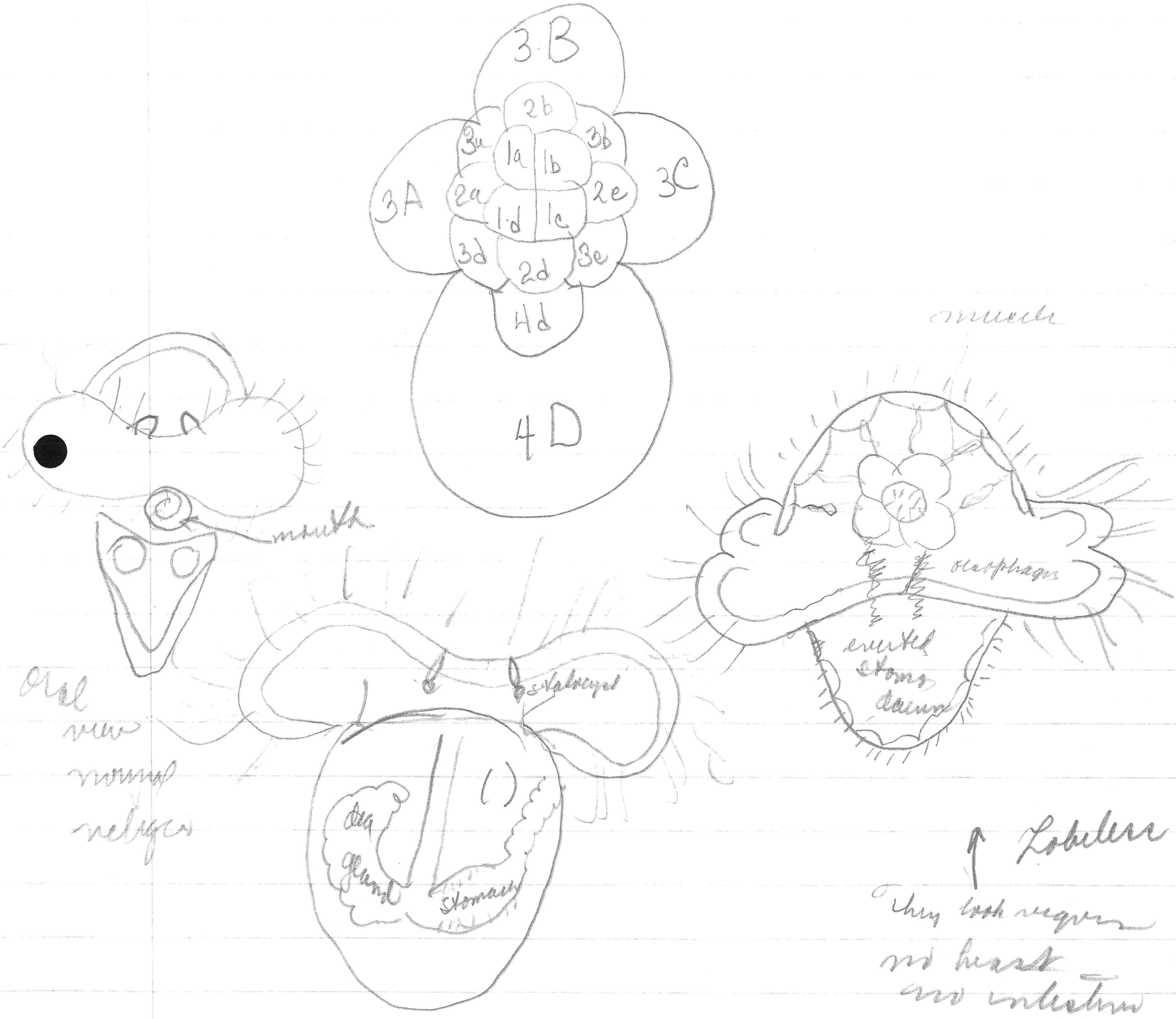
1. Now transfer, after root begins, to an agar medium –

Get stem & leaves & eventually an adult plant

Embryos of these plants can be put in cell free culture (contrast to normal embryos) & these will recapitulate normal embryology

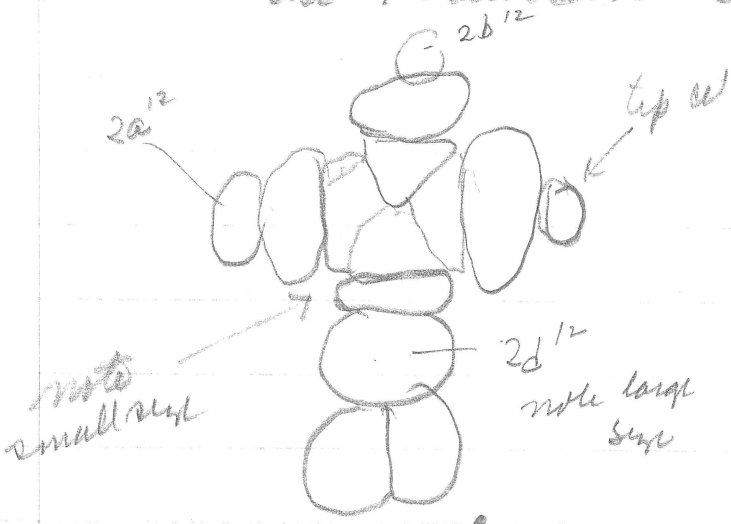
Tony Clement – June 9, 1965

– Analyse of Development of Styanassa –

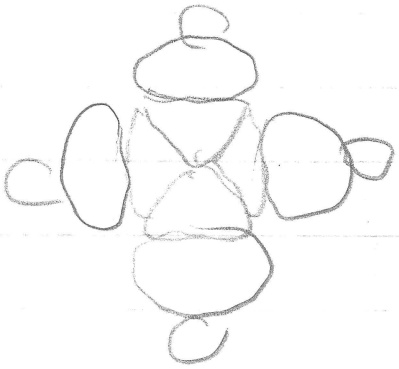


A. Effects of removing polar lobe

1. 1d is same size as others of 1st quartette instead of smaller as in normal
2. In normal, 1d lags in cleavage but in lobeless, it is synchronous
3. The Nolluscan cross is different



Normal embryo cross



Lobeless cross

4. Separate AB from CD
   a - AB is essentially a "lobeless" in development
   b - CD gives a fairly good one -
5. Isolate at 4 all stage
   None develop well but D, if it survives, does best.

B. At what stage is lobe stuff active? How does it act?

a Remove D macromere after 4d formed
Get small, but normal larva
Conclude all lobe stuff now out of D.

b. Remove D at various stages

i 4 cell → "lobeless" embryo

ii 8 " → " "

iii 16 cell → " "

(NB – 2d is now form but these seldom give rise to shell in absence of D.

iv after 3d form → get a better on eyes present – but heart not present nor intestines. (NB 4d gives rise to endoderm cells which go to intestine.

		Shell	Eyes	Velum	foot	heart	intestine
–D	aBC						
–1D	aBC + 1d						
–2D	aBC + 1d + 2d	(+)(–)					
–3D	aBC + 1d + 2d + 3d	+	+	+	+		
–4D	aBC + 1d + 2d + 3d + 4d	+	+	+	+	+	+

JEB 149, 1962

to effect due to cytoplasmic segregation or induction.

a) Remove 3d only - get variable results - but invariably only 1 statocyst & left half of foot missing -

b) Remove 3c & get right statocyst & right half of foot missing - known variable in other experts.

c) Remove 3c & 3d - get eyes - velum & but no foot

d Remove 2d - get eyes - velum - foot, but shell very poor - red fairly good -

e) Remove 2a & eliminate correlatively left eye & left velum -

f) Remove 2b has given normal

g) Remove 2c - No heart - shell reduced -

h) Remove 4d - No heart & no intestine but eyes & statocyst -
Conclude heart & intestine materials by cytoplasmic segregation.

Left eye, by cell lineage comes from 1a & right from 1b - but wont form without 3d micromere → induction

Have know lobe stuff as what gets in to d?

C. What is distinctive about lobe cytoplasm?

1. Lehmann & Clement – Janus green – mitochondria stain in animal pole – very few in lobe. Zone of Mitochondria below 1st quartette in 8 cell stage – Eventually all micromeres get a load of mitochondria.

muscle needs mito?

2. Reverberi – Dentalium – found a lot of mitochondria in polar lobe.

3. Look with E.M. – Reverberi compares using Mytilus – no real difference detected.

4. Compare O_2 uptake of lobes, & segregated AB & CD. Show 25% lower uptake by lobes Bell Berg

5. Collier – alanyl-glycine peptidase – – no significant difference between regions of egg on basis of fluid cytoplasm content

6 Berg & Kato 1959 – Lobe region has 2X the content of low mol wt nucleotides as has rest of egg

7 Collier, studying phosphorus, confirms.

D Collier & Collier –

Ovarian egg has 32X amt of DNA as expected. New synthesis does not begin until about 25 cell stage. – Lobeless synthesizes less DNA than normal

Noles – 1961 – impaired protein synthesis in lobeless.

Morgan centrifuged Ilyanassa (?) eggs upside down & centrifuged -- Lobe formed in normal plane.

Porter -- July 13

Ribosomes on membranes - synthesis for export
Smooth ER. considered to be assoc c̄ carbohydrate metabolism
and transport.
Porter considers nuclear envelope as ER

Notes in embryonic stages here is little ER & little ultrastructural
sign of polarity -
3 Problem to be taken up
1. Increase in bulk - cytoplasm ie myofibrils / Hemoglobin / Keratin } etc

2. Achievement of Organization

3 Fine structure expression of origin of asymmetry

Difficulties - reconstruction of dynamic events from static
sections -

Erythroblast - ~~Fo~~ lots of ribosomes in clusters & loose polysomes
nucleus. - Mature erythrocyte shows no clusters - it
is uniformly dense - polysomes not seen in mature cell
- probably gone!

In liver, development of all clusters of ribosomes disappear & progressively, ribosomes are found chiefly on rough ER.

In myoblasts Hay describes strings of ribosomes appear in strand which may be beginnings of myofibrils — are the ribosomes along strands of messenger or myofibrils — Ribosome population depleted in mature muscle cell — practically none left.

Regeneration of cytoplasm in ~~cytolyzing~~ chromatolyzing neurons shows strings of ribosomes — possibly spirally around a central axis.

Where do ribosomes come from? — from nucleolus? — Do the ribosomes move thru nucleus & out nuclear pores? — No one has ever photographed a ribosome in a nuclear pore.

Tapetal cells of microsporangium — Cytoplasm loaded with ribosomes — Later, however, very few ribosomes left as membrane system develops a bit —

In plant, ribosomes in cyto resemble strongly similar particles in nucleus — Are thru ribosomes & do they get out in mitosis?

Since spindle largely comes from intranuclear material, if we find ~~nucleolus~~ ribosomes in the spindle

it would suggest they get out in mitosis

E.R. has a morphology which is characteristic of each cell type. In muscle it is a system of systemae wrapped around each fiber & interrupted at each Z band!

When cell is damaged, the morphology is lost. Some time it will repair.

Look at tracheid with its spiral thickenings. The photo shown has a large ER profile in between each turn of the spiral — as the thickening formed at interruption of E.R — Highly speculative!

Monday - Spiegelman & Ebert
" PM Hastings Cohen Halvorson

Mon & Tues at 1:30 Monroy

Tues AM Leventhal
Wed AM Pollack, Keynan & Sussman

Porter — July 14 —

I Origin of E.R. —

A Undifferentiated cell — infrequent profiles of vesicles & cisternae + nuclear envelope. — Does it unfold from nuclear envelope? — Evidence for continuity is clear cut in many cases. — Showed picture of amphib notochord cell which shows this — also in African violet.

B. In oogonia & spermatogonia. One frequently sees layers of annulate lamellae which seem to have come off in sequence from nuclear envelope. — pinching off of vesicles from nuclear membrane & their fusion ~~[illegible]~~ — Texture of material in annulae similar in nuc & in lamellae.

C nuclear Envelope behavior in mitosis. — Plant.

Plant cells
Fragments of nuc. env persist in the spindle and distributed to daughter cells. — At metaphase, fragments collect near spindles & seem to proliferate (did old E.R. invade, rather? — Spindle seems to exclude material from outside).

All this illustrated in a plant.

At Telophase, new nuc. env. seems to form out of fragments around nucleus.

2. Animal cells -- is the story different?



as centrioles divide, nuclear envelope is drawn into peaks as illustrated.

These peaks can give the appearance of double cisternal profiles! assoc near nucleus.



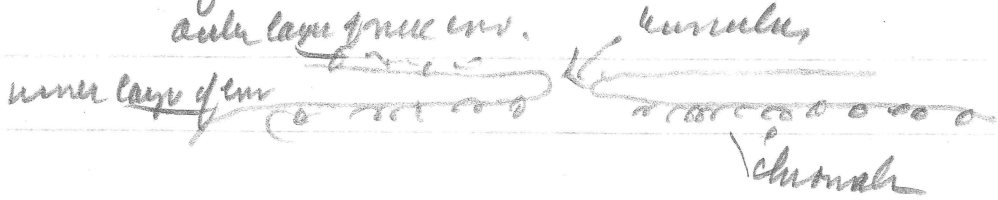
← no granules in here! granules on outside & also on outside of nuc envelope.

These peaks, too, contribute fragments as shown for plant. & they may also contribute ER of new cell.



no granule here.

3. Chromatin material against inner layer of nuclear membrane —

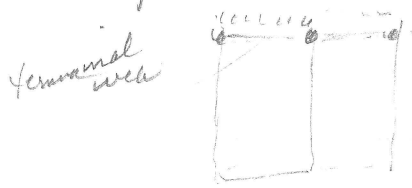


Differentiation of nucleus seen in # of pores & relationship of chromatin!

II Maintenance of cell shape — Surface tension properties alone will not account for cell shape.

A. 1960 Leblond recognizes c̄ light microscope a "fibrous" cortex — Terminal web, so called.

eg.



epithel cell might show it here — as in kidney tubule.

B. Leblond missed the peripheral band of nucleated erythrocyte — described 60 yrs ago in amphibia by M—— (?)

1. Fawcett saw similar thing in erythrocyte of toadfish — appear in EM as tiny tubules of uniform size.

2 Philpot, in Porter's lab gets the same! Diameter ca 200 Å — 35 or 36 of them in an X sec

C. Cortical fibers in ∥ distribution in trypanosomes seen by Porter — Steinert & Novikoff found these very prettily just inside & attached to plasma membrane



D. J.

Spermatogenesis.

A. 1. Manchette, or collar, in spermatelessis — made up of fine tubules in ∥ array.



— it is transient

2 Planarian sperm - shows these tubules around the margin of head. - Seem to be same size & appearance as tubules of the flagellar of this sperm

3. Note this same filamenture in cilia of various forms. — these are the skeletal elements & locomotor elements of cilia

E. Spindle - Note that the spindle fibers likewise are in tubular form - exist in bundles — Found in both plant and animal cells -- only recently in plant — Porter's lab. -- See similar ones in timothy adjacent to plasma membrane but only in interphase - Do they play a role in synthesis of cell wall. - Cellulose fibers parallel them - parallel in long dimension - mixed up at ends.

F. Neurotubules - especially found in dendrites & extend into cytoplasm of neuron, then taking on a more random distribution.

G - Note that the filaments of the muscle cell - (is the myosin on the outside of tubule?) - they have the right size & morphology to be related to the other tubules we've been talking about.

Colchicine abolishes these tubules in many cells -- Is this the reason colchicine affects mitoses?

Rehbein comment - Hetrozoan spikes show bundles of these tubules running length of spike

Porter seems to think that as fixation methods improve well see more of them -

During talk mentioned preparation in glutaraldehyde wet post osmication.

DEVELOPMENTAL CYTOLOGY

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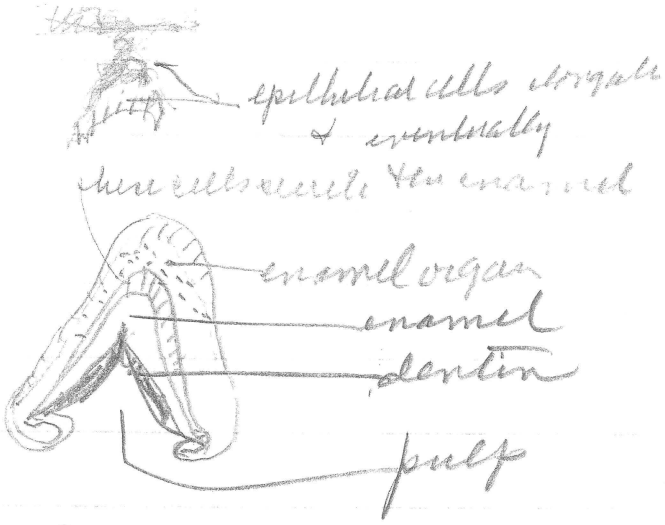
Rhodnius - passage of material from nurse cells to egg

Anderson & Beams

Glimcher Mass General — July 12, 1963
— Devel Cytol of Tooth —



oldest enamel at junction with dentin

as thin as 6° but usually

Enamel crystals are thin elongate platelets 15-20 Å thick — 350 Å wide — 1200 to 1500 Å long.
Enamel "prisms" oriented ⊥ to tooth surface — crystals are stacked in prisms. —

As enamel ages, the orientation of the crystals is progressively more uniform — better oriented — thus closer to the dentin. Also crystals grow in thickness — 150 Å
Interprismatic crystals is at right ∠'s ~~to~~ to those in the prisms. —

Each prism has a sheath of organic material around it — it never becomes mineralized.

At dentin - enamel junction —
Dentin is mineralized collagen.
At junction, the crystals in dentin & in enamel
retain their order & integrity.

What does organic matrix of enamel look like. — Decalcify on grid — find protein fibers about 40Å wide — oriented ∥ to crystals. — Probably orientation of matrix orders the crystals —
— the 40Å fibers are made of 2 parallel fibers of 12Å with 17Å spacetechnic.

Proline makes up about 55% of the protein & it puts definite restrictions on configuration —
You can't have an α helix with this much proline.

Decalcify & cut sections — ~~[illegible]~~ extract — find the neutral soluble fraction comes out from prisms leaving behind only the sheath protein.

In early tooth the matrix is 20%-30% of tooth.
Beyond 7th month & into adult tooth, the protein is only 0.1%.

Suggest that something diffuses thru sheaths & digests out the protein, leaving very little left.

Analyze AA composition & find that ~~stu~~ aa composition is quite different.

Clencher notes that prisms are put out by the amelobladts - probably putting out one prism in one direction & another at rt LS to it.

In ricketts, does the matrix organize itself & mineralization fail? Not tried yet. - This is what happens in Bone.

Saunders -- July 13, 1963 -

Microsymposium on Keratin and Collagen

Introduction -- Specificity in Feather Development

I Introduction

A. General -- Distribution of keratin and collagen

1. Keratin and collagen are fibrous proteins which enjoy an ubiquitous distribution in the animal kingdom,

a) Keratin, in several forms, is the characteristic product of the epidermal layer of the skin: comprising the protective outer layer, stratum corneum, of the epidermis, the chief substance of hair, feathers, claws, epidermal scales, etc.

b. Collagen is a major component of the dermal portion of the integument and, indeed, a major component of connective and supporting tissues throughout the vertebrate body.

2 My colleagues will, I hope, have a good deal to say about the synthesis of the keratin & collagen molecules and the way in which they are built into complex fabrics.

B. My own remarks will concern the integument of the vertebrate, more particularly the bird, which shows remarkable regionally specific differences in the character of its keratinized derivatives and their substratum of collagenous connective tissue in the dermis.

II Regional specificity of integumentary derivatives in birds.

A. Appreciate regional differences in the integument of own bodies - e.g.

a) Scalp -- Thin dermal layer with its collagenous fibers covered with thin epidermis with thin keratinized layer and abundant keratinized derivatives - hair.

b) Soles of feet - palms of hands -- Thicker dermal layer with ridges interdigitating with thickenings of epidermis -

c) Thick dermis and epidermis of back & shoulders contrasted with thin skin of chest - etc -

2. But differences are much more dramatic in birds. —

Slide 109

a) Illustrate different derivatives

b) Note dual nature [illegible] derivatives from

c) Note regional differences known from differences in prominence of
   i Keratinized component of epidermis
   ii The dermis with its blood vessels and (largely) collagenous connective tissue.

d) Special attention to feathers.
   Slide 42 — i arranged in tracts
   Slide 152+108 — ii each tract produces distinctive feathers
   Slide 37 — iii tracts form pattern of intersecting coordinates
   Slide 104 — iv Graded changes in structure and physiology from feather to feather along each coordinate.

B Residence of specificity in adult feather.

DRAW 1. Structure of adult feather in follicle -
SLIDE (Plastic) Draw - Show slide noting dual structure

2. Regeneration - continuity of follicle-specific properties

3. Role of 2 components in regeneration - DRAW
   a) no papilla - no regeneration
   b) rotate papilla - rotate feather, but get follicle specific one.
   c) Conclude papilla induces ectoderm to form feather

4. Test for site of specificity – Wang.
   a) Exchange papillae between follicles of different tracts (autograft)
   b) Obtain feather characteristic of host tract
   c) Conclude:
      i Specificity resides in ecto
      ii Dermal papilla give non-specific stimulus so far as tract properties are concerned.
      iii Since:
         A Each feather unique ecto structure and
         B. Each forms in response to the same mesodermal stimulus
         C Therefore the ectoderm of each follicle has an unique set of properties – response competence -- which it expresses upon appropriate stimulation.

C. Origin of specific response competence.
   1. Embryonic origin of feather
      a) rows of dermal condensations – alternating to give staggered rows
      b) thickening of epidermis – sinking in etc

DRAW

2. Various experiments we will not take time to discuss here show that there is a reciprocal inductive interaction between ectoderm and mesoderm in origin of feather germ.

3. Hypothesis — Mesoderm supplies to the ectoderm inductive cues which specify the kind of feather it will form.

Slide 351 a) Test — Wing & thigh feathers distinctive and tracts accessible in embryo.

Slide 46 Graft prospective thigh to wing

b) Result -

Slide 117 i Thigh feathers from wing ectoderm

Slide 114 ii Pattern of variation from feather to feather corresponds to normal.

4 Conclusion. — During embryonic development the mesoderm specifies in the ectoderm what shall be the pattern in which it shall respond to non-specific inductive cues. This specification not only determines what kinds of feathers shall be formed by ectoderm in each tract but it even extends to the most intimate details of feather structure -- minute variations in structure from follicle to follic

5. What makes each feather distinctive is a spatio temporal variation in morphogenetic patterns and synthetic activities during growth of the feather. What is astonishing is the fact that the same "collar" proliferates a pattern of rachis - barbs - calamus which varies not only morphologically - but also in the composition of the feather keratin

III Summary Slide

Slide -23-

A. Point out that scheme holds for all integumentary derivatives that have been investigated. Scales & Claws - Saunders; Comb - Lawrence; Beaks - Rawles

B. Note that ectoderm necessary & mesodermal differentiation but morphogenetic pattern of mesodermal components determined independently by regional factors - these largely determine & connective tissue pattern

C. Ectoderm likewise dependent on mesoderm, but mesoderm specifies what the ectoderm shall do -

a) Pattern in which it synthesizes

keratin in feathers

b Synthesis of oil – in uropygial gland.

D. Comment on complex job the induction of specificity carries out.

1 Sets up a sequence of feather types

2 sets up a morphogenetic pattern which is spatiotemporally different from apex to base of feather -- and different in proteins synthesized

3. Which provides for cyclical repeat after each moult.

4. If protein synthetic pattern is due to ~~expo~~ particular gene sites exposed, the original induction must set up sequence of exposures — —

IV Time of Induction action – Problem! When during development is the regionally specific induction action exercised?

A. All Saunders experiments suggest that induction occur early – altho they do not prove it

B. Sengel's work suggests feather induction (no knowledge of specificity) occurs 6½ – 7 days

C. Rawles has shown that epidermis of chick foot as late as 14 days of incubation will form feathers when combined with 8-9 day skin dermis!!

also – beak ~~[illegible]~~ induces beak epidermis from chorionic epithelium

D. Trust that next speaker will have some biochemical evidence to address to this question

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SPECIFICITY IN DEVELOPMENT OF INTEGUMENTARY DERIVATIVES

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See also Ebert's review of the recent investigations of Dr. M.C. Rawles reported in the "Annual Report of the Director of the Department of Embryology" in the Carnegie Institution of Washington Yearbook, #'s 60& 61.

Saunders
July 13, 1963

Ebert – July 15

I Role to set stage for wider activities – will ask a group of questions –

A Wilson – "The Cell" 1896 – recognized modern role of nucleus & cytoplasm – even tho he did not know mechanisms

B – Meaning of "Differentiation"
A – Seen as a whole – diversification
B – Seen at cell level – homogeneity – specific protein synthesis

II We know little about cells
~~A. little about origin of the egg~~ –
II Exceptions in Xenopus Brown & Caston etc
A. When oocyte begins to form – there is intense synthesis of RNA – does on in adjoining cells as well as in oocyte.
B. are they concerned with yolk formation?
B When egg is ready to be shed, formation of ribosomes has ceased – at fertilization there are very few ribosomes.

c There is no new ribosomal synthesis until neurula - abundant only at tail-bud stage.
This confirms EM Work -

D - DNA — in amphibs.
In early stages of maturation there is much more DNA in nucleus + ~~cytoplasm~~ there is about 4 x the haploid conc of DNA. Don't know how it is organized. - Drops to normal level by fertilization - Also DNA in cytoplasm - what does it do?

III Early development is very DNA dependent -
~~[illegible]~~

II A tissue seems to send out signals which persist during life - thus a homograft is tolerated, ~~[illegible]~~ ~~spleen like~~ ~~[illegible]~~ under proper condition, but when tolerance is broken, the distinctive signals of the graft are recognized as foreign & the graft is rejected! - (use inbred mice - spleen injection

Hemoglobin first found in nucleus in bug & amphibian —
Is this usual?

Is stability of a differentiation characterized by loss of DNA dependence? All info now fixed on ribosome? — Might this be different in tissue which regenerate?

Is there direct evidence of nuclear change during drift —

1. Puffs — at a given stage in given organ there is activity at given locus
2. Nuclear transplants
   a) Briggs + King — with gastrulation & restriction in capacity of nuclei to support full gamut of differentiations — based chiefly on work c̄ endodermal nuclei

Rana & axolotl

   b) Fishberg & Gurdon — rarely but really get good larvae from transplants of gut nuclei — they emphasize the normality —

Xenopus

Is success in getting normal development a measure of experimenter skill in transferring nuclei sans damage!

Xenopus has fewer nucleases & other possible damaging agents than Rana

So Gurdon possibly picking up primordial germ cells — Slim, very slim chance.

Laying down of molecular arrays is a more complex level which — How controlled? Know nothing of how pattern is achieved. — Possibility in the "pre-patterning" in insects & Curt Stern —

What is relationship between reading of genome & origin of specific enzyme —
Hypoxanthine dehydrogenase (Croisette) appears in 3 different tissues at 3 different times — 1st in mesonephros in chickembry at st 5 — Goes down precipitously at day 13-14 — —
Starts in metanephros & reaches peak at 16 days — drops out at hatching — then starts in liver.
This is the identical enzyme in each tissue. So same gene sites turned on at diff times in diff tissue.

Shows great lability of CAM

Ecto → beak synthesis under influence of beak dermis.

Mesenchyme → cartilage when stimulated by lymphoma

Muscle with Rous sarcoma + ~~[illegible]~~ RNP of adult heart

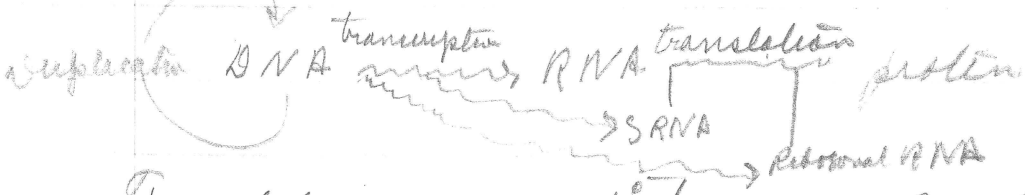
Endoderm → gut epithelium – by what means?

Now get at mechanical problems – cell movement, for example – primordial germ cells migrate – Melanoblasts – Blood cells – formed first in extraembryonic region, then spleen, liver, bone marrow. Do the production arise by new differentiation in each site or by infection?

Single mutation affects all 3 types of cells above in Mouse – W locus affects those which need to be transported –

Spiegelman – July 15 –

Indonesia flow



Translation requires a dictionary, S-RNA,
Machine required to translate – the ribosomes.
Info in DNA prescribes composition of S RNA & R RNA

Controls can be imposed at various places.
Operon is name given to coordinated group of DNA sites –

Do you get messages from both strands of DNA?
In test tube, both strands are transcribed? But in the cell, Ribosomal RNA does not show G = C and A = U. Therefore, copies of both strands are not represented.

G – C
A U

In β-?? virus, can show messenger RNA reads only 1 strand.

Is there order in the reading of the cistrons?
or ordered use of randomly read info?

In T-2 infection, host immediately turns off its own synthesis of messenger

Label identical samples - one with C^{14} labeled purine or pyrimide & other with tritium
Let one look for 2 minutes & other for 15 - Find differences in Base ratios of the 2 samples, showing that what is read early is differ from that read later -

Chloramphenicol prevents message ~~destruction~~
If it ~~interrupts~~ is given early to both samples, but one is allowed to go on, then, later, they show identical base ratios in the messages.

Where does an inducer act -

"Pi phage is a transducing phage which has the lac locus stolen from a ~~coli~~ [also has some other coli sequences]

Pulse label induced & non induced Coli
Co-chromatograph & see if there are
discrepancies in the Base ratios
For control, take lac deletion mutant
Do mock expt using inducer in one
sample & no inducer in other, these
then give identical Co-chromatograph
profiles. — with 2 samples of wild
type, also get correspondence

~~Use + and~~ - lac
Use induced & non induced la
(lost hm).

Inducer does turn on message production

July 16 –

Pollock – Stability of extrachromosomal Characters

"Differentiation" implies a degree, at least, of irreversibility
Suggests that to some degree, at least, there is a relationship between stability of differentiated state and cell multiplication.
What sort of systems in a cell are likely to remain stable thru persisting cell divisions.

It is conceivable that there is produced non replicating DNA which has only the function of coding messenger RNA

This does appear to be the case before the lac+ gene is brought into coli by phage –

Can you increase the amt of a character (protein) by increasing the amt of DNA which codes for that character

alpha + gamma
+ + ~~Beta~~ penicillinase are coded by the same structural gene, and are interconvertible – thus are different in tertiary structure – Activity of γ (sthick) is much greater than that of α. – to way to control enz activity sans gene.

glutamic dehydrogenase — 2 chains involved
Single chain is alanine dehydrogenase — in
combined form it is glutamic dehydrogenase

Stability genzymes — once formed, they vary
greatly in "turnover" — Some much more
susceptible to breakdown than others.

L-forms of bacteria — induced to grow
without cell walls (by penicillin? — is this
selection or stripping off F.) probably
not selection — can get 40% of heritable
change to L-form after brief exposure. — Maybe
the penicillin removes the "primer" — No
cell wall — ∴ none synthesized thereafter — Adding
the polysaccharide to L-forms does not revert
them — — Some have reported very low rate
— or frequency — of reversion.

Discussion

Ebert brings up when & where does one find
extrachromosomal DNA in embryonic systems

Chloroplasts contain DNA of different
base composition from Nuclear DNA.
— Someone point out chloroplasts can mutate
independently.

Do "bleached" chloroplasts offer anything here?

Grant remarks on widespread distribution of cytoplasmic DNA in egg -- sponges, coelenterates, insects etc -- In many cases this comes from nurse cells & follicle cells -

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If ribosomes from regenerating liver are purified not too carefully & are provided with all else in excess they synthesize proteins much more rapidly than do ribosomes from non regenerating liver -- not stimulated by GTP as are ribosomes from non regen - if

Sussman – July 14

A differentiated cell is one with a change in phenotype – The alteration is strictly programmed with respect to time, place etc – & lead to a considerable stability

In Dictyostelium discoideum – Differentiation (aggregation, culmination etc) accompanied by 70% loss of dry wt of protein, RNA etc –
Aggregateless mutants likewise do this, so this is not a significant aspect of differentiation.
In normals, Hexose does not diminish proportionately, but in aggregateless, the carbohydrate goes down.

3 polysaccharides isolated –
1st one goes up, then down during differentiation in normal – Not synthesized at all in aggregateless – its something like glycogen

A TCA insol, alkali insol fraction goes up & levels off – probably cellulose
– Aggregateless does not synthesize.

A third is a mucopolysaccharide — is antigenic & cross reacts with several muco-polysacch. — pneumococcus for ex — Again — not formed by aggregateless.

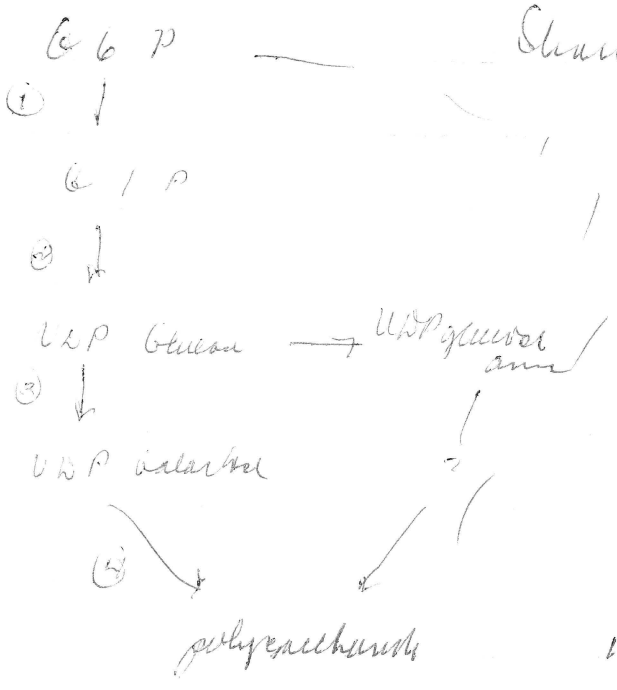
Another mutant of fruitless kind (FR-17) makes a peculiar aggregate but does not fruit — it makes, however, all cell types in the aggregate makes all compounds & antigens (and products) of normal morphogenesis without going thru normal morphogenesis

stalk antigen

Apparently the timing of these events is all off — the specialized products all are produced too soon
Stalk antigen at 13 hrs, in contrast to 25 hrs in wild type.

Primer — the mucopolysaccharide

UDP-Galactose & primer $\xrightarrow{\text{Enzyme}}$ Gal primer + UDP
The enzyme activity appears abruptly during stalk formation & disappears at 32 hrs just before spore formation begins



Shows a scheme of involving 13 enzymes going by 3 routes from glucose 6 p to polysaccharide - proposes to test which enzymes are of morphogen signif.

Cy Leventhal - July 17, 1963
RNA - the kinetics of its production amounts and its stability in Bacterial Cells.

What happens to messenger RNA in cells - is it broken down

Actinomycin D stops nucleic acid synthesis if conc high enough
it knocks out both DNA & RNA synthesis
~~with RNA p~~

Actinomycin binds to guanine of DNA and stops it from being a template for RNA.
Seems to behave as a reversible competitive inhibitor but its inhibitor constant is 10X as high for RNA as DNA -
So far as we know, protein synthesis per se is not inhibited by actinomycin

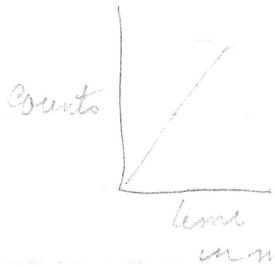
RNA viruses replicate - their synthesis not interfered with by actinomycin

If feed uracil - used for Messenger RNA, and for S- (transfer) RNA (the 4S stuff) and Ribosome RNA (23 S & 50S particles) (Evosomes are stages on way to Ribosome).

[illegible] happens to S-rna

If actinomycin added, M RNA decays — but anything on path to ribosomes continues on to synthesis. — SRNA stable also.

Add uracil to Log phase Bubbles — look at TCA precipitable activity



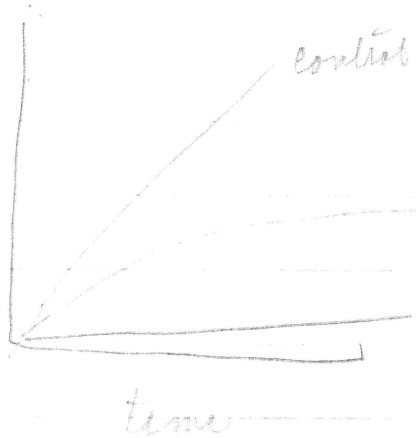
no lag — uracil gets into high Mol wt stuff rapidly — no large pool (feed cold uracil first & get lag & indicating pool).

If sufficient actinomycin added, there is no uptake use 10 γ/ml.
(Must be sure actinomycin gets in! Won't enter cols, for ex unless cell walls removed)

Give very brief pulse of hot uracil RNA — most goes to messenger, but some goes to all forms. With longer ~~pulses~~ wait before sampling, more shows up in the stable RNA (transfer & Ribosome)
Uracil is reused 4 or 5 times in messenger before being used in stable RNA.
Can only measure half-life of messenger by stopping RNA synthesis

Regardless of rate of growth the messenger fraction is 8 to 9 % of the total RNA — Decay time is same in different conditions.

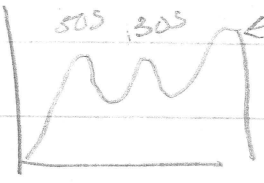
Protein synthesis (Serine incorp) after addition of actinomycin



Stop synthesis at the level of the peptide bond.

Rate of protein synthesis after actinomycin is function of how much messenger left — Beyond this: however, there is some weak evidence that with actinomycin there can be a little copying into messenger between guanine residues & then get some "junk" protein synthesis.

Sucrose gradient after 3 minutes ~~after Actin~~ incubation hot ~~[illegible]~~ ~~[illegible]~~ ~~[illegible]~~



crude extract

Can't remove actinomycin from mammalian cells.

23 S corresp X Mol wt of 1,000,000
16 S " " " " " 500,000

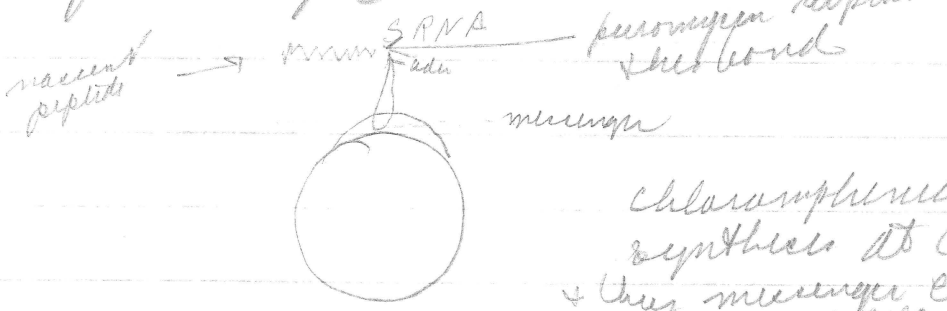
In presence of chloramphenicol, the ribosomes do not mature [illegible] ribosomes do not get their protein coat, becoming 30 & 50 S Ribosomes, and instead decay!

It is thus a single polypeptide chain

[illegible] — Says [illegible] — if so, it is made without use of messenger RNA
This is still an assumption.

Is breakdown of M RNA assoc c̄ protein synthesis
— In bacteria it is used 10-15 times first.

Stop protein synthesis with anaerobiosis & with allenomycin, there is some stabilization of Messenger — with puromycin stop of prot synthesis, no diminution of decay rate of messenger



chloramphenicol prevents synthesis at the bond, & thus messenger can slip out of the SRNA loop.

Add puromycin to reticulocytes & ribosomes come off the messenger — Stop prot synthesis by other means does not release messenger

Cereus

Hela Cells - Penman & Darnell — Polyribosomal structure found o—o—o—o — Uracil quickly goes to polysomes. Add actino-myxin & polysome structure decays with half-life of about 3 hours.

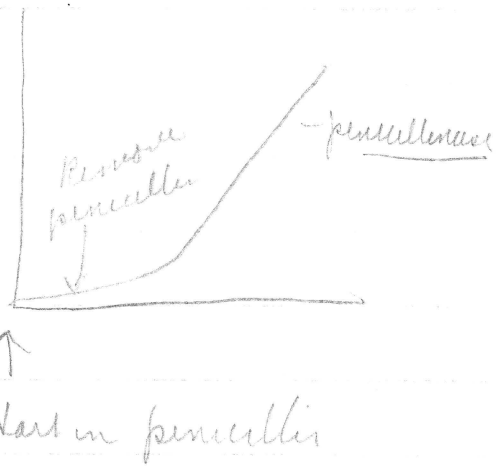
Sea Urchin — Wilt — Nemmer —
Before fertilization, little or no C^{14} uracilliplake or of labeled aa —
At fertilization, is the first job to make messenger?
Yes, according to Wilt — both messenger & SRNA are made first! — Mostly messenger.

Note that for differentiation we still.

Pollock – January 17

Effect of actinomycin on Penicillinase Development

Bacillus cereus – penicillinase production

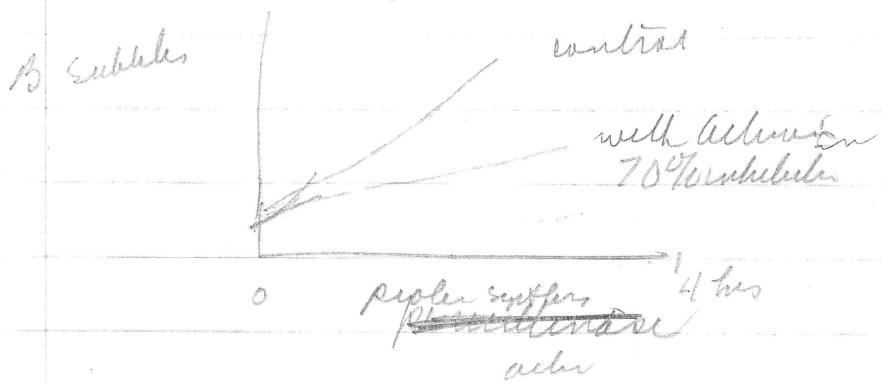


Unusual (?) – note perpetuation of induction response. this goes on indefinitely!

Will penicillin [illegible] fixed state in cell & continue to act as inducer?

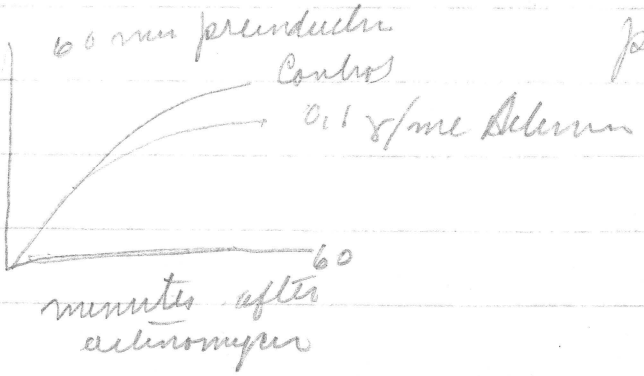
Explanation not known – Is there something which persists – Will actinomycin help us?

Add actinomycin D at different stages.



0.05 γ/ml

Similar for B. cereus

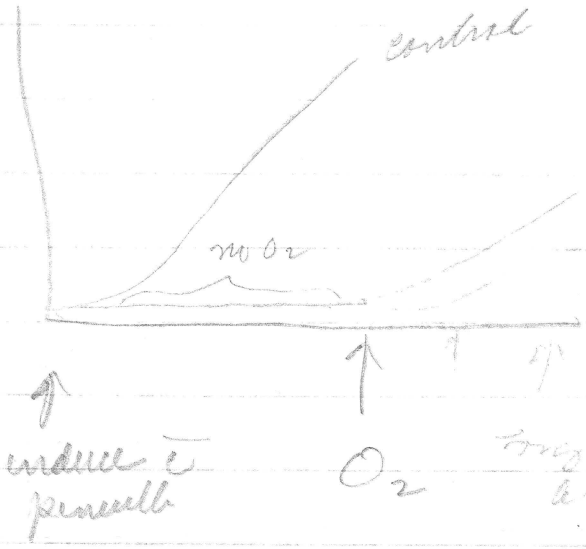


penicillinase activity

Actinomycin has no effect on activity of penicillinase for 60 minutes in strains with constitutive enzyme. — Clearly here we are dealing with a long half life messenger — Even in same species some messengers may be more stable than others.

In cereus anaerobioses turns off almost all synthetic systems.

If induction has gone on before O_2 deprivation, when O_2 is restored penicillinase production continues at original rate



The longer the anaerobioses, the more feeble the induction of penicillinase

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Discussion — Leventhal suggests his concentration of actinomycin are too low.

Leventhal suggests that all messenger is equally susceptible & "messengerase" the reticulocyte may have little messengerase (messengerase may be polynucleotide phosphorylase).

P. Cross – July 17

(messenger molecules)
Do templates present in fert egg? – Any reason we should expect them to be there.

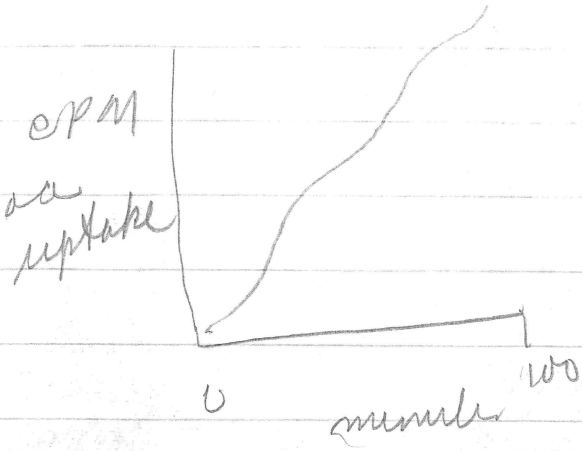
① Embryo reasons – ⓐ almost any thing you do to sea urchin doesn't affect it to blastula except FUDR

FUDR stop DNA synthesis by blocking thymidylate reverse by thymidine & various nucleosides – some abnormal ones!

(b) Enucleate parthenogenesis

i Hultin – puromycin (?) has parallel ~~same~~ eff on cleavage & protein synthesis

ii See urchin enucleate halves cleave! Protein synthesis in absence of genes! What about messenger RNA – – Is it there without genes?



uptake of radioactive amino acids
Begins at least by 10 minutes after fert!

Nemmer showed that RNA made right after fertilization — short pulse — label shows up in 4 S RNA. — None in ribosomes.

Hultin — prepare ribosomes from fert & from unfert eggs. Those from fert eggs are more effective at incorp of aa's in artificial systems than are those from unfert.

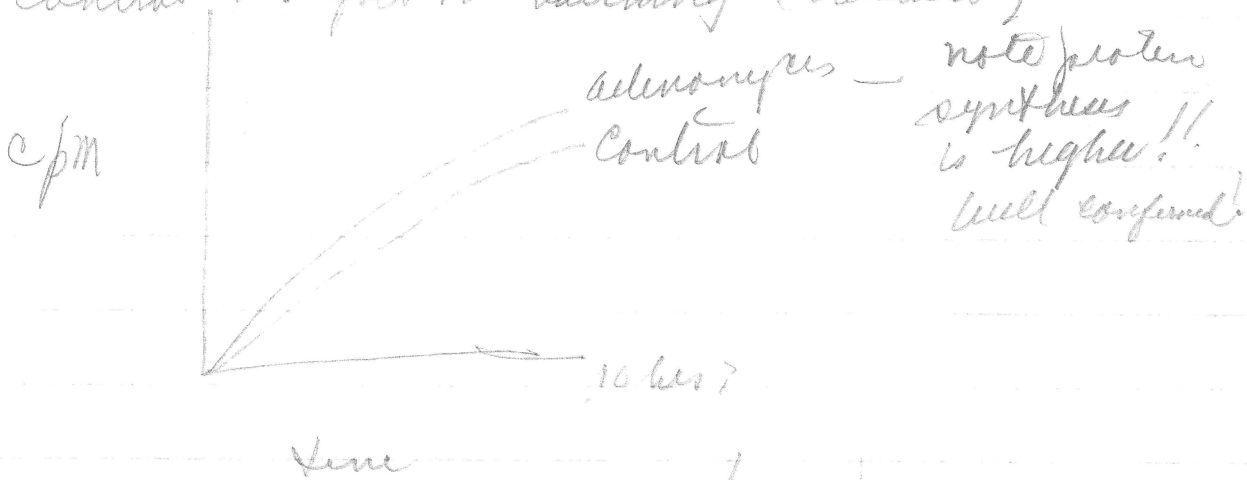
Holt, Wilt, Nemmer showed that feeding poly U & Poly C to 2 kinds of ribosomes. Those from unfert made better incorp of aa's than from fert. — Possible that ~~after~~ before fert, the ~~messenger~~ ribosomes are naked. After fert, messenger preempts the ribosomes.

Data of Tyler (?) sea urchin ~~parthenogene~~ merogones

	untreated	-CPM- artifactive c bulgrie area	% activated
enucleated	19 17	98 92	50%
nucleated	19 [illegible]	91 94	100%

c hot uracil actinomycin balls RNA synthesis in sea urchin. — Co cut it, however, requires 20γ/ml.

Protein synthesis, however is same as control !! goes to balching (blastula)



Messages necessary for protein synthesis to blastula thus may be present in fert egg — Contrast to prior argument with Nemmer etc

But — actinomycin does not completely shut down RNA synthesis in sea urchin

~~P Dent~~

Does development start with just a few proteins & then spectrum of proteins increases? If one labels with aa's during early divisions, essentially all of activity appears in the

spindle!. Isolated spindles show X, too after uptake by whole egg.

Spindle shown by E.M. has essentially all other cell constituents - fibers of spindle comprise 0.3% of total protein of cell.

Cross drew conclusion that only a few are being synthesized at first, but I couldn't follow his reasoning

July 18, 1963 – Leventhal – Genetic complementation

E. coli Mol Wt 80000

Alk p-ase – Made of 2 subunits with same aa's by same cistron – each 40,000

Run on electrophoresis gives several bands – Don't know why! Guess something attached to peptide chain, possibly a carbohydrate which alters charge.

Mutants accumulated which are deficient in enzymes. All mutants in same region of genome – make a deficiency in the enzyme protein, not absence – may or may not have activity

Can the coli gene be transferred to another organism? Try E. coli to Serratia marcescens. – Does gene get read in the same way?

Serratia normally makes a alk p-ase very much like that of coli in enzymatic behavior, but physical properties (antigenicity, isoelectric pain, fingerprints, aa composition) are quite distinct.

If coli gene is transferred to Serratia, it is the coli alk p-ase in every respect. The genetic info is read the same way in each.

Ex: the coli DNA is carried & replicated in Serratia but Serratia reads it the same way coli does.

Code must be the same — surprising in view of evolutionary divergence.

To do this expt, required a Serratia mutant which had no alk p-ase.

Genetic complementation

Backgr — Tried to make alk p-ase in vitro — in course of study, it was learned that it was a dimer  made ~~of~~ same cistron — heat stable, resistant to proteolysis —

Separate dimer by treatment with combination of urea and thioglycolic acid — (neither alone)

Monomer then stabilized of iodoacetate used to block its exposed SH groups. (4 SH's titratable in monomer)

See PNAS 1962 Page 1230

Union of monomers gives variable % of normal activity depending on concentration! No dimers formed at low conc. Were formed they have original phys properties

Can we get active enzyme by hybridizing monomers of different mutants?

Reduce monomers, mix & reactivate & get electrophoretically distinct bands different from parent ~~sp~~ proteins.

Can make hybrid between Serratia protein & coli protein — this is an active dimer.

Return to in vitro synthesis problem.
Use ribosomes, messenger, etc — got incorp of radioactivity, presumably in monomers but too low conc to make dimers. Added cold monomer to permit dimerization. But when added proteolytic enzyme, all radioactivity was lost from the active enzyme, indicating that synthesis of alk p-ase monomer had not occurred. — thus in the Artificial Nurnberg system used, peptides were made, but not phosphatase monomer

---

Monomers "find each other" easily, even in presence of high [s] of irrelevant protein

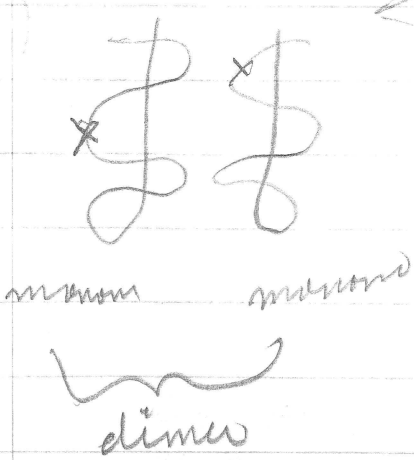
Simple genetic complementation — 2 cistrons, each makes peptide chain — basis of cis-trans test but occasionally get a different type of genetic complementation when 2 mutations in

same cistron

With alk p-ase mutants of coli - got complementation resulting in a protein with slightly different characteristics from parental protein - Enzyme activity is much less than wild type, but much more than either parental type.

Formation of dimers from monomers originating by complementation goes on in similar pattern to that shown by wild type.

Shows slide representing activities of enzyme activities obtained by combining monomers of different alk p-ase mutants - All mutants within the same cistron



can get active enzyme in some cases by complementation if defects in monomers do not correspond

Pardee - July 15 - Cell Regulatory Mechanisms

I. What kind of machinery regulates rate of enzyme reaction in cells. - Will discuss regulation chiefly in higher organisms - Bacterial authors today -

A. Feedback regulation by end product compound which is distant - Biosynthesis of pyrimidines -

carbamyl phosp + aspartate $\xrightarrow{\text{aspartic transcarbamylase}}$ carbamyl aspartate

can be shown in test tube

inhibits reaction

cytidine triphosphate

B Control by enzyme induction & repression

Control of enzyme - forming machinery

1. Induction of tryptophanase in E. coli

Requires continuous presence of tryptophan

B. Do we find examples of this control in higher organisms?

1. Schimke J Biol Chem, 1962 237: 459

Enzyme of arginine metabolism - Levels of these enzymes in liver is proportional

& amt of protein in diet.

2. Walker & B 1962. The Molecular Biology of Neoplasia, 1962

Liver transaminase activity

Enzyme repressed in diet containing creatine. — the enzyme is responsible for production of creatine (Not sure if in liver enzyme are made de novo — changes in enz activity is very fast)

3. Knox et al. J Biol Chem 235: 2662 (1960) — enzyme of tyrosine metabolism — enzyme poisoned by products of tyrosine metabolism.

4. Phosphorylase — exists in active & inactive form — phosphatase converts enzyme from by lopping off phosphate groups Phosphorylase kinase & activate phosphorylase by putting the p back on.

Krebs & Fischer Adv Enz 1962 24: 263

Case of control by activation & inactivation rather than induction & repression

5 tryptophan pyrollase. Greengard & Fiegelson, 1962, J Biol Chem 237: 1983- 1962.
Activated by tryptophan via haematin - gives induction and activation.

6. Ceska & Fisher 1959. Biol Bull 117: 611 -
Arginase activity of chick heart - 21 day heart extract inhibits activity of 12 day heart. - thus there are inhibitors which change during development.

7 J Biol Chem 234: 1492, 1959

for cell death →

Purine ribonucleotides inhibit first step in purine metabolism

8 Inhib. of thymidine tryptophan

Biochemistry 1: 347 - 1962
Maley & Maley. 547?

a Phosphofructokinase – needs ATP, but enzyme inhibited by high concs of ATP – Can consider ATP a feedback inhibitor of glycolysis

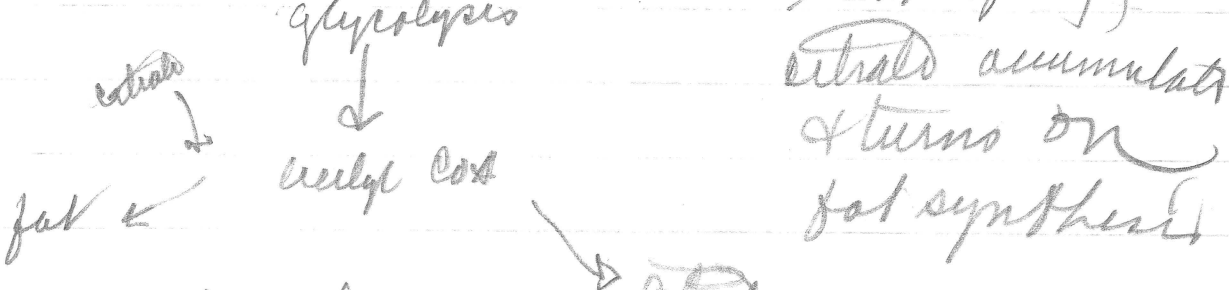
D - Feedback stimulation by small molecules

(1) DPN pyrophosphorylase stimulated by ATP

(2) How determine if acetyl CoA goes to fat synthesis or to Krebs.

Key enzyme forms malonyl CoA only if citrate is present – When plenty of citrate present, fat ~~[illegible]~~ will be synthesized – When Krebs runs rapidly, citrate accumulates & turns on fat synthesis

glycolysis
↓
acetyl CoA
citrate → fat
→ citrate



malonyl CoA synthetase is the enzyme – it actually changes physical config in presence of citrate – later changes enzyme from monomer (inactive) to trimer (active)

Koshland et al Fed Proc 21: 1031 (1962)

Suggest enzyme wraps itself around substrate. Many more possibilities for activation and inhibition of enzyme is flexible. Maybe this is the way control is exercised by non-related metabolites

Finally - for higher organisms lets look beyond the Jacob Monod theory to other mechanisms of control of synthesis.

Szabo - July 19

Fitzpatrick -

Separation on sucrose gradient

Show:

① Mitochondria & melanosome separate

② Succinoxidase activity goes with the mitochondria

3. Tyrosinase activity with the melanosome fraction

Considers this refute Burke & Woods & collaborators at N/H -

Also shows ribosomes from pigment-producing cells have T-ase activity. - Not used, however, until the enzyme put on the melanosome.

Tyrosinase activity seems to have an inverse relationship with the degree of blackening of the melanosome -- Note that ① retina pigment loses T-ase activity as hatching approaches in chick

② "Brown" mutants have more active T-ase than black! --

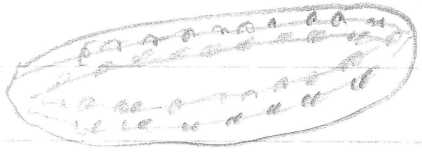
3. Harding Passey melanoma (brown) has higher T-ase activity than B-16 (black).

In albinos, the melanocytes & melanosomes are there—

In mouse albinos examined, there seemed to be no "beaded" appearance — In human albino, melanosomes with beaded strands used.

Pheomelanin granules as found in agouti are round & with the strands on which T-ase deposited all tangled apparently, whereas in Eumelanin granules





Summary

Have not been able to elute or remove tyrosinase from ribosome - Question raised -- are other ribosomes? Fitzpatrick answers that this same ribosome pick up has occurred.

Friday

Cadu-Cell Regulatory Mechanism II - July 19, 1963

I Induction systems in bacteria --

A, Definitions & explanations -

1 assume every function of a cell has a genetic basis -

2 Mutation can occur & affect each function - ie building a structural gene - Thus can have available form of a gene and an inactive or modified form

3 Dominance

B galactosidase gene - call Z

Z^+ dominant over Z^- Z^+ can make

Suggests dominant gene makes something & recessive does not -

4 Cis & trans action -

If we have a regulatory gene it might be next to the active one in a hybrid zygote (cis) or next to the inactive gene (trans) - If regulation acts both cis and trans, then active regulator (in cyto) produces something which moves & regulates the active gene. If it acts only cis, then its action is entirely in the genome

B. β-galactosidase in Coli

1. Recall

a) Wild type does not make (in appreciable units) unless approp. galactoside is provided eg lactose



2. Analysis by genetic techniques — look at lac region



z is str gene.
i^+ is wild type & enzyme is inducible —
i^- = enzyme is constitutive present regardless of lactose
i^S = no enzyme

o = a controlling locus — with similarities to i

i & o are regulatory gens

These are other structural genes { y controls permease
x controls an acetylase structure —

i & o also act on x & y — the group of structural gens all controlled as a unit, an operon.

3 Analyses of i

a) Apply cis - trans to i

b) $\boxed{\begin{array}{c|c} i^+ & z^+ \\ \hline i^- & z^- \end{array}}$ = inducible

i^+ dominant over i^- in cis

$\boxed{\begin{array}{c|c} i^+ & z^- \\ \hline i^- & z^+ \end{array}}$ = inducible in trans / no constitutive enzyme

conclude
i^+ acts thru cytoplasm - prevents z^+ from producing B galactosidase unless inducer present
i^+ makes a repressor of z^+ action

b) try a deletion mutation - eliminate i region - this makes the organism have the enzyme constitutively - prove that i^+ makes an inhibitor

c) i^s produces a super repressor which is not eliminated by repressor

Ap

4d. Examine what O gene does - assume that repressor from i attaches to O + this blocks copying of Z gene -

O^c

a) get mutants of O which do not accept repressor substance — these then become constitutive! O mutants act only Cis

b - another O mutant is O^o - it blocks synthesis of enzyme — Imagine the O region is a switch — in O^o, the switch is broken — z, y & x can not be turned on at al -

O^o works only Cis.

4. How does the Inducer fit? — No good evidence, but we imagine that lactose blocks action of repressor — assume R has a site to which lactose binds, this R can not turn O off — ie switch is on & z can be copied.

Review: Jacob & Monod. 1961. J Mol Biol 3: 318

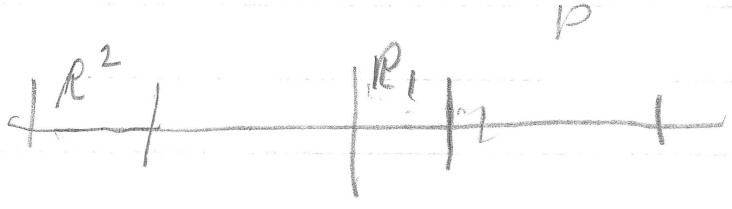
See also CSH symp of 1961.

(A) Repression of enzyme action — nutritional repressor

1. General — add — p and little alk p-ase is produced — E. Coli — note this is opposite of enzyme induction.
ie inorganic p combines c̄ R & makes it a better repressor!

a. All mutations map in same region for structural gene —

b. 2 regulatory genes known — but no equivalent of O, the operator or switching gene



P determines aa sequence of enzyme

R^2 — similar to i

R_1 is close to P.

2 Mutations —

a) R_2^- — cant make repressor.

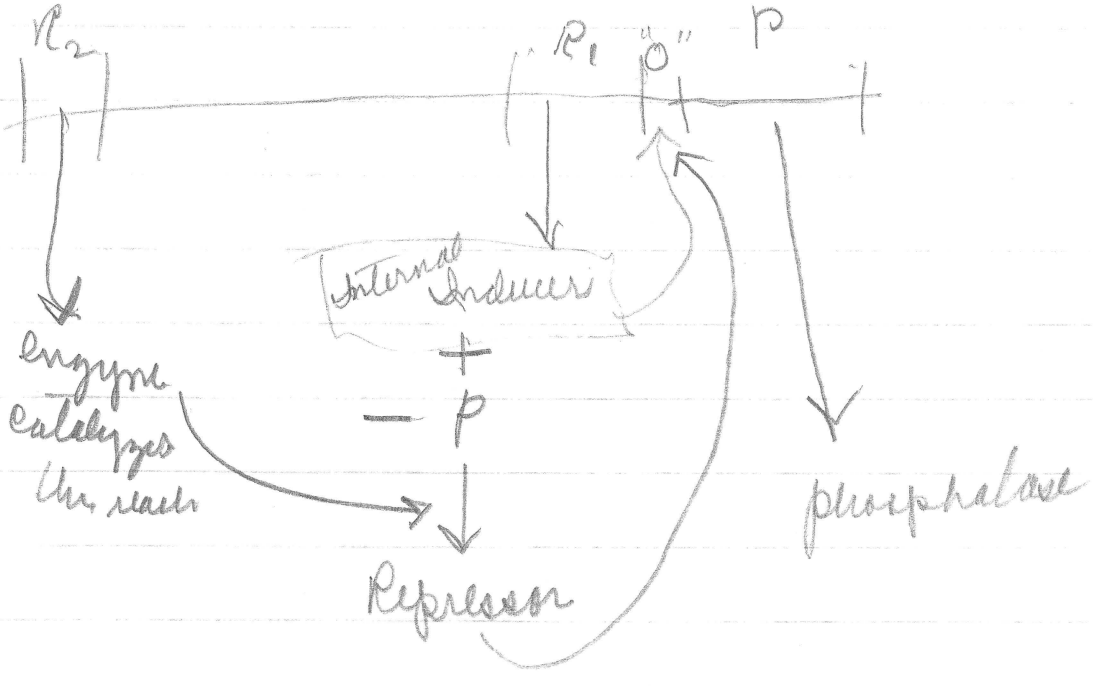
Strain	low P	high P
$R_1^+ R_2^+ P^+$	9	< 0.1
$R_1^+ R_2^- P^+$	12	10

NB – with R_2^- high P is not inhibitory

R_2^+ acts both cis & trans thus R_2^+ makes something

3 General Conclusions of rest of work – Dola Labs

"O" is assumed



R_1

4) You would expect R_1 mutants to have various effects

a) R_1 locus in the C configuration – little enzyme in either absence or presence of phosphate

	low P	high P
$C\ R_2^+\ P^+$	0.2	< 0.1

Apparently the C mutation of R_1 locus makes a modified inducer which isn't much good, but combines well with phosp to make a good repressor –

Read → PNAS 48: 1398 (1962)

Has Cis trans properties

b) $R1_a^-$ – makes same amt enzyme in high or low P.

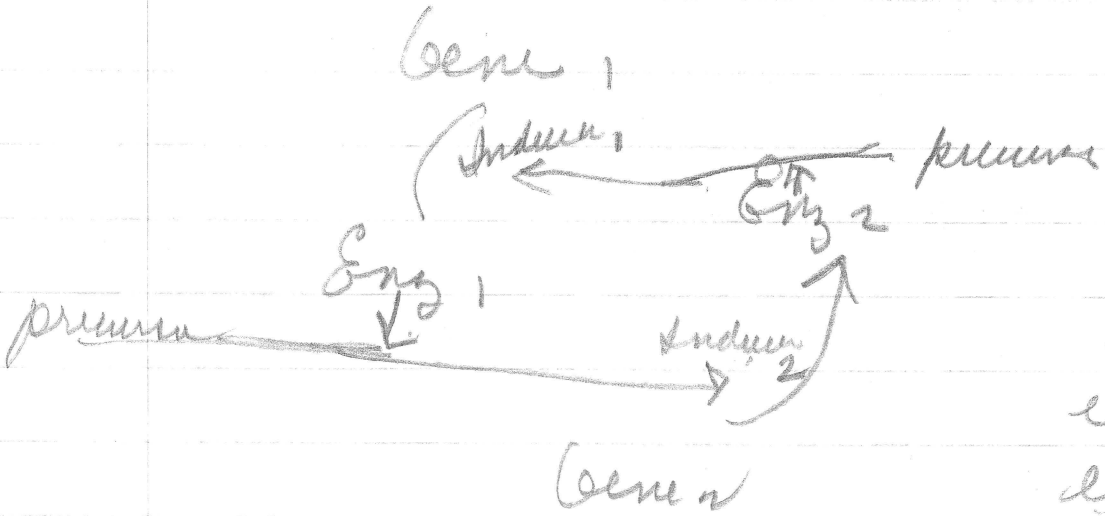
Assume they make a poor inducer which also makes a ~~poor~~ no repressor – phosphate conc has no effect on it

e R_{1_b} — make maximum amt of enzyme with low P & little

ie makes good inducer but in combination with P, makes poor repressor

	low P	high P
$R_{1_b}^-$ R_2^+ P^+	12	3

III How make these sorts of changes permanent & not dependent on presence or absence of inducer?



this could lead to stabilization

provides for entrance of external influences

Tryptophan pyrollase inducible in adult rat liver

Greengard give adult rat ~~puromycin~~ & actinomycin

Check c Spiegel

Does one have to copy an entire operon at one time — NB Galactosedase & acetylase enzyme not synthesized in equal amts (#'s of molecules) — is this due to fact that copying turned off after galactosedase & before acetylase code copied — or is it due to different translation rates later on?

Saturday

Vincent – July 20 Microsymposium.

I Introduction

Nucleolus of starfish oocyte – Function suggested by recent observations – see below –

NB – that nucleoli are found only where one has organized chromosomes & a nuclear envelope.

Parameters for function:

1. Involved in nucleic apparatus? – No so far as we know
2. Protein synthesis? Yes – we'll be concerned with this

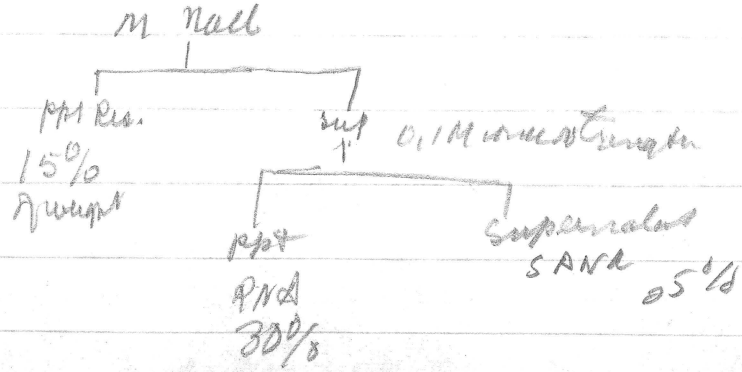
Each of these parameters has DNA

a) RNA of nucleus concentrated there – look at accumulation of SRNA
b) Ribosomes & Ribosomal RNA
c) – m RNA

3. Involved in organization/formation of chromosomes? Plant material suggests yes –

Fractionating Scheme –

Nuclei in M NaCl – @ 0°C – Stir 1 hr –
grind & ppt. – Dilute supernate to 0.1 M conc strength. Ppt is "L-protein" supernate contains 5 RNA – 5% of wt of nucleus RNA



SRNA seems definitely present in nucleolus and may be synthesized there. Amino acid activation goes on

Re ribosomes - nucleolus made up of many granules approx size of ribosomes

"The 1.M residue is ribosomal RNA

Appears to be new synthesis of SRNA + Ribosomal RNA (0.1M super) in nucleolus - suggest cistrons for synthesis of SRNA in or on nucleolus organizing region.

With nucleole, SRNA and poly U incent got skin amino acid incorp - data not real good but strong indication!

Now look at 0.1M ppt - this is the L-protein (lots of lysine) made antibody to it - RNA is associated with the L protein. It is different from the others.

Doesn't store information while its being formed. This works during cleavage - Ribosomes formed later show no cross reaction with L-protein

Vincent suggests ribosome of vocyte has messenger fastened to it & protected by protein. (so messenger is stable?) at fertilization, there is a burst of proteolyses (does this take away protection so later synthesized messenger can get in?) (JMS comments in parenthyses - didn't quite follow).

Discussion

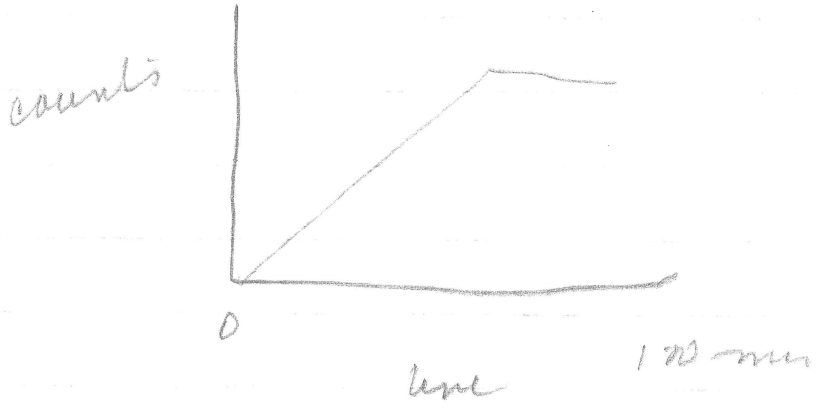
NB at fertilization, the "germinal vesicle breaks down - the nucleolar contents dispersed to the cytoplasm -

Grant - suggests connection between nucleolar components & fertilizability of egg -- Recall enucleat halves made prior to g.v. breakdown are not fertilizable.

Wilt — July 20 -

Dramatic increase of aa incorporation after fertilization or artificial activation. — In vitro incorporation parallels, if one uses ribosomes of the sea urchin

RNA synthesis - fertilized urchins



Kedes asks why = See Nemer in Science recently
Ribosomes are at fault that no increase
of aa occurs until after fert

Nemer shows that ribosomes of fert &
unfert egg act identically in poly U

Ribosomal RNA of unfert egg + fert
are identical in sedimentation

RNA synthesized in Cleavage is very low
Lots of RNase present - so you might
lose what you've got - better use
bentonite (inhibitor of RNase) in
extraction - Use hot (60°) phenol

Get 27, 18 & 4-6 S synthesized in early
cleavage - just like that produced
elsewhere. - Will label a count
peak is at 4-6 S - Some correspondence
at 18 S - & none at 27 -

Early blastula eggs - couldn't follow

Immediately after Fert there is incorp of uridine
in non ribosomal RNA & there
is polysome formation - thus there is
new synthesis of RNA - - Is there
also some pre-existing RNA

Collier – July 20 (Saturday) – Kyanassa –

Zash July 20, 1963
Chondroitin sulfate induction

the Induction event occurs in vitro trans filter in first few hours, but response not until 3 1/2 – 4 days later.

Sulfate begins to be incorporated as soon as induction takes place – Chondroitin sulfate is not measurable until ~~cartilage~~ 3 1/2 to 4 days later!!

PAPS – the "active sulfate principle"
Phospho adenosine phospho sulfate – transfers ~~phosphate~~ sulfate to chondroitin

Somites grown with induction have PAPS – PAPS not present in non induced Somites.

PAPS not detected until 3rd day, so far – Are enzymes present prior to induction.

Fresh tissues seem to have the sulfate activating principle by chem test.
Correlate with metachromasia of early chick tissue? May be sulfated mucopolysaccharides

Beginning Cultures have no sulfate activating principles & show no detectable metachromasia ~~until some~~
Activity & metachromasia show up later in culture.

Possibly the trypsin takes out the stuff responsible for metachromasia when cultures are prepared.

NB - Zwillings lab shows pile up cols of lateral plate from unsegmented region in culture & get cartilage!

Edgar - July 25 - 1963

Reviews: Bacterial Viruses

Kellenberger 1960 Advances in Virus Research 8

Champe 1963 (not out yet) Annual Reviews of Microbiology

I Intro

A Characteristics of Bacterial viruses

1. Many with tadpole-like shape - & special anatomy required?

not necessarily - for there are some without these special structures

B Will be examined with T "even" viruses.

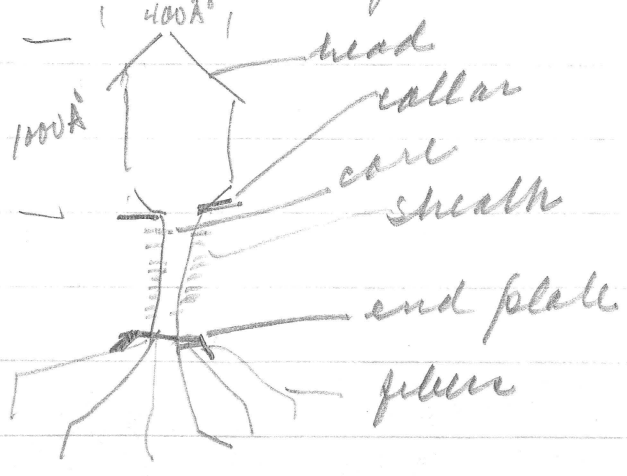
II Characteristics of T viruses

A T4 Anatomy - Head - Tail & kinked tail fibers

1 Head hexagonal

2 Neck region complex - but poorly resolved in future slides

B2.



T4 →

sheath contracts & acts as syringe, squirting DNA into bacterial host

Head has about 1000 identical (?) protein subunits — different from sheath protein

DNA in head — has hydroxymethyl cytosine instead of cytosine (latter absent!) — there are glucose residues attached to the Hydroxymethylcytosine. — In T4, glucose has α linkage in 70% + 30% β linkage —
Glucosylation pattern varies from phage to phage

There is but one DNA molecule in the head. Has to be folded in a special way to fit in the head — 3% of total protein of phage is in head also & enter bacterium along with DNA — about 20 polypeptide chains — may be of different kinds.

Hole in core is 25 Å in diameter (DNA filament is 20 Å)

Sheath made of ca 150 identical protein subunits of Mol WT 150,000. — It is contractile, shortening from 900 Å to 350 Å — It is a helical structure
ATP is associated — 150 molecules per phage and 150 molecules of Ca^{++}

End plate looks like a star of David with points folded down.—

Tail fibers 1200 Å long & 20 Å wide.

Possibly some enzymes — Lysozyme possibly an integral part of the phage particle — But, phage mutants known without lysozyme & these infect nicely.

Phosphatase seems to be present.

III Response of infected cell.

A. Eclipse period — No phage particles recognized in cell for 12 minutes after initial infection —

B. Thereafter for next 12-15' # of particles increases logarithmically & lysis occurs.
At 37° the total period is 20-25 min
at 15° " " " " 9½ hours.
Proportion of eclipse period & maturation period remains the same.

C. Changes in cell

1. In 1-2 minutes, the bacterial

(nucleoid)

nuclei break down! –
– New areas of DNA then appear – Pools of phage DNA increase in size during eclipse period
Phage is not infective until fully built!

Chloramphenicol blocks assembly of DNA into the form of a "head" – Chlor – must block synthesis of protein responsible for assembly of DNA into head.

In cell, within 1 minute after infection Host DNA synthesis stops & within 5 minutes phage DNA synthesis begins. All host protein & RNA synthesis stops when phage enters – Maybe one of internal phage proteins knocks out hosts synthetic system –

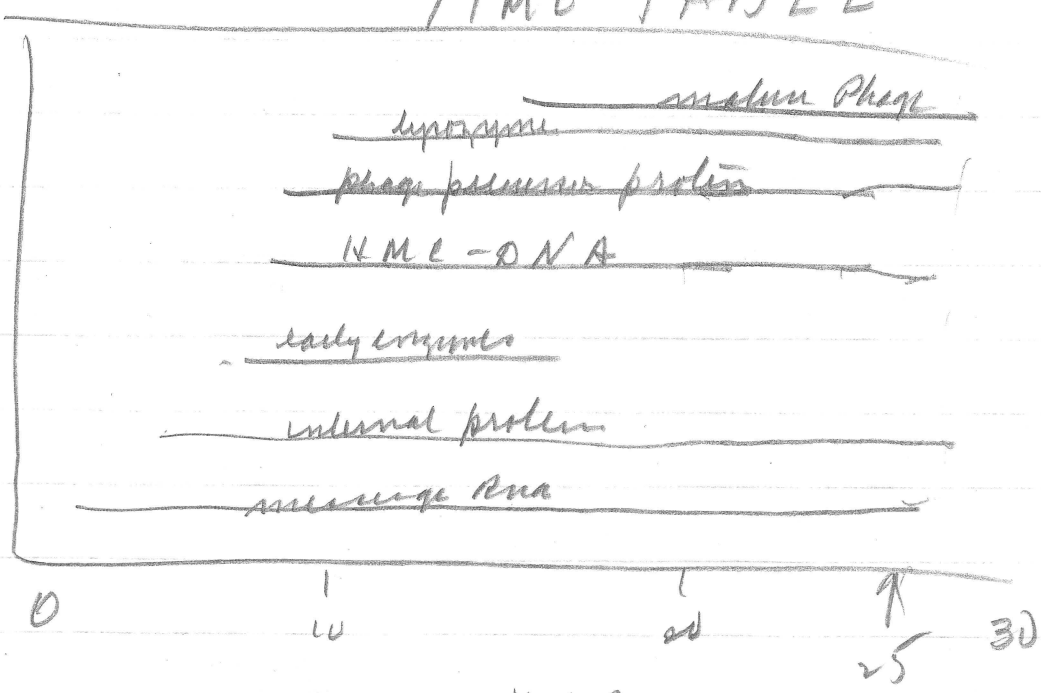
not true of all viruses

Soon new RNA is made in bacterial cell. It hybridizes c̄ phage DNA. It uses the hosts' ribosomes!! – there is phage messenger RNA.

Reviews enzymes required for phage synthesis.
These stop being made by 10 minutes after infection —
Lysozyme synthesized at 7 minutes, but not used until the cell explodes — In lysozyme-less mutants, phage can't get out unless cell is artificially broken.

Superinfected, the period before lysis is prolonged.
(Slept thru some interesting possibilities of combined lysozymeless & normal T4 —

TIME TABLE



THE PHAGE CLOCK

From above, genes must act at different time in course of infection — activations and deactivations — —

Infected bacterium + phage thus behaves like a differentiating system in higher organisms — contrast a non-infected bacterium which is more of a steady-state system.

Can phage help us in understanding way in which structures are built — how components assembled from macromolecules.

There are about 25 proteins synthesized after infection which can be attributed to phage genes. — naively figure about 200 - 300 genes — What are all the rest doing that aren't making 25 proteins.

With mutants, gene functions will show up!

Conceivably + reasonably one could

get a mutant of each of the 300 genes.
But - the effect must be visible -- assume
if mutations are severe, we can detect them
as lethals & will have a chance to learn
how each fails.

90% of
Antibodies which block infection are anti tail fibers.

Edgar — July 26, 1963

Can't idea of yesterday — find lethal mutations — But have to propagate — so, get Conditional lethals — ie those that will not survive under restricted conditions —

Edgar has sought temperature - sensitive mutants Also can use Host dependent mutants — Many isolated by Epstein in T4 — Survive in strain K of coli but not in b — These are called the "amber" mutation — The hosts differ in a "suppressor" gene — Mutant gene of phage can not be transcribed in the "restrictive" host.

Both Edgar & Epstein have isolated several mutants of these respective kinds — Map the genes with complimentation test — No deletions; all point mutations.

ie "trans" combination

Circular map — have mapped about 48 genes of temp sensitive and amber mutations. At some loci, both temp sensitive and host sensitive mutations have been found.

Genes vary in size — some are very long —

With "amber" mutant there is no intergenic complementation!?
but with temp. sensitive, complementation can occur unless the temp sensitive is in an "amber" region — ie when lots of amber. ???

When we deal with different genes making different proteins then complementation occurs regardless of amber & sensitive.

Now how find out what processes the mutant control?

First check for whosts nuclear breakdown
Do they induce DNA synthesis
Can mutant lyse the cells?
Is tail fiber antigen synthesized
Are there any morphological changes in the phage.

Just a few functions.

D.O. — DNA Zero do not synthesize DNA or activity in "late functions"

D.A. — DNA arrested — DNA synthesis starts normally but stops in 10 minutes.

DA's do synthesize tail fibers antigens etc -

DD. DNA delay — DNA synthesis late in starting — not until 20 minutes — lysozyme produced + other proteins correspondingly delayed — but can get many phage particles

MD - maturation defective — late functions do not occur.

Others - Not viable - presumed all some which fail to make tail fibers. Amber tail fiber mutants make no tail fiber antigen — temp sensitive ones make protein but can't assemble it into fibers

morphog mutants - Heads only; Tails only; Heads and Tails, but can't be assembled — Heads empty (DNA leaks out); Head & Tail fall apart. "Triggered" sheath (ie phage already fired) but head still filled.

Each mutant now known for specific effect, except one.

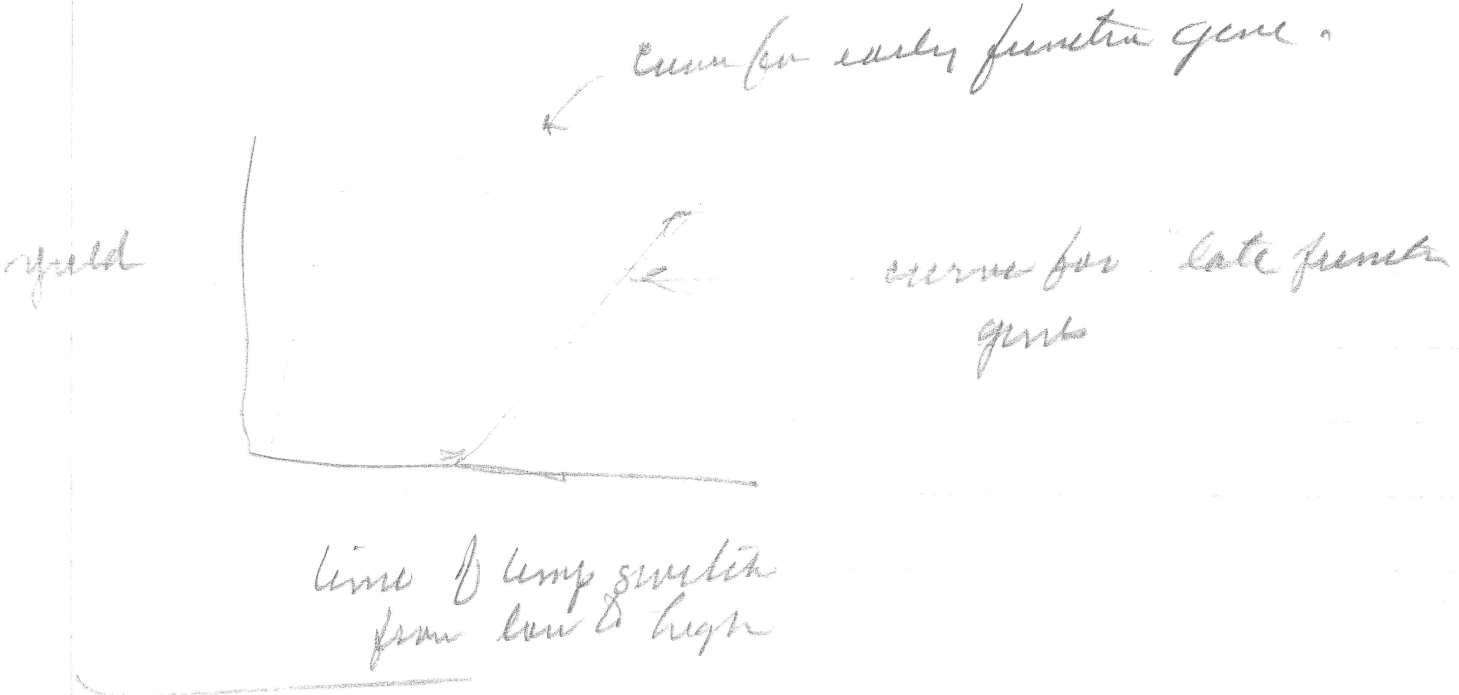
Genes seem to be clustered with common "phenotype"
Is genome organized into operons?

Back to question of turning on & off the genes — Assume 2 blocks of genes: early functions & late functions & these are completely separate — Early function finished at 10 min —

When early functions do not occur, late functions do not occur

In mutants which fail to synthesize DNA, the early functions stay turned on and late ones are never turned on.

With temp sensitive mutants, you can switch temps during growth — thus find out at what time genes function.



Epstein has been able to give 5 fluorouracil to some temp sensitive mutants & get the faulty message corrected — this is speculative — but if true, should tell when messenger is made — — Indeed early mutants do seem to make messenger early & vice versa. —

Morphogenesis of phage particle. — More genes located for maturation of particle than for other functions — More genes than components of particle — 35 of them found —
Some, therefore, are not engaged in producing components of the particle. Seems to violate principle (from TMV studies) of spontaneous crystallization of form from subunits

Edgar considers that some genes may be concerned with producing something important to the aggregation of subunits. — Can we get evidence to bear on this —

Lots of loci concerned with head — is only one concerned with producing head protein? etc.

Mutants resistant to osmotic shock — no peptide differences from normal.

Mutant ~~Gene~~ 23 found not to make head protein — Other headless phage mutants, however have the protein, but apparently can't assemble it. — ~~Have one~~

Other genes ① release head from membrane, ② control osmotic resistance, etc —

Bodenstein -

I Intro -

A. Why cockroaches

1. Tough

2 - Regenerate easily - cut off an appendage & it regenerates in next moult -

II General considerations about regen

A Organ stump only carries potencies of regeneration -

B always regenerate only what was lost.

a) Sometimes regenerate is smaller - Why? Depends on how near to next moult it is when you cut off appendage. - If moult is soon, the new leg will be smaller. -

Next, must have enough cells.

III Nerves & regeneration - Do insects require nerves for regeneration?

A. In walking-stick report that nerveless regenerate has no muscles

B In Cockroach, contrary to what people said, nerves regenerate & severed legs regenerate beautifully - Have found no way to stop regeneration of nerves into stump - even when ganglion cells are removed.

Regeneration in Adults

IV ~~Failure~~ of regeneration in Adult.

A. Are Hormones at fault - Give adult some hormones from nymph (transplant ~~glands~~. prothoracic gland.

Do So - Get regeneration in adult - of adult type

Run into trouble with adult, - he has lost his zipper & cant get out of his moult skin

B. Does adult require higher hormone titer for regen than does nymph. -

1. Parabiose a nymph & adult to see - Nymph leg regeneration proceeds much more rapidly - Eventually adult, also, builds a leg.

C Can an adult tissue moult more than once? Transplant adult leg to back of young nymph which has several moults to go - By cutting new leg, have made regenerates of adult leg 5 times.

D - It's hormone & not wound influence which stimulate regeneration in absence of a leg. Let wound heal 4 weeks after cutting. Add hormone & get regeneration

But!

D. Remove Prothoracic gland of nymph & nymph leg will regenerate!!

1. Let such a nymph moult to adulthood. Remove all other glands also. Parabrose a nymph also lacking its complete hormone system & brain —
The adult now still can regenerate!!

2 Accidental observations — adult ~~[illegible]~~ for electrophysiology — electrodes into leg nerves cut. — Legs broke off and in 2 cases of many a leg regenerated. Tried to control the mechanical & electrical variables — no success in repeating!

moult?

3 Postulate maybe there is a brain hormone which is activated by ecl.

Transplant 3 nymphal brains to adult & got 6 cases in which animal moulted & regenerated leg.

4 Clara study showed that in these cases the prothoracic glands (which normally degenerate in adult) had regenerated!

5 Now have removed prothoracic glands and get a very few cases of degeneration & then in all females!!

Now all is a mess! — Where do we go from here.

Discussion —

In insects, sensory nerves originate on periphery — So you can never get a regenerate completely nerve free.

What is source of insect blastema? — Possibly from muscle, for if corp depleted of muscle, regeneration of femur etc. is depressed — ie, smaller regenerate.

August 2 – Weissman

I Intro – How we started

A. Floppy ears with Vitamin A in excess.

B Reversible with withdrawal

C – Histology

Metachromasia of cartilage throughout body, as well as in ears, knocked out –

Papain, injected I.V. does same thing

D Lysosomes

1 Acid hydrolyases

and RNase

RNase

and P ase

B glucuronidase

B N-acetylglucosaminamidase

B galactosidase

Aryl sulfatases

and lipases

" proteases

" lysozyme

" α mannosidase

2 Released in vitro by variety

2 Released in vivo by – ischemia, autotypic, shock, endotoxin, etc

pp-L = legally sedimenting chondromucoprotein of rabbit ear —
Its viscosity is lower ~~when isolated from~~ when treated with liver homogenates of Vit A injected or UV-treated animals.

Cortisone protects completely the effect of hypervitaminosis A.

Soluble enzymes of mitochondria also released by vitamin A — e.g. malic dehydrogenase. — membrane bound enzymes of mitochondria not released

Mitochondrial "swelling" agents also release malic dehydrogenase. Streptolysin, cysteine & vit a release malic Dehydrog as well as lysosomal enzymes.

With many agents the lysosomal changes in enz activity can be reversed, indicating that lysosomal rupture does not always occur when the enzymes are released! — Control, in tissue, of lytic activity?

Xenopus tadpoles – treat with Vit A –
1. "Tentacles" resorb after drooping & collapsing
2. Hemorrhage in toes
3. Tails resorb in 3 weeks or more
a Melachromasia of tail fin demonstrating

In Gut – many more mucous cells and resulting diarrhea.

Tadpole protected against Vitamin A acid – (not against Vit A alcohol – latter stored in liver as palmitate.)

In Metamorphoses, β glucuronidase activity of tail increases ~~like~~ cf. Weber's increase in catheptic activity.

Cells containing lysosomes flood the area of amputation, cleaning up debris.

2 hr pulse of Vit A will inhibit regeneration.

What would be effect on metamorphoses by pre-rupturing

lysosomes — those which survive pulse, usually die when metamorphosis almost reached

A rudeu shows at metamorphosis certain protein produced which bind lysosomal enzyme

Thyroxin — does it have direct effect on lysosomes?

thyroxin promotes swelling of mitochondria from animals with hypervitaminosis A but not from hypo vit animals

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Discussion —

Are there different lysosomes for different enzymes — Not known — Staining does not show all in same particle, but is this a staining artefact —

Eosinophils

Leucocytes loaded with granules which are lysosomes — go to wound areas —

IRS Drinking back

mucoplasia on one hand from Hyper A, — ie metachromasia — But loss of metachromasia in cartilage.