THE EXPLOSION OF THE SPERMATOZOA OF THE CRAB *LOPHOPANOPEUS BELLUS* (STIMPSON) RATHBUN.

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(FORTY-SIX FIGURES.)

CONTENTS.

INTRODUCTION.

For a number of years the writer has been studying the male germ cells of the Decapoda with two purposes in mind: (1) to discover the means by which the mature, dormant spermatozoa of the Decapoda become activated, in order to shed light on the problem of fertilization in this order of Crustacea, and (2) to trace more clearly the process of spermatogenesis. The present paper on the explosion of the spermatozoa of the black-clawed crab, *Lophopanopeus bellus*, is a contribution involving the former of these problems.

MATERIAL AND METHODS.

The material for this study consisted of the living spermatozoa of *Lophopanopeus bellus*, common in certain localities around the Puget Sound Biological Station, Friday Harbor, Wash. The spermatozoa of this crab are very favorable for study in that they are not enclosed by the numerous spermatophores so common in other brachyura. As pointed out in another paper (Fasten, 1917), "in *Lophopanopeus bellus* it doesn't seem as if numerous spermatophores are developed. Here it appears that one large spermatophore is formed in which the spermatozoa are tightly packed." Since this is the condition all that was necessary to

obtain a plentiful supply of living spermatozoa was to rupture the deferent ducts and the male gametes oozed out in tremendous numbers.

The living spermatozoa were studied in the same manner as described in my earlier paper on the spermatogenesis of the edible crab, *Cancer magister* Dana (Fasten, 1918). Numerous spermatozoa suspended in the crab's body fluid, or in sea water which is isotonic with the crab's body fluid, were placed on a slide and covered with a cover glass. These could then be studied with the high power oil-immersion lenses. By allowing various chemical solutions to diffuse under the cover glass all changes in the spermatozoa could be observed and outlined with the aid of the camera lucida.

The living spermatozoa were studied in the following solutions:

1. Crab's body fluid.
2. Sea water.
3. Sodium chloride (NaCl)—M/2 NaCl and less.
4. Sodium nitrate ($NaNO_3$)—M/2 $NaNO_3$ and less.
5. Calcium chloride ($CaCl_2$)—3/8M $CaCl_2$ and less.
6. Potassium chloride (KCl)—M/2 KCl and less.
7. Potassium nitrate (KNO_3)—M/2 KNO_3 and less.
8. Potassium hydroxide (KOH)—very dilute solution.
9. Distilled water.
10. Cane sugar ($C_{12}H_{22}O_{11}$)—M/1 $C_{12}H_{22}O_{11}$.
11. Ovarian fluid.
12. Acidulated sea water. Various small amounts of acids were added to sea water, such as: glacial acetic, salicylic, saponin, sodium glycocholate, nitric, hydrochloric, oxalic, tannic, picric, and chromic acids.

Many of the spermatozoa in all stages of explosion were fixed on the slide with either osmic acid fumes, or Bouin's fluid, or Flemming's mixture, and then stained with Heidenhain's iron-hæmatoxylin and acid-fuchsin. Those fixed with osmic acid fumes gave beautiful results, so that the stained elements were perfect representations of the living structures. This can be clearly seen when one examines Figs. 3–7, which are from stained preparations fixed with osmic acid fumes, and compares them with figures 1 and 2 which are from living spermatozoa suspended in the body fluid of the crab.

Normal Appearance of Spermatozoa.

The living spermatozoa of *Lophopanopeus bellus* when studied in the cœlomic fluids of the crab are found to be small, greenish bodies, which appear like spheroids when seen from the top or bottom (Fig. 1), and like ellipsoids when viewed from the side (Fig. 2). In structure they seem to be similar to those of *Cancer magister*. Within the centre there is a clear central body (Figs. 1 and 2, *b*) and surrounding this are two vesicles; a uniform, darkly green secondary vesicle (Figs. 1 and 2, *v'*), and a clear, transparent primary vesicle (Figs. 1 and 2, *v*). Outside of these vesicles is a granular and vacuolated protoplasmic cup (Figs. 1 and 2, *h*) of a lighter greenish hue than the secondary vesicle. If the spermatozoa remain suspended in the crab's body fluids for some time their protoplasmic cups open up and liberate the radial arms (Figs. 3–7). It is thus seen that the protoplasmic cup of the spermatozoön consists of a nuclear cup (Fig. 3, *n*) and radiating radial arms (Fig. 3, *r*).

When the spermatozoa are fixed with osmic acid fumes and stained by the iron-hæmatoxylin and acid-fuchsin methods, then the nuclear cup, radial arms and the central body stain black (see Figs. 3–7), the second vesicle stains a dark amber, whereas the primary vesicle remains transparent.

Four types of spermatozoa are produced, depending on their number of rays. There is a three (Fig. 4), four (Fig. 5), five (Fig. 6), and a six (Fig. 7) rayed type. The four (Fig. 5) and five (Fig. 6) rayed types, however, are produced in largest numbers. These rays are not pseudopodia-like processes similar to those which Binford ('13) pictures for the spermatozoa of *Menippe mercenaria*. They are distinct arms similar to those found in the crayfish *Cambarus virilis* and *Cambarus immunis*, as pictured by the writer in a previous paper on the spermatogenesis of these forms (Fasten, 1914).

Effects of Chemical Agents on Spermatozoa.

1. *Sea Water.*—Sea water produces no change in the normal appearance of the spermatozoa. The protoplasmic cup, however, swells slightly and liberates the radial arms (Figs. 8 and 9).

2. *Sodium Chloride.*—An M/2 NaCl solution which is isotonic

with sea water produces no change in the normal appearance of the spermatozoa (Figs. 10 and 11). An M/4 NaCl solution brings about a slight shrinkage in the nuclear cup, otherwise there is no further change. In an M/6 NaCl solution the secondary vesicle is very slowly everted. First of all it squeezes out in the form of a small bubble (Fig. 12), until very gradually it assumes the appearance shown in Fig. 13. In an M/7 NaCl solution the eversion of the secondary vesicle is much faster. Figs. 14, 15 and 16 show successive stages in the eversion process. Most of the spermatozoa proceed to the stage shown in Fig. 16 and then cease. An M/8 NaCl solution brings about a complete and rapid explosion of all the spermatozoa. Figs. 17, 18 and 19 show respectively the beginning, middle and end of the process. In Fig. 19 the secondary and primary vesicles, as well as the central body are seen completely everted.

3. *Sodium Nitrate.*—An M/2 $NaNO_3$ solution which is isotonic with sea water brings about no appreciable change in the normal appearance of the spermatozoa. In an M/4 $NaNO_3$ solution the only change noticed in the spermatozoa is a slight swelling of the nuclear cup. An M/8 $NaNO_3$ solution causes a slow eversion of the secondary vesicle, producing figures similar to those shown in Figs. 15 and 16. In an M/16 $NaNO_3$ solution the eversion of the two vesicles occurs rapidly and with considerable force, so that all the spermatozoa soon take on the appearance shown in Fig. 19.

4. *Calcium Chloride.*—A 3/8M $CaCl_2$ solution is isotonic with sea water and this brings about no change in the normal spermatozoa. A 3/11M $CaCl_2$ solution brings forth a partial eversion of the secondary vesicle (Fig. 20). In a 3/16M $CaCl_2$ solution the spermatozoa explode completely. The vesicles are entirely everted and at the same time the nuclear cup shrinks considerably and becomes irregular. Figs. 21–24 show various stages in the explosion process. In the $CaCl_2$ solutions the detailed structure of the spermatozoa can be clearly distinguished.

5. *Potassium Chloride.*—In an M/2 KCl solution which is isotonic with sea water the spermatozoa remain normal. In M/4 and M/8 solutions of KCl the only perceptible change produced in the spermatozoa is a disappearance of the granules

and vacuoles in the nuclear cup making it become more homogeneously green. Also the secondary vesicle shrinks somewhat, thereby leaving the clear primary vesicle to show more prominently (Fig. 25). An M/16 KCl solution produces swelling and explosion of the spermatozoa (Fig. 26). In many instances the explosion is so violent that the nuclear cup ruptures completely.

6. *Potassium Nitrate.*—An M/2 KNO_3 solution which is isotonic with sea water does not produce any explosion. However, the protoplasmic cup swells and becomes more homogeneous in appearance. Also the primary and secondary vesicles become more distinctly marked off from each other (Fig. 27). An M/4 KNO_3 mixture has a similar effect. An M/8 KNO_3 solution brings about a swelling of the protoplasmic cup and a slow eversion of the second vesicle so that the spermatozoa resemble Fig. 28. In an M/16 KNO_3 solution the spermatozoa explode very rapidly and they come to look like Fig. 26.

7. *Potassium Hydroxide.*—Very dilute solutions of KOH bring forth a violent reaction in the spermatozoa. The protoplasmic cup swells, becomes homogeneous and at the same time pushes the vesicles upward (Figs. 29–31). The secondary vesicle undergoes a rotation and is pushed to one side. Finally the vesicles explode with great violence and the entire spermatozoön soon goes to pieces.

8. *Distilled Water.*—Distilled water produces a rapid eversion of the vesicles so that in a very short time the spermatozoa come to resemble Figs. 32 and 33.

9. *Cane Sugar.*—From the above experiments two conclusions might be inferred regarding the explosion of the spermatozoa, one is that it is due to lack of electrolytes, and the other is that the explosion is due to a reduction of the osmotic pressure produced by surrounding the spermatozoa with a hypotonic solution. In order to determine which factor we have to deal with, the spermatozoa were surrounded with an M/1 cane sugar solution which is approximately isotonic with sea water. If the factor involved were due to lack of electrolytes then, since the sugar solution contains no electrolytes, the spermatozoa ought to explode. But the M/1 cane sugar solution did not produce any

change in the normal appearance of the spermatozoa, thereby pointing to the second factor, namely, osmotic pressure, as the one which undoubtedly operates in bringing about the eversion of the vesicles.

10. *Ovarian Fluid.*—Since a reduction in osmotic pressure produces the explosion of the spermatozoa, the next question which naturally arises is whether the female gonads at the time of fertilization produce a hypotonic substance which, when coming in contact with the spermatozoa, causes them to explode, thereby bringing about fertilization of the ova. In order to test this out, the ovaries and oviducts were mashed up in sea water and the living spermatozoa were then surrounded by this mixture. In some cases (not all), a few of the spermatozoa exploded violently. The nuclear cup at first swelled and became homogeneous (Fig. 34). Then the vesicles were everted with considerable force and in many instances, the primary vesicle, or both the primary and secondary vesicles completely disintegrated, leaving stages like those shown in Figs. 35–39. Whether this was due to some agent produced by the female gonads or to some other agent cannot be definitely stated, for not all of the spermatozoa were affected in the same manner as those mentioned above. However, it is also significant that the ovaries used during the months of the year when the investigations were conducted (June and July), were past maturity. They were small and immature and this might account for the results obtained. Another significant fact to be taken into consideration is that in control experiments in which living spermatozoa from the same males as those used in the experiments with the ovarian fluids, were surrounded with sea water alone, none of the spermatozoa exploded. Now, the question arises, why should we get a violent explosion of even a few spermatozoa when ovarian contents are used and no explosion when the ovarian fluids are lacking? I am strongly of the opinion that the female gonads produce some substance which is responsible for the explosion. Also, it seems very probable that at the time of sexual maturity of the female this specific substance must be present in such quantities as to activate all of the living spermatozoa.

11. *Acidulated Sea Water.*—In all cases weak dilutions of the

acids were used. If the acid was a liquid, the dilution used was 1 part of the concentrated acid dissolved in 25 parts of sea water. A drop of this was then added to the edge of the cover glass under which the living spermatozoa were held suspended in sea water. If the acid used was crystalline in texture, then a few of the crystals were placed at the edge of the cover glass and allowed to dissolve slowly under it.

(*a*) *Glacial Acetic Acid.*—Causes the protoplasmic cups to lose their granular and vacuolated appearance. Usually two or three dark granules remain in the nuclear cup. The nuclear cups and radial arms swell and lose their color (Figs. 40 and 41). The spermatozoa in many instances are thrown together into aggregates (Fig. 42). After remaining exposed to the action of the acid for some time many of the spermatozoa explode (Fig. 43) and disintegrate completely.

(*b*) *Salicylic Acid.*—Reaction here is similar to that caused by glacial acetic acid.

(*c*) *Saponin.*—Causes considerable swelling (Fig. 44). Nuclear cup and radial arms become more homogeneous and much paler in color. They appear almost transparent. A few of the spermatozoa explode after being exposed for some time.

(*d*) *Sodium Glycocholate.*—Causes swelling similar to that produced by saponin or glacial acetic acid. During this swelling the vacuoles of the nuclear cup at first enlarge and then disappear, giving the nuclear cup a homogeneous appearance. Soon a violent explosion of vesicles takes place. Nuclear cup now loses its greenish color, becomes ragged and transparent with small dark spots. Very shortly the spermatozoa disintegrate.

(*e*) *Nitric Acid.*—This brings about a homogeneity of appearance in protoplasmic cup with considerable shrinkage (Fig. 45). The second vesicle in many cases is everted (Fig. 46).

(*f*) *Hydrochloric Acid.*—The reaction here is very similar to that caused by nitric acid.

(*g*) *Oxalic Acid.*—Reaction is similar to that produced by nitric acid.

(*h*) *Tannic Acid.*—Reaction is similar to that of nitric acid, with the exception that none of the vesicles are everted.

(*i*) *Picric Acid.*—The reaction produced in the spermatozoa

is the same as that brought about by tannic acid. The spermatozoa are soon killed and stained a yellowish-green.

(*j*) *Chromic Acid.*—This produces a similar result to that obtained with either tannic or picric acids. Here the spermatozoa are fixed a yellowish-brown.

Discussion.

A careful examination of the data presented in this paper shows quite clearly that a lowering of the osmotic pressure in the medium which surrounds the spermatozoa is responsible for their explosion. In this respect the present research bears out what Koltzoff ('06) first suggested for the explosion of the spermatozoa of other decapods. Also, Binford ('13) in *Menippe mercenaria* and the present writer in *Cambarus virilis* (Fasten, '14), and *Cancer magister* (Fasten, '18), have found that osmotic pressure accounts for the explosion of the spermatozoa. In the light of all this accumulated evidence it seems quite certain that the stimulating agent which brings about the explosion in the spermatozoa of the Decapoda, is one which reduces the osmotic pressure in the medium that surrounds them.

Since this is the operating factor, the question which naturally suggests itself is where in the Decapoda is such a stimulating agent produced? The writer is strongly of the opinion that the mature gonads of the female decapod produce some chemical substance which, when it comes in contact with the spermatozoa, brings about their explosion. The experiments with the ovarian fluids seem to point to such a conclusion. The work of Koltzoff ('06) and Binford ('13) also suggests a similar conclusion.

Concerning the function of the explosion, it, undoubtedly, acts as the force or the motive power which drives the spermatozoön into the egg during the process of fertilization. What parts of the spermatozoön actually penetrate the ovum during fertilization is still a debated question. Koltzoff ('06) and Spitschakoff ('09) are in agreement that the nuclear cup (derived from the nucleus of the spermatid) is the only structure which enters the ovum. Binford ('13), on the other hand, claims that the everted vesicles (cytoplasmic structures) of the exploded spermatozoön are driven into the egg, whereas the nuclear cup

remains on the outside where it soon disintegrates. In order to bring this mode of fertilization in harmony with the idea of the continuity of the chromosomes, Binford suggests that "the contents of the capsule (vesicles) may be derived from the nucleus of the spermatid and is probably oxychromatin which deposits basichromatin after it enters the egg and so gives rise to the chromosomes in the male pronucleus."

It is thus obvious that more research along this line is essential before any definite conclusions regarding fertilization in the Decapoda can be formulated. If we accept Binford's results then we must admit that they are contrary to everything that we know regarding fertilization in animals.

Summary.

1. The spermatozoa of the black-clawed crab, *Lophopanopeus bellus* (Stimpson) Rathbun, are minute, greenish cells, which appear like spheroids when seen from the top or bottom and like ellipsoids when seen from the side.

2. The structure of these spermatozoa is very similar to that of the edible crab, *Cancer magister* Dana. In the centre there is a tube-like central body, and surrounding this in order of sequence is a secondary vesicle, a primary vesicle and a nuclear cup with slender radiating arms.

3. There are four types of spermatozoa produced in *Lophopanopeus bellus*, depending on the number of radial arms which they contain. There are three-, four-, five- and six-rayed spermatozoa, with the four- and five-rayed types predominating in numbers.

4. In sea water and isotonic solutions of various salts, no change occurs in the normal appearance of the spermatozoa. In hypotonic solutions of these salts the spermatozoa explode by an eversion of the two vesicles and the central body.

5. In ovarian fluids some of the spermatozoa explode violently, with a rupture and disintegration of one or both vesicles.

6. Acidulated sea water has a harmful effect on the spermatozoa, either causing swelling or shrinkage, with subsequent disintegration.

7. A lowering of the osmotic pressure in the medium that

surrounds the spermatozoa, undoubtedly brings about their explosion.

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DESCRIPTION OF PLATES.

All figures in the accompanying plates were made with the aid of the camera lucida. All figures, except Figs. 3–7, were made from living spermatozoa. Figs 3–7 are drawings of spermatozoa which were fixed by osmic acid fumes and stained with Heidenhain's iron-hæmatoxylin and acid-fuchsin. The magnification of Figs. 1–7 is 3,300 times; that of Figs. 8–33 is 1,400 times, and that of Figs. 34–46. is 1,700 times.

Explanation of Plate I.

Figs. 1 and 2. Bottom and side views of living spermatozoa suspended in crab's body fluid. *b*, central body; *h*, protoplasmic cup; *v*, primary vesicle; *v'*, secondary vesicle.

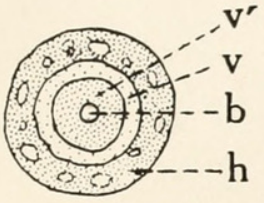
Fig. 3. Side view of spermatozoön fixed in osmic acid fumes and stained with iron-hæmatoxylin and acid-fuchsin. *b*, central body; *n*, nuclear cup; *r*, radial arms; *v*, primary vesicle; *v'*, secondary vesicle.

Figs. 4 to 7. Bottom views of spermatozoa fixed in osmic acid fumes and stained with iron-hæmatoxylin and acid-fuchsin, showing, respectively, three-, four-, five- and six-rayed types.

Figs. 8 and 9. Spermatozoa in sea water.

Figs. 10 to 19. Spermatozoa in various concentrations of sodium chloride.

Figs. 20 to 23. Spermatozoa in various concentrations of calcium chloride.



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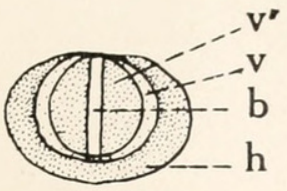

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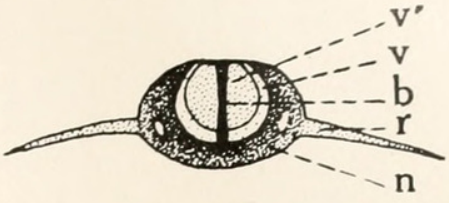

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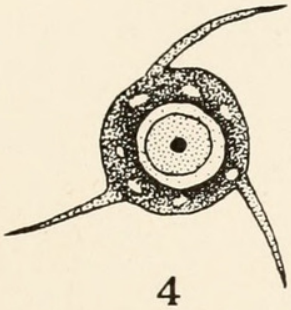

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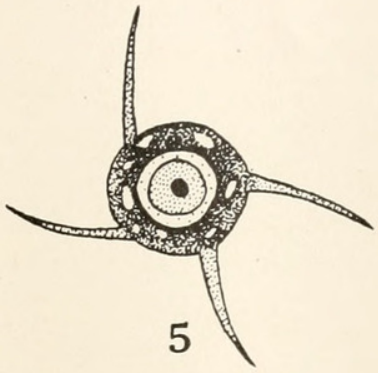

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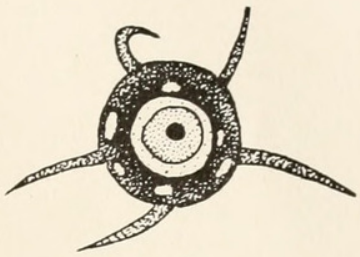

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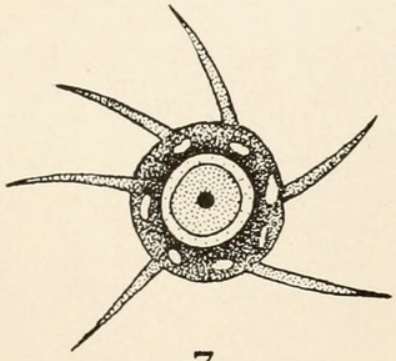

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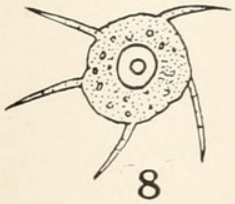

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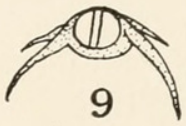

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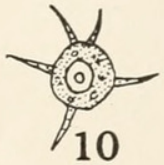

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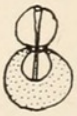

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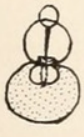

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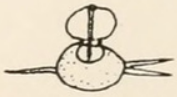

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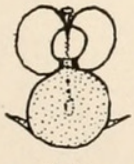

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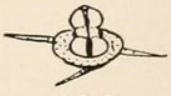

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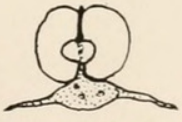

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Explanation of Plate II.

Fig. 24. Spermatozoön which has exploded in a hypotonic solution of calcium chloride.

Figs. 25 to 28. Spermatozoa which have been exposed to various concentrations of potassium chloride.

Figs. 29 to 31. Spermatozoa in weak solutions of potassium hydroxide.

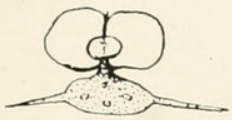
Figs. 32 and 33. Spermatozoa which have exploded in distilled water.

Figs. 34 to 39. Spermatozoa which have been subjected to the effects of ovarian fluids.

Figs. 40 to 43. Spermatozoa which have been acted on by glacial acetic acid in sea water.

Fig. 44. Bottom view of spermatozoön which has been exposed to saponin in sea water.

Figs. 45 and 46. Spermatozoa which have been acted on by nitric acid in sea water.



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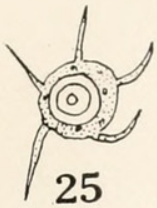

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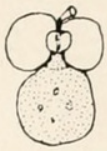

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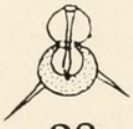

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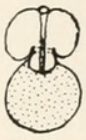

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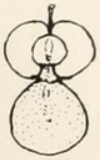

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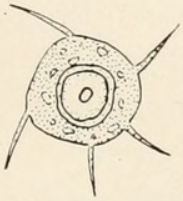

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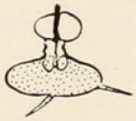

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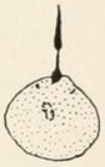

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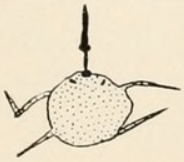

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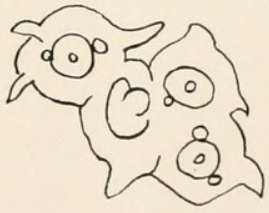

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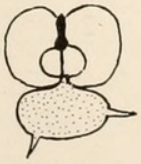

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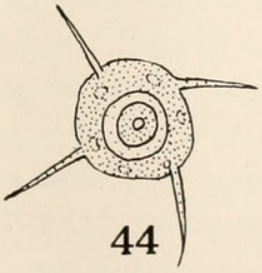

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NATHAN FASTEN.

A LEAF MIMICKING FISH.[1]

CARL H. EIGENMANN AND WILLIAM RAY ALLEN.

Heckel, in Johann Natterer's "Neue Flussfische Brasilien's nach den Beobachtungen und Mittheilungen des Entdeckers,"[2] described a small fish 3.5 inches long from a forest pool along the Rio Negro. He named it *Monocirrhus polyacanthus* and stated that at Marabitanas it was called *pirá-cáa* which means leaf-fish. Marabitanas is less than one degree north of the equator near the fiftieth degree of west latitude, in other words about sixty miles south of the southern end of the Rio Cassiquiare. No other specimens have been recorded. Günther placed it with *Polycentrus* to constitute the family Polycentridæ. The leaf-like appearance evidently impressed the Indians about Marabitanas who were acquainted with it and had a name for it.

During the Centennial Expedition of Indiana University, Dr. Allen secured three specimens of the same, or of a similar species from a brook near the Rio Itaya at Iquitos on the Peruvian Amazon.

The junior author reports that this fish was collected on September 19, 1920, while a guest of Don Antonio Layet at his hacienda, about six kilometers up the Rio Itaya from Iquitos. It was found in a small, sluggish brook which flows over very flat second bottom land, seldom inundated, and in the midst of dense forest.

There had not been much recent local rain, and there was only a slightly perceptible trickle of current at the riffles. Most of the brook was now reduced to quiet pools ten to fifteen feet across, densely overhung by vegetation, and shaded except for an hour or two at mid-day. The water was clear and of a slightly brown color, the bottom brushy, and matted with fallen leaves.

"Sr. Layet's servants had just introduced poison for me at the riffles, allowing it to flow slowly into the pools. Others with their long knives had made paths by which the low bank could

[1] Contribution from the Zoölogical Laboratory of Indiana University, No. 183.

[2] Ann. Wien Mus., 1840, II., p. 439.

be followed, The poison used was the milky sap washed from the pounded roots of *cube* (or *barbasco*), a plant cultivated as a fish poison and insecticide wash for cattle.

"I was beginning to grow impatient at the slowness of the poison, and to wonder if our long wait was going to be useless. I had observed several different species of fishes but they did not seem to be yielding to the usual respiratory difficulties following *cube*-poisoning, nor even to be trying to escape past the seines which we had stretched across the brook above and below.

"In order to know if there was sufficient current to carry the poison to every part of the pool, I began tossing broken twigs on the water to observe their course with the current. One such twig had reached a standstill, when directly beneath it I saw what was apparently a dead leaf being wafted past the twig. I couldn't understand why the twig was not moving too. At about that moment the leaf moved out into a path of sunlight, and toward the surface. There the resemblance to a fish became apparent, especially to one in search of the same. Its behavior, too, was like that of a poisoned fish struggling for oxygen."

The outline of the fish is similar to that of an asymmetrical leaf. The erected spinous dorsal and anal with their serrated character are not unlike the toothed edge of a leaf. The mimicry in color and markings is very close, the photograph and drawing of the dead specimens scarcely doing it justice. The lateral band has a position like that of a midrib of an asymmetric leaf. Like a midrib it fades away before reaching the distal margin. A petiole is not lacking, for the sharp, elongated snout and the protractile barbel carry out the resemblance.

While this fish may fall short of the perfection in mimicry exhibited by Kallima, it does take due account of the fact that few perfect leaves exsist, especially by the time they have reached the water. The transparent dorsal and soft anal between the spinous fins and caudal peduncle resemble breaks in the margin of a leaf. Futhermore the faded and discolored portions of many leaves, due to fungi, have their counterpart in the more ashy triangular area in the forward half of the fish.

The mimicry of *M. mimophyllus* has a physiological side. When swimming it moves in a gliding manner (like a seahorse)

that resembles a drifting leaf. This movement is due chiefly to the rapid beating of the small transparent soft dorsal and soft anal. These fins are set within the outline of the body, their bases being transverse to the body length. They have the direct forward push of the screws of a ship. Being hyaline their motion does not attract attention.

Several other species of fishes in the forest pools have the color of dead leaves. The others were seen before yielding to the poison, while *M. mimophyllus*, with a much more complete mimicry, was not.

A technical diagnosis of the species follows.

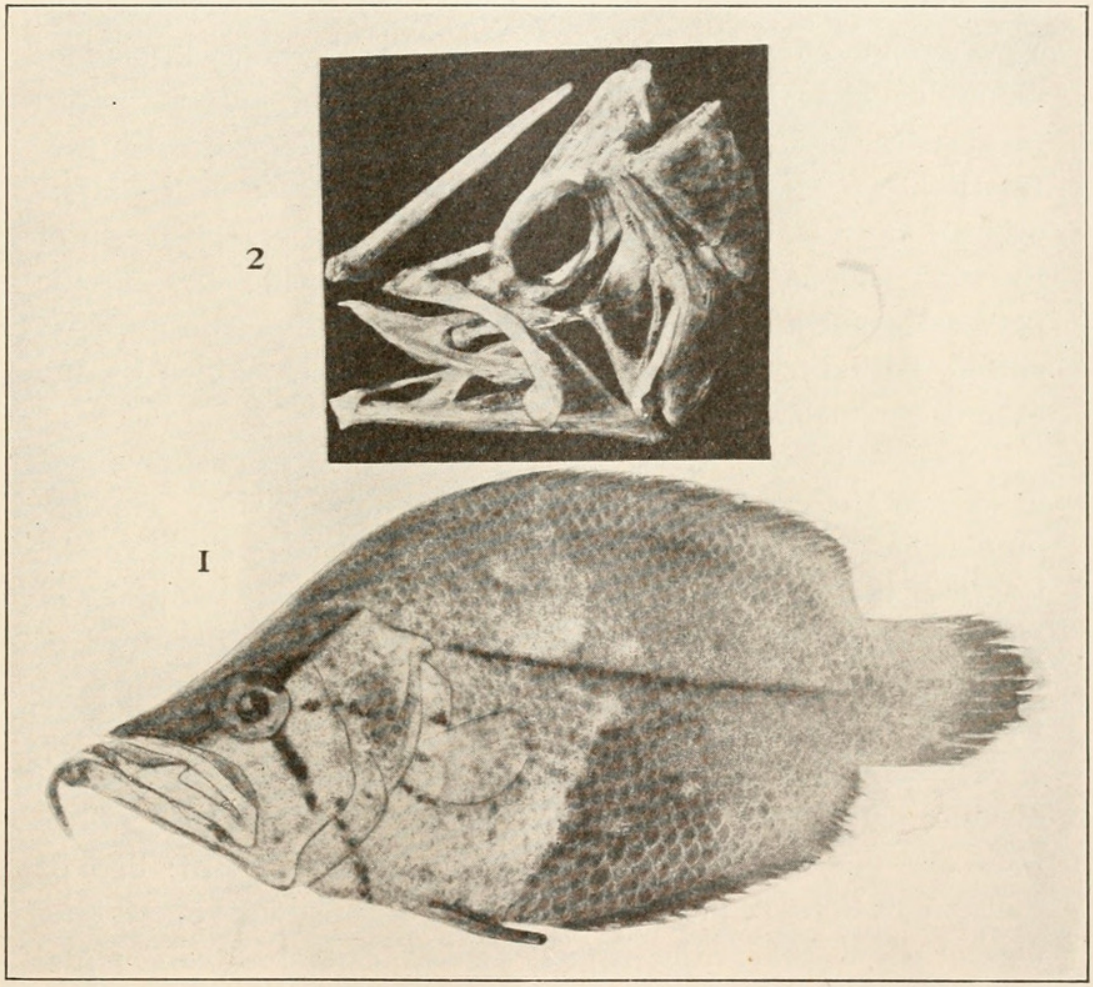

FIG. 1. Photograph of a specimen 61 mm. over all.

FIG. 2. Photograph of the skeleton of the head. The premaxillary spine is broken off from the rest of the bone, and the posterior end of the premaxillary has slipped upward a little away from its original position.

MONOCIRRHUS MIMOPHYLLUS Eigenmann & Allen spec. nov.

15715, I., 3, 44, 47, and 51 mm. long to base of caudal (65 mm. over all). Brooks near the Rio Itaya, Iquitos. Collected by Dr. W. R. Allen.

Evidently closely allied to *M. polyacanthus* Heckel, if distinct. In *M. polyacanthus* the caudal is said to be emarginate, the lateral band is said to run through the lower half of the tail, and the edge of the dorsal, anal, and tip of the ventrals are said to be blackish, the end of the caudal white.

Head 2.5; depth 1.92; D. XVI or XVII, 13; A. XII or XIII, 12 to 14.

Greatly compressed, the snout very sharp, the chin projecting, with a goatee barbel; the two rami of the mandible in contact below, equal in length to the head behind the anterior nares; maxillaries equal to snout and eye; premaxillaries greatly protractile; eye 1.5 in snout, 4 in the head, about .8 in the interorbital; opercular spine on a line between the upper margin of the orbit and the upper margin of the caudal peduncle. Profile between snout and occiput concave; gill-membranes somewhat united, entirely free from the isthmus, entirely hidden by the rami of the mandible.

Tongue very long and slender, rod-like, the free portion about as long as the eye, its tip soft, curved up and slightly cupped; premaxillary spine extending far beyond the eye, equal to the length of the mandible; mandible with one, in part two series of minute, recurved teeth; premaxillary with a single series of teeth on the sides, a triangular patch of teeth at the tip; no teeth on roof of mouth.

Pectoral broad, its length about 3 in the head, soft-rayed; distance between tip of the snout and origin of the dorsal a little more or a little less than 2 in the length without caudal; base of the spinous dorsal 2 in the length; base of soft dorsal about one-fifth of the length of the spinous dorsal; caudal *rounded*, equal to snout and eye or a little shorter; origin of anal and third dorsal spine equidistant from tip of snout; base of spinous portion of the anal about three in the length; base of soft part of anal a trifle longer than base of soft dorsal; ventrals reaching origin of anal, their inner ray adnate. First ray stout and spinous. Cheeks, opercle, and top of head to tip of snout scaled; preorbital

the only portion of the head naked. Scales of sides regularly imbricate, without lateral line pores; dorsal and anal partially depressible into a scaly sheath, the spines alternating when depressed. The scales of the sides roughened on half their exposed part, margined with very fine hyaline spinules.

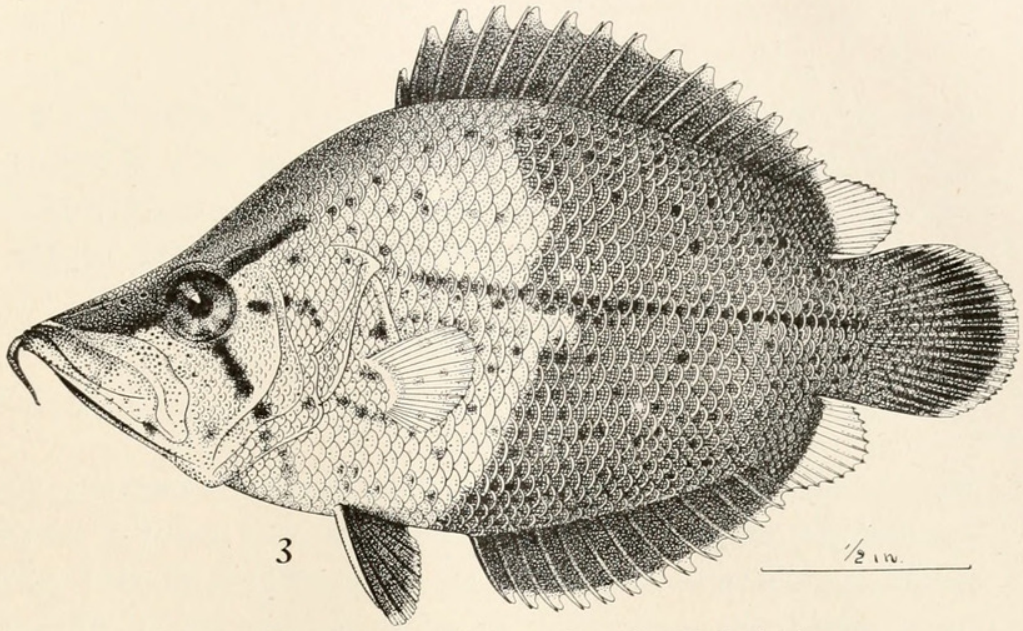

FIG. 3. Drawing of another specimen by W. S. Atkinson.

Four gill arches, lower arch of the first one with eleven rakers, the first a spinulous patch without projection, graduated to the last one which is about two thirds as long as the eye; all of them with numerous small spines; only two spinulous cushions on the upper arch; pharyngeal teeth similar to those on the gill-rakers.

Pectorals, soft dorsal, soft anal, margins of spinous dorsal, spinous anal and caudal and to a less extent of the ventrals hyaline; the hyaline of all but the soft dorsal, soft anal and pectoral bordered by black; a dark line from above the upper angle of the gill opening through the middle of the eye to the maxillary, a similar line from the eye through the cheeks crossing the breast half way between the ventrals and the gill opening, another one extending straight back from the eye; a similar dark line extending from the point of the opercle to the middle of the caudal peduncle; area from the middle of the ventrals up to the dorsal, and then forward below the line through the eye to the mandible several shades lighter than the back or the area behind this line. Slightly coppery color in living fish, this shade lost in alcohol.

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BIOLOGICAL BULLETIN

THE EFFECT OF IODINE AND IODOTHYRINE ON THE LARVÆ OF SALAMANDERS. II. THE RELATION BETWEEN METAMORPHOSIS AND LIMB DEVELOPMENT IN SALAMANDER LARVÆ.

E. UHLENHUTH.

(From the Laboratories of the Rockefeller Institute for Medical Research.)

In previous experiments (1) on the larvæ of *Ambystoma opacum* I found that iodothyrine did not accelerate the development of the limbs, although it caused rapid metamorphosis. Consequently, if the administration of iodothyrine was begun at an early larval stage, the metamorphosed salamanders possessed hind limbs which did not have the full number of toes. In agreement with these observations is the fact that feeding of thymus gland, although it resulted in an inhibition of metamorphosis, did not retard the development of the limbs of the thymus-fed salamander larvæ (2). Hence it is evident that in salamander larvæ the development of the limbs is independent of the substance (thyroid hormone) which causes metamorphosis.

The relation between limb development and metamorphosis as it exists in salamander larvæ is of especial interest, since apparently it is just the opposite of what should have been expected from the experiments performed on the anuran tadpoles. Through the work of Gudernatsch (3) and many other investigators it is well known that in tadpoles administration of thyroid gland, iodothyrine and other thyroid preparations accelerates not only metamorphosis, but also the development of the limbs. Lately Swingle (4) found that the administration of inorganic iodine which causes precocious metamorphosis of the tadpoles likewise accelerates development of the limbs.

Recent investigations have shown that the thyroid hormone and probably other morphogenic hormones, by increasing the rate of certain fundamental reactions, have the ability of causing structural changes throughout the entire organism, bringing thus about morphological expressions of a wide range affecting nearly the whole body. It seems that these hormonic substances, as far as their immediate effect is concerned, act chiefly by inducing a general histolysis throughout the various organs of the organism. There can be no doubt, however, that, besides these hormones referred to above, other substances play an important rôle in the development of an organism; these substances seem to possess a more localized action, effect the development of only certain organs and are concerned chiefly with the building up of the structures of these organs. Certainly, in the evolution of the organisms, the acquirement of the ability of elaborating the latter kind of substances must have played a rôle equally important as that played by substances such as the thyroid hormone. The limbs of the amphibians are apparently organs whose development seems to depend chiefly on the action of substances belonging to the latter group of substances and not on the activity of the thyroid hormone.

On account of the increased importance which, in the light of such considerations, seemed to attach itself to the finding that the development of the salamander limbs is independent of the thyroid hormone, it appeared necessary to repeat my previous experiments on the relation between limb development and metamorphosis. The present article will be devoted to reporting these new experiments. They consisted in causing precocious metamorphosis of the larvæ of *Ambystoma maculatum* by keeping them in iodothyrine, whereby special attention was paid to a possible acceleration of limb development. The result was the same as in the experiments on *A. opacum;* the rate of the development of the limbs remained unchanged, although metamorphosis took place at an early date. Not only larvæ, but also embryos, at early stages, were exposed to the influence of iodothyrine, in order to avoid the objection that failure of the iodothyrine to cause accelerated limb development was due to the experiments having been started at a stage at which limb development was too far advanced. Again

these experiments were completely negative as to an acceleration of limb development. In order to be certain that the method employed in my experiments on salamanders was correct, several tests were carried out on tadpoles; these were positive.

In addition to these experiments, several experiments were made to test the influence of inorganic iodine on the development of the amphibian limbs. Like Swingle (4), I found a distinct acceleration of the limb development in tadpoles; in the salamander larvæ, however, iodine had no effect on the development of the limbs. The bearing of this result, which is different from that of the experiments with iodothyrine will be referred to in the discussion.

Experiments on Tadpoles.

The experiments on tadpoles, which were intended to serve as a check to the experiments on salamanders, were not advanced beyond a merely preliminary stage, since they gave positive results from the very beginning. As they are confirmative of the observations reported by other investigators, they will be reported only briefly.

In one experiment on the larvæ of *Rana sylvatica* the controls were kept under observation till the 66th day after hatching. At this time the hind limbs of the control larva furthest advanced possessed 3 distinctly differentiated toes, while the 2 other toes were still rudimentary. In the iodothyrine series (kept from the 18th to the 26th day in water which contained 0.005 gm. Bayer's iodothyrine per 1,000 c.c. of water) the fore limbs broke through the walls of the gill chamber on the 33d day after hatching in every one of the 3 larvæ surviving, at this date, from the 6 larvæ composing the series at the beginning of the experiment. The inorganic iodine, in the concentration used in this experiment (2 to 3 drops of a 1/20 M solution of iodine per 1,000 c.c. of water), as well as in other experiments, proved to be considerably less effective than the iodothyrine, as 66 days after hatching the fore limbs had not perforated the gill chamber in a single instance. Yet the limbs, the hind limbs as well as the fore limbs, were distinctly further differentiated than in the controls of the same age; the hind limbs possessed 5 fully differentiated toes and in shape

were much like the hind limbs of an adult frog. Moreover, in 2 larvæ of the iodine series, which died at an age of 50 and 56 days respectively, the hind limbs possessed already at that date 5 fully differentiated toes.

In another experiment 5 series of the tadpoles of *Rana sylvatica* were kept in different concentrations of iodine (varying from 1 to 10 drops of a 1/20 M solution of iodine per 1,000 c.c. of water). The controls were kept under observation for 83 days; at the end of this period the hind limbs had remained undifferentiated, whitish buds in 5 of the 8 larvæ, while in the other 3 larvæ differentiation had taken place, the best differentiation being represented by 4 toes on the foot of the hind limbs. Many of the larvæ kept in inorganic iodine died; none of these was further advanced than the surviving larvæ. Among the surviving larvæ none had metamorphosed at the termination of the experiment, nor had the fore limbs broken through in a single instance; yet the limbs were considerably further differentiated than in the controls. In one larva of the iodine series, at an age of 73 days, the foot of the hind limbs was differentiated into 5 toes; in another larva, at an age of only 59 days, the hind limbs possessed 5 toes, and the fore limbs, which could be seen vigorously moving in the gill chamber, had developed 3 toes.

There can be no doubt that iodothyrine when administered to tadpoles greatly accelerates development of the limbs. Inorganic iodine, although it seemed less efficient in these experiments than iodothyrine, likewise increases the rate of the development of the limbs.

Experiments on Salamander Larvæ.

As pointed out in the introduction, my previous experiments on the larvæ of *Ambystoma opacum,* in which the administration of iodothyrine did not accelerate development of the limbs, were open to the criticism that the administration of iodothyrine was started at a stage at which limb development was fairly advanced (the toes having begun to differentiate), and that for this reason the iodothyrine may have been incapable of accelerating limb development. Therefore it seemed necessary to start the experiment at a very early stage. Two experiments were carried out, both on the embryos of *Ambystoma maculatum.*

Experiment I.—One egg mass of *Ambystoma maculatum* was collected on April 18, 1920. The eggs were not only freed from the general mass of jelly, but also the individual egg envelopes were removed, in order to assure ready access of the iodothyrine and iodine to the developing embryos.

Beginning of the experiment: 28 embryos selected; 9 of them placed into iodine-free water (10,000 c.c. H_2O, 0.16 gm. Na_2CO_3, 0.04 gm. K_2CO_3, 0.4 gm. $MgSO_4$–$7H_2O$, 0.6 gm. $CaCl_2$), 9 into iodine (2 drops of a 1/20 M solution of inorganic iodine per 1,000 c.c. of iodine-free water) and 10 into iodothyrine (0.01 gm. Bayer's iodothyrine per 1,000 c.c. of iodine-free water). In all embryos the first four visceral arches are formed; the fore-limb rudiments not yet differentiated from the pronephridial protuberance; no hind limbs.

The concentration of the inorganic iodine was increased to 8 drops per 1,000 c.c. 5 days, decreased to 6 drops 7 days, and decreased to 4 drops 11 days after the beginning of the experiment.

Sixteen days after the beginning of the experiment: Neither the iodine nor the iodothyrine had produced any influence on the development of the limbs (see Table I.).

TABLE I.

EXPERIMENT I.: 16 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.	Development of Toes.		
		3.5	3.0	2.5
Water	8	7		1
Iodine	8	5	1	2
Iodothyrin	10	9		1

Twenty days after the beginning of the experiment: The concentration of the iodine is decreased to 3 drops per 1,000 c.c. of water, the concentration of the iodothyrine increased to 0.1 gm. per 1,000 c.c. of water.

Twenty-seven days after the beginning of the experiment: Hind limbs commenced to differentiate into toes; but neither iodothyrine nor iodine accelerated limb development as compared to limb development of controls kept in iodine-free water (see Table II.).

Table II.

Experiment I.: 27 Days after Beginning of Experiment.

	Total Number.	Development of Toes.		
		6.0	4.5	4.0
Water	8	3	5	
Iodine	7		4	3
Iodothyrin	10		2	8

Yet the influence of the iodothyrine on metamorphosis had become noticeable in spite of the early stage of the larvæ, as the gills as well as the fin of the tail are found to be greatly atrophied.

Experiment II.—In this experiment one series of the embryos of *Ambystoma maculatum* was kept in inorganic iodine and one in iodine-free water. The concentration of iodine was 1 drop of a 1/20 M iodine solution per 1,000 c.c. of iodine-free water in the beginning, was increased later on to 8 drops and then gradually decreased to 3 drops. The embryos were at an early stage (formation of neural folds), when the experiment started; only the common jelly mass was removed.

Table III.

Experiment II.: 10 Days after Beginning of Experiment.

	Total Number.	Fore Limb Buds Present, No Indication of Toes.
Water	15	15
Iodine	14	14

Table IV.

Experiment II.: 18 Days after Beginning of Experiment.

	Total Number.[1]	Development of Toes.	
		3.5	2.5
Water	14	14	
Iodine	13	7	6

[1] Several eggs and embryos were attacked by moulds and as they disintegrated or developed abnormally, they had to be discarded causing thus the decreases in the total numbers.

TABLE V.

EXPERIMENT II.: 30 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.[1]	Development of Toes.		
		6.0	4.5	4.0
Water	14	5	7	2
Iodine	12	1	10	1

As Tables III., IV. and V. show, the inorganic iodine had no influence whatsoever on the development of the limbs.

The relation between limb development and metamorphosis was further tested in two experiments in which larvæ of *Ambystoma maculatum* at a more advanced stage were employed. Concerning the action of iodothyrine, the results were in complete accordance with those obtained in the larvæ of *Ambystoma opacum;* rapid metamorphosis, but no influence on limb development was observed. In each experiment one series was devoted to the study of the influence of inorganic iodine; this substance likewise had no influence on limb development, but unlike iodothyrine it did not cause precocious metamorphosis. Both iodine experiments as regards the influence of iodine on metamorphosis were described in detail in a previous article (5); they will be only briefly reported in this article.

In Experiment III. the larvæ were placed into iodine-free water containing 0.1 gm. iodothyrine per 1,000 c.c. of water at an age of 20 days, at which date nearly all larvæ had developed 4 toes in the fore limbs and several had commenced to develop the first 2 toes in the hind limbs. Thirty-three days after hatching—*i.e.,* 13 days after the first administration of iodothyrine—every one larva metamorphosed (as compared to 101 days in the controls), but in none of them the number of toes was more than 7.5, and in one it was only 6.0, this stage of limb development corresponding to the control series kept in iodine-free water without the addition of iodothyrine.

In Experiment IV. a smaller dosis of iodothyrine (0.01 gm. per 1,000 c.c. of iodine-free water) was administered. Precocious metamorphosis was caused also by this dosis, but the development of the limbs again remained completely unaffected as compared to

the controls. Since, however, metamorphosis took place at an age at which normally the limbs are fully developed (as shown by the controls), the precociously metamorphosed salamanders possessed in this experiment fully developed limbs.

Concerning the influence of inorganic iodine in these experiments, it was shown in a previous article (5) that administration of iodine does not result in precocious metamorphosis of salamander larvæ. In this article it should be added that it did have no effect also on the development of the limbs.

Discussion.

The experiments reported in this article confirm fully the observations made in my previous experiments. In the larvæ of salamanders the development of the limbs can not be accelerated by the administration of iodothyrine. Therefore, if iodothyrine is administered in doses which cause metamorphosis before the time at which, under normal conditions, the limbs are fully developed, metamorphosis takes place before the completion of limb development.

These facts demonstrate that in salamanders limb development is independent of the substance (thyroid hormone) which causes metamorphosis. This conclusion has recently been supported by several other facts. *Typhlomolge rathbuni,* the Texan cave salamander, does not possess a thyroid gland (6) and consequently does not metamorphose; yet its limbs develop in a normal manner. Hoskins and Hoskins (7) have shown that in the larvæ of *Ambystoma* the limbs develop normally, if the larvæ are deprived of their thyroid glands in early embryonic stages. This season I have repeated these experiments. Larvæ of *Ambystoma maculatum* were thyroidectomized at an early embryonic stage; these larvæ which are believed to have been successfully operated on (histological examination has not been made as yet) showed the same rate of limb development as the controls. Several larvæ of *Ambystoma tigrinum* were thyroidectomized at a stage at which 3 toes of the hind limbs were developed; the two other toes developed at a normal rate after thyroidectomy. These facts prove that in salamanders the substances causing limb development are not identical with those causing metamorphosis and consequently are not identical with the thyroid hormone.

In tadpoles substances which cause precocious metamorphosis also accelerate the development of the limbs. I have suggested, in a previous article (8), that in spite of this external difference existing between the larvæ of *Anura* and *Urodela* the primary effect of the thyroid hormone may be the same in both groups of animals, and the difference may not be a fundamentally different reaction upon the thyroid hormone, but merely a different mode of limb development.

There is no doubt that, except for the development of the limbs in tadpoles, the immediate effect of the thyroid hormone is, in both groups, predominantly a breaking down of tissues throughout the whole organism, not a building up of new organs. It is possible that in tadpoles the same substances endowed with a merely localized action cause limb development as in salamanders, but that in tadpoles these substances can not commence to build up the structures of the limbs before some obstacle has been cleared away by the action of the thyroid hormone. That the thyroid hormone controls limb development in the tadpoles does not necessarily mean that it has any part in the constructive processes of limb development. If we consider the advanced stages of the development of the fore limbs in tadpoles, we find conditions which make it indeed very probable that the thyroid hormone, in this process, plays merely the rôle of removing an obstacle external to the tissues of the limb itself. In salamanders both hind and fore limbs develop freely, while in tadpoles the fore limbs are inclosed in the gill chamber. In order that they may break through the walls of the gill chamber, certain changes of the skin and the tissues underlying it must take place; these changes are not caused by the legs themselves, but take place even in the absence of the limbs (9) at the time at which metamorphosis occurs. I have pointed out repeatedly that in salamanders one of the most conspicuous effects of the thyroid hormone is a certain change of the skin which finally results in the shedding of the skin and may be identical with the process which leads to the atrophy of the gills. A similar change is brought about in the skin of the tadpoles; in the tadpoles, too, the skin is shed for the first time when metamorphosis takes place. It is possible that the change of the skin which is necessary to

permit of the freeing of the fore limbs is identical with the change that causes the first shedding of the skin.

In support of this view is the fact that the freeing of the fore limbs is clearly the only step in the development of the limbs, which in not one single instance has been observed to take place in tadpoles which had been deprived of the thyroid secretion, while the developmental processes preceding the freeing of the limb may take place in thyroidectomized tadpoles. Allen (10) states that in tadpoles of *Bufo,* in the complete absence of a thyroid, both fore and hind limbs develop normally (and even grow larger than the limbs of normal larvæ)—*i.e.,* behave exactly like the limbs of salamander larvæ—yet the fore limbs fail to break through the walls of the gill chamber. Apparently in this anuran species the development of the limbs, except for the freeing of the fore limbs, is independent of the thyroid hormone as it is in the urodelan larvæ.

Should these views prove to be correct, it would seem probable that the mode of limb development in amphibians is the morphologic expression of the existence of two kinds of morphogenic substances; one group of these substances serves to procure the actual building stones of the morphological structures of the organ, while the other group of substances merely brings about a general histolysis of old structures, removing thus obstacles to the action of the substances belonging to the first group of substances.

As has been mentioned above, the ineffectiveness of inorganic iodine in the limb development of salamanders has a reason different from that of the ineffectiveness of iodothyrine. In a previous article (5) I have shown that, in contradistinction to iodothyrine, the administration of inorganic iodine does not produce precocious metamorphosis in salamander larvæ. The inorganic iodine has no effect on the salamander metamorphosis, because the thyroid hormone in salamanders is not released during the greater part of the larval period, and a greater supply of inorganic iodine, even if it should result in the elaboration of an excess of thyroid hormone, as it actually does in tadpoles (11), can not make itself felt in the salamander larvæ in consequence of the retention of the hormone. As has been shown in this article, inorganic iodine, like iodothyrine, has no effect on limb development of salamanders. But it must

be borne in mind that the ineffectiveness of inorganic iodine in limb development of salamanders is merely due to the above-mentioned peculiarity of the thyroid apparatus of the salamander larvæ and not to the fact that limb development in salamanders is independent of the thyroid hormone and of metamorphosis. Inorganic iodine could not accelerate limb development of salamanders, even if iodothyrine would have an accelerating effect.

Summary.

1. Tadpoles of *Rana sylvatica* were kept in iodothyrine and in solutions of inorganic iodine. Both substances were found to accelerate limb development. This result confirms the observations of previous investigations.

2. Embryos as well as larvæ, in early stages, of *Ambystoma maculatum* were kept in iodothyrine and in inorganic iodine. Neither of these substances accelerated the development of the limbs, although iodothyrine caused rapid metamorphosis of the salamander larvæ.

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MICRODISSECTION STUDIES, III. SOME PROBLEMS IN THE MATURATION AND FERTILIZATION OF THE ECHINODERM EGG.

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This paper is a record of operative work on the starfish, sea-urchin and sand-dollar eggs to ascertain the morphological nature of changes which take place in the egg during its maturation and fertilization. Results were obtained on the effect of nuclear material on cytoplasm, the nature of cortical changes in the maturing and fertilized egg and the difference between cortex and medulla of the egg with respect to fertilizability and to other life activities. The dissection and injection of the living eggs were carried out at first by means of Barber's ('14) apparatus and later with an improved micromanipulator of my own design ('21[b]). A description of the technique as applied to microdissection has already been published (Chambers, '18[a]). A detailed description of the new micromanipulator will appear both in the *Journal of Bacteriology* and in the *Anatomical Record*.

1. THE GERMINAL VESICLE IN THE MATURING STARFISH EGG.

Starfish eggs, on being shed naturally, have already begun maturing. In order, however, to secure large quantities of eggs, it has been the general custom to remove the ovaries bodily from a ripe female and to cut them up in a bowl of sea water. This procedure brings the eggs into the sea water in the immature condition with germinal vesicles intact. The germinal vesicle begins to disappear anywhere from thirty to fifty minutes after the eggs come into contact with the sea water and maturation usually proceeds in a normal manner (Wilson and Mathews, '95).

The undisturbed germinal vesicle or nucleus of a fully grown

immature egg is a hyaline sphere containing a sharply differentiated nucleolus and occupying about one fifth the volume of the egg. With the microdissection needle the vesicle may be moved about in the fluid cytoplasm without injury to the egg. With the needle one may considerably indent the surface of the vesicle. On removal of the needle the vesicle reverts again to the spherical shape (Fig. 1). The vesicle possesses a morphologically definite surface membrane inclosing an optically homogeneous liquid (cf. Chambers, '18[b]). Within this liquid lies a visible body, the nucleolus. By agitating the vesicle the nucleolus may be made to occupy any position within the nuclear fluid. The nuclear membrane is very easily injured. If, however, a microneedle be carefully inserted into the nucleus, the membrane about the puncture adheres to the body of the needle and the tip of the needle may push the nucleolus about with no apparent injury. The existence of considerable tension in the nuclear membrane is shown in the following experiment. An egg was cut into three fragments in such a way that the surface film forming over the cut surfaces of the middle fragment pressed upon the nucleus, deforming it considerably (Fig. 2). The attempt of the nucleus to return to a spherical shape bulged out one end of the egg fragment until it was constricted off from the remainder of the fragment (Fig. 2*b–f*).

Tearing the nuclear membrane in most cases results in a destruction of the nucleus. In a few cases it was possible to produce a slight rupture with no noticeable injurious effects. Such a case is recorded in Fig. 3. At 10:44 A.M. undue pressure on the germinal vesicle when cutting an immature egg in two resulted in its rupture followed by a lobular extrusion bounded by a very delicate film. During the following ten minutes the vesicle began slowly to revert to its original shape (Fig. 3*b* and *c*). Before that was attained the maturation process began and, at 10:55, the outline of the vesicle had disappeared. The nucleated egg fragment maturated normally and five hours and a half after insemination it had segmented in two. At 8:40 P.M. it had developed into a swimming blastula.

The cytoplasm of the egg allows of considerable tearing without

apparent injury (Chambers, '17-*a*). If, however, the nuclear membrane be torn, a very striking phenomenon occurs. The cytoplasm immediately surrounding the nucleus disintegrates and

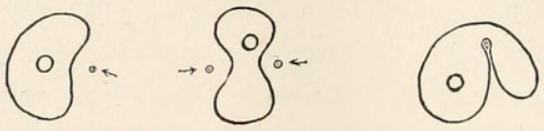

Fig. 1

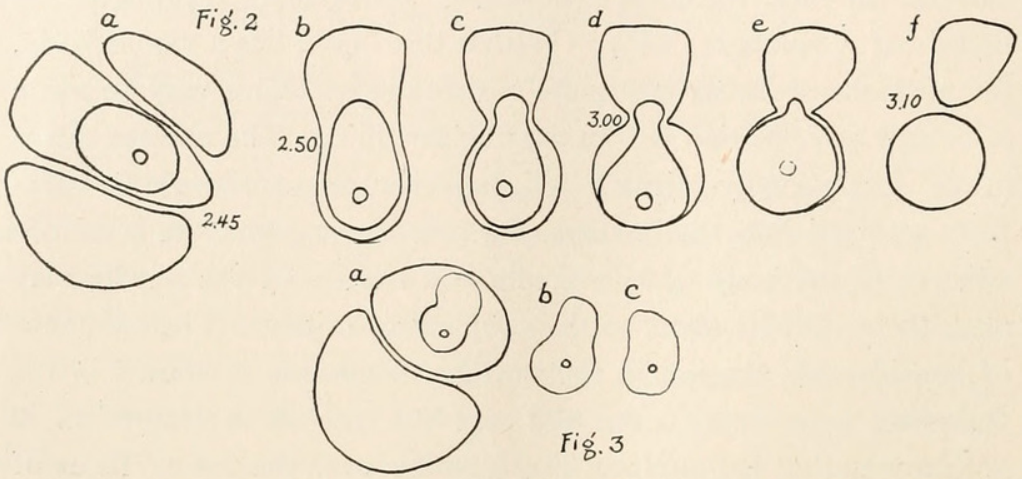

FIG. 1. Figures showing the extent to which the nucleus (germinal vesicle) of an immature starfish egg may be indented on one or both sides without rupture. On removing the needle the nucleus reverts to its original spherical shape.

FIG. 2. *a*, immature starfish egg cut at 2:45 P.M. into three parts; the nucleus has remained intact but is laterally compressed in the middle fragment. *b*, *c*, *d*, *e* and *f*, successive steps in attempt of nucleus to round up; *b*, 2:50 P.M.; *d*, 3:00 P.M.; *f*, 3:10 P.M.

FIG. 3. *a*, partial rupture of nucleus followed by a repair of its membrane. *b* and *c*, successive changes in the shape of the nucleus within the following ten minutes after which time it disappeared.

liquefies. If the rupture of the nucleus be violent, the disintegration of the cytoplasm spreads rapidly until the entire egg is involved. If the rupture be slight, the disintegrative process is quickly limited by a surface film which forms on the boundary between the disintegrating and the surrounding healthy cytoplasm (Fig. 4). This film tends to prevent any further spread of the destructive process. The destruction of the cytoplasm is evidently due to something which emanates from the injured nucleus. The injury to the cytoplasm does not start where the nuclear membrane is first torn, but from the entire surface of the injured nucleus.

This is analogous to results obtained by injuring red blood corpuscles with a needle upon which hemoglobin escapes immediately from the entire surface (Chambers, '15).

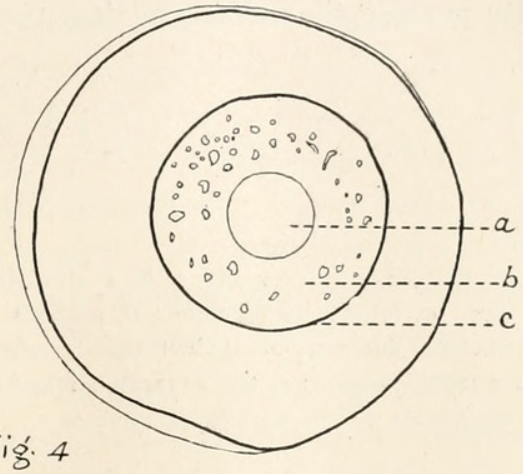

Fig. 4

FIG. 4. Disintegration of cytoplasm surrounding the nucleus on tearing the nucleus with a needle. (*a*) Faint hyaline sphere, a remnant of the destroyed nucleus. (*b*) Disintegrated cytoplasm. (*c*) Cytoplasmic surface film separating disintegrated from healthy cytoplasm.

Within the nucleus itself the immediate effect of the injury is a dissolution of the nucleolus. A nuclear remnant tends to persist after the injury as a hyaline sphere lying within the disintegration products of the cytoplasm. On being touched with the needle it fades from view.

In permanently immature eggs, such as eggs which have been standing in sea water for an hour or more without maturing, the disintegrative effect on the cytoplasm by injuring the nucleus tends to be much more restricted, and the nuclear sphere which persists after the injury can be shown to possess a morphologically definite membrane. Such a sphere is easily dissected out of the egg. Frequently, when the germinal vesicle lies close to the periphery of the egg, the disintegration of the cytoplasm quickly reaches the surface. With the formation of a surface film over the healthy cytoplasm the disintegrative area lies in a deep bay on one side of the egg. This hollow is slowly obliterated as the semi-fluid substance of the egg strives to assume a spherical shape. In this way the disintegrated material is forced out of the egg together with the persisting nuclear sphere. This nuclear sphere persists for some time in the sea water. It can be deformed by means of the needle and, on

tearing its surface, the fluid contents escape, leaving behind a collapsed membrane which disappears within 10 to 15 seconds.

Fig. 5 shows the effect of cutting the mature egg nucleus of the starfish egg. By pushing the nucleus against the inner surface of

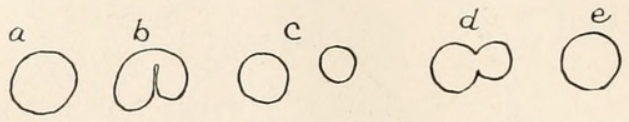

Fig. 5

FIG. 5. Effect of cutting mature nucleus of a starfish (*Asterias*) or sea-urchin (*Arbacia*) egg. *a*, intact egg nucleus; *b*, nucleus in process of being cut in two. The nucleus was pushed against the periphery of the egg as it was being cut by a vertical needle; *c*, the separated fragments of the nucleus; *d*, reunion of the fragments; *e*, reconstituted nucleus.

the egg it is possible to pinch it into two pieces. Each piece rounds up but, if the two are allowed to come into contact, they will fuse into a single nucleus again. The same result obtains in the sand-dollar and sea-urchin eggs. If, however, the nuclear membrane be torn, a disintegration of the cytoplasm results analogous to that produced on rupturing the germinal vesicle. The extent of disintegration is much more limited, owing doubtless to the much smaller amount of nuclear material present. Similar results were obtained on tearing the nucleus of the *Arbacia* egg.

It was found possible to destroy the cytoplasm of one egg by injecting into it nuclear material obtained from another egg. This experiment has to be performed very rapidly, for if the nuclear material be allowed to remain longer than ten seconds within the pipette it has no effect whatever when injected into the cytoplasm of an egg. If it be injected within that time the destructive effect is very pronounced.

If an egg be allowed to undergo normal maturation, the germinal vesicle disappears except for a small remnant which becomes the definite egg nucleus. This egg nucleus moves to the surface of the egg, where it gives off the two polar bodies. It then constitutes the female pronucleus, which remains quiescent until fertilization occurs. The disappearance of the germinal vesicle is a well-known phenomenon. In order, however, to locate definite stages selected for my operations I introduce the following sum-

mary. The germinal vesicle with an intact membrane is shown in Fig. 6. Within thirty to forty-five minutes after standing in sea water the nuclear membrane exhibits wrinkles and its outline begins to fade from view. Within a few minutes no membrane is visible and cytoplasmic granules can be seen moving into the region hitherto occupied by the nucleus, while the nuclear sap appears to be diffusing out (Fig. 6-*c*). As the nuclear membrane disappears the nucleolus fades from view. The invasion of the nuclear area by cytoplasmic granules continues until all of the area except a small portion is rendered indistinguishable from the general cytoplasm of the egg. This small portion persists as the egg nucleus (Fig 6*e* and *f*). In Fig. 6-*g* two consecutive positions of the nucleus are shown. At 1 : 13 P.M. it lay deep in the substance of the egg. In twenty minutes it had moved to the periphery of the egg preparatory to the formation of the polar bodies.

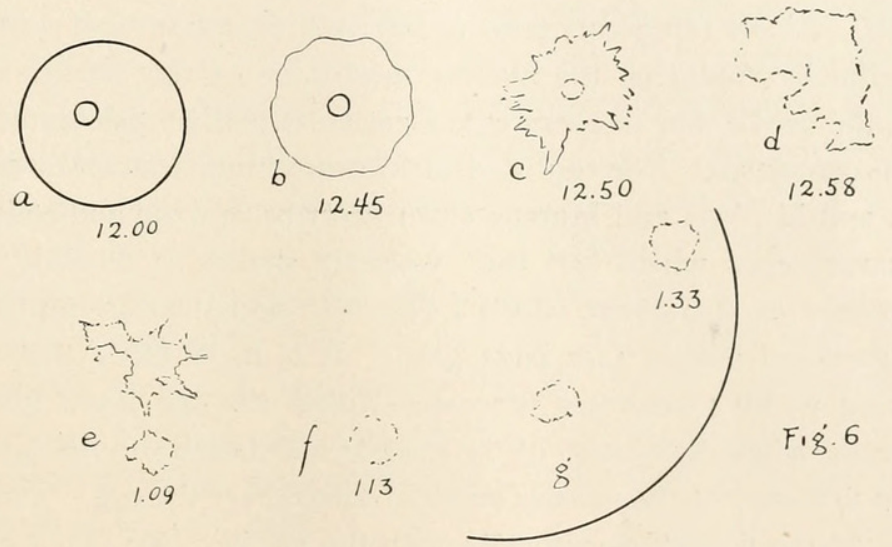

Fig. 6

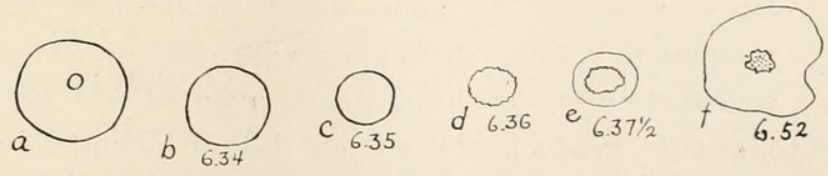

Fig. 7

Fig. 6. Camera lucida drawings of the successive steps in the normal dissolution of the germinal vesicle in the maturing starfish egg. The process was somewhat slowed down owing possibly to the compressed condition of the egg necessary for detailed observation.

Fig. 7. *a*, intact germinal vesicle within the egg. *b*, nucleus after having been torn out of the egg and brought into sea water. *c*, *d*, *e* and *f*, successive changes undergone by the nucleus lying in sea water.

By means of the microdissection needle it is possible to show, at the stage shown in Fig. 6-*d*, that the membrane of the germinal vesicle no longer exists. By careful manipulation it was possible to push the cytoplasmic granules into the nuclear area. A slight rapid movement of the needle, however, was sufficient to give rise to disintegrative processes similar to those on tearing an intact germinal vesicle. In the normal maturation process the mingling of the nuclear sap with the cytoplasm is very gradual, being completed in the case recorded not under ten minutes. It is this gradual mixing which apparently prevents disintegration.

Morgan ('93) and Mathews (Wilson and Mathews, '95) found that maturation was accelerated by shaking starfish eggs shortly after they were placed in sea water. They concluded that the shaking ruptured the membrane of the germinal vesicle and so allowed the nuclear material to mix more quickly with the cytoplasm. I have repeatedly tried to intermix cytoplasm and nuclear material by rupturing the nuclear membrane of the starfish egg with the needle, but in every case I get an explosive disintegration of the cytoplasm. The ruptured nuclear membrane which Mathews (W. and M., '95) and Marcus ('07) describe in fixed and stained immature eggs which had been violently shaken is possibly the membrane of the sphere which I found to persist after injury to the germinal vesicle (see page 321). It is more likely that the shaking which accelerates processes within the egg leads to the normal gradual dissolution of the nuclear membrane and the subsequent diffusion of the nuclear material throughout the egg. I have been able to do this occasionally with the needle. An intact germinal vesicle which to all appearances should take fifteen to twenty minutes to go into dissolution will often immediately exhibit a wrinkled outline on being gently agitated with the needle. Then follows the gradual fading from view of its outline with the subsequent changes as shown in Fig. 6.

The intact germinal vesicle may be brought into the sea water by tearing away the surrounding cytoplasm. During the process the nucleolus fades from view. The slightest tearing of the nuclear surface then causes the entire liquid vesicle to disappear in the water. If, however, the nucleus be left alone, it shrinks for a

time and then swells. The changes appreciable to the eye are shown in Fig. 7. During the swelling of the nucleus a substance apparently separates out which collects into a small mass and persists as a gelatinous body. It is possible that this abnormal separating out is analogous to the formation of the definitive egg nucleus in the normal process of maturation. This separating out of a gelatinous material from a liquid nucleus upon injury may be similar to the method of precociously inducing chromosomes in spermatocytes of the grasshopper (Chambers, '14).

2. The Existence of an Extraneous Membrane About the Unfertilized Egg.

The existence of a membrane about the unfertilized egg rising off as the fertilization membrane upon insemination was first suggested by the earlier investigators (*e.g.*, Hertwig, '76; Herbst, '93). Kite ('12) and Glaser ('13) agreed with them whereas McClendon ('14), Harvey ('14) and Elder ('13) claimed that the fertilization membrane is a new formation consequent to fertilization. Heilbrunn ('13) also identifies it with the actual protoplasmic surface of the egg, which he considers to be in a state of a gel and which lifts off as the fertilization membrane, a new surface film forming over the egg underneath it.

My experiments indicate that the unfertilized eggs of the starfish, sea-urchin and sand-dollar all possess a membrane extraneous to their true protoplasmic surface, and that it is this membrane which, upon insemination, is lifted off as the well-known fertilization membrane.

In the unfertilized egg the membrane is more or less tightly glued to the surface of the egg just as Kite ('12) described it. In the sea-urchin egg it is extremely delicate and can be demonstrated only as follows (Fig. 8) : The needle is inserted as nearly as possible through the periphery of the egg and left there. Within a few seconds the protoplasm, lying immediately under the egg membrane and distal to the needle, flow away from the needle until the needle lies in a small protuberance which is formed by a very slightly lifted portion of the egg membrane.

The existence of the egg membrane is easily demonstrated in the

starfish egg. In Fig. 9 the disintegration of the cytoplasm following injury to the germinal vesicle has reached the surface of the egg. The disintegrated area is quickly localized by a surface film bounding a cup-shaped depression on the surface of the egg. Roofing over the depression is the egg membrane. The egg membrane can also be shown by cutting an egg in two by pressing the egg against the coverslip with the side of a needle. The pressure of the needle cuts the egg in two without rupturing the membrane, which, on releasing the egg, bridges the gap between the pieces and holds them together (cf. Figs. 11 and 12, page 329).

The difference between the consistency of the egg membrane in the starfish and the sea-urchin egg is strikingly shown in the fol-

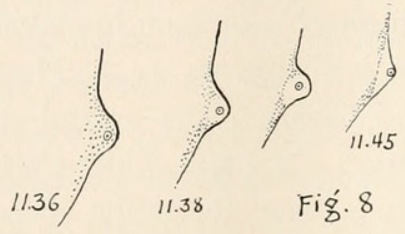

Fig. 8

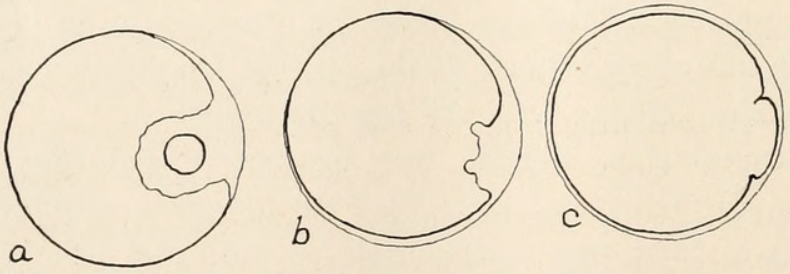

Fig. 9

FIG. 8. Needle inserted at 11:36 A.M. through periphery of a sea-urchin egg and left there. At 11:38 the cytoplasmic granules have been flowing away from the needle. A new surface film begins to appear with the needle left outside. At 11:45 the original egg membrane appears as a delicate membrane partially lifted off the surface of the egg by the needle.

FIG. 9. Lifting of a membrane from the surface of an immature starfish egg following injury to the egg. *a*, local disintegration of cytoplasm following destruction of the germinal vesicle (cf. Fig. 4). An egg membrane becomes apparent as the cytoplasm retreats from it. *b* and *c*, gradual separation of the membrane all over the surface of the egg.

lowing experiments. With the eggs in a hanging drop the egg is pressed against the coverslip with the side of a glass needle until

the pressure divides the egg into two pieces. In the sea-urchin egg the two pieces immediately round up and roll away from one another. In the starfish egg the tougher membrane is not ruptured, but holds the two pieces together.

The membrane of the sea-urchin egg is so delicate that it is also possible to cut the egg in two in the following manner: In a hanging drop the horizontal end of the needle is brought *over* the egg (Fig. 10). The needle is now lowered. This brings the needle

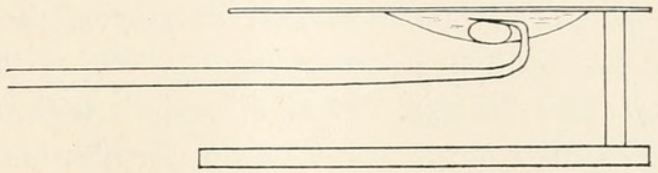

Fig. 10

FIG. 10. Side view of moist chamber to show one method of cutting an egg in two with the microdissection needle.

against the upper surface of the egg and presses the egg down against the surface film of the hanging drop. On lowering the needle still further it passes through the egg and out of the drop, cutting the egg cleanly in two. In the case of the starfish egg this procedure would drag the egg out of the drop along with the needle. The membrane of the sand-dollar egg is weaker than that of the starfish and stronger than that of the sea-urchin egg.

The consistency of the membrane varies with the age of the egg. The full-grown immature egg of the starfish has a relatively tough membrane. On the other hand, young ovarian eggs possess very delicate membranes and they can be cut in two with the same ease as mature sea-urchin eggs.

The strongest argument regarding the existence of a membrane about the unfertilized egg is that a membrane may be stripped off the egg whereupon the egg, which was previously non-adherent, now sticks to everything it touches. The fertilizability of such naked eggs is discussed under the next heading.

The existence of egg membranes is a fairly universal feature and it is, therefore, not surprising that we should find them in the

echinoderm eggs which have generally been considered as naked. The unfertilized *Cumingia* egg has an extremely tough membrane, so tough that it is difficult to rupture it without completely destroying the egg contents. The vitelline membranes in the frog and in the chick are undoubtedly analogous structures.

3. The Egg Membrane and the Fertilization Membrane are Identical.

Prior to fertilization no membrane enveloping the egg is visible. Upon fertilization a membrane lifts off which can easily be cut away from the egg. Figs. 11 and 12 indicate the identity of a preexisting membrane with the fertilization membrane. Fig. 11-*a* shows an egg cut in two with an investing membrane holding the pieces together. Upon fertilization the membrane lifts off, enclosing the two pieces in a single cavity (Fig. 11-*b*). One only of the pieces happened to segment, and the fact that the two pieces lie in one cavity is shown in Fig. 11-*c*, where the blastomeres of the segmented portion have encroached on the area around the nonsegmented piece. In Fig. 12 an egg was cut into three pieces, the egg nucleus lying in one of the pieces. Upon fertilization the membrane lifted off the pieces, each of which received sperm and developed into swimming larvæ. Fig. 12-*c* shows the empty fertilization membrane after the three larvæ had escaped. In Fig. 13 is shown an egg which, on being cut in two, was rolled about in an attempt to separate the pieces. The egg membrane between the two pieces was twisted into a thread joining the two. Upon fertilization each piece exhibited a complete fertilization membrane, but the fact that the two investing membranes are portions of one common membrane is shown by the connecting thread.

A conclusive test for the starfish and sand-dollar egg is the removal of the egg membrane prior to insemination. Occasionally, pricking the egg is sufficient to elevate the membrane. No subsequent development takes place. It is possible, however, to remove this membrane by tearing it and the egg then be made to slip out. This is more easily done on eggs which have been standing for some time in seawater. On catching at the sur-

face of such eggs with the needle, the membrane is often torn in such way that the egg slips out leaving the membrane stuck to the needle. Such an egg, when inseminated, is fertilized and subsequently segments with no investing membrane whatever.

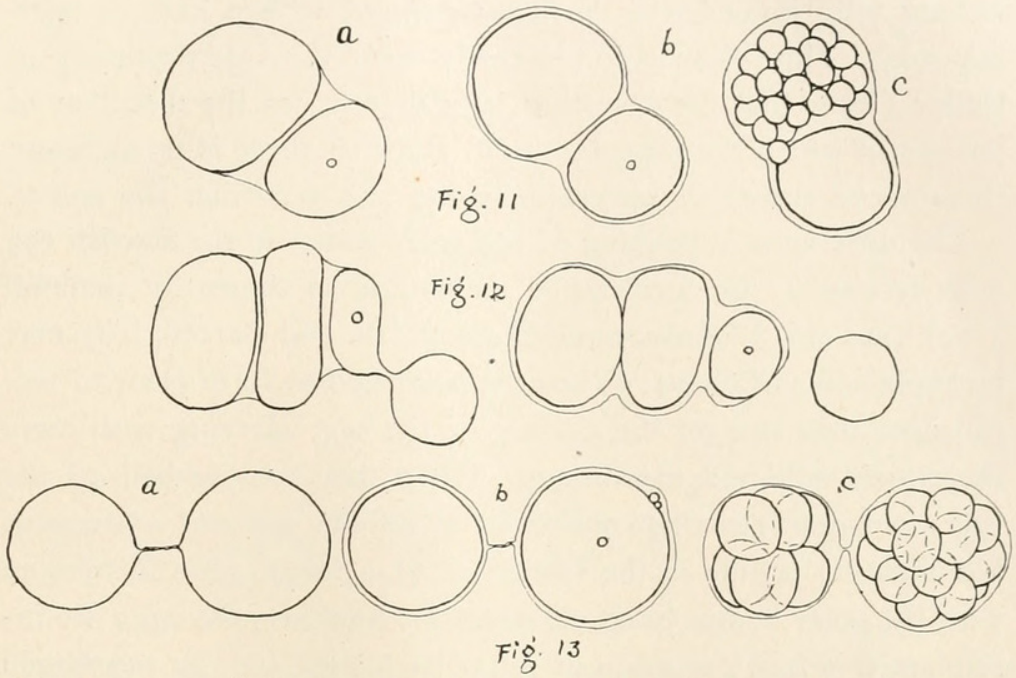

FIG. 11. *a*, starfish egg cut in two without destroying the investing membrane. *b*, after insemination the investing membrane lifts off both fragments as the fertilization membrane. *c*, one of the fragments segmented, the other did not. That both fragments lie in a common cavity is shown by the encroaching of blastomeres of one fragment into the region of the unsegmented fragment.

FIG. 12. *a*, starfish egg cut into three pieces. One piece was squashed and produced an exovate. *b*, on being fertilized the exovate was pinched off as an endoplasmic sphere (cf. Fig. 25). The rest of the fragments produced a common fertilization membrane. Each of the three enclosed fragments developed into a swimming larva.

FIG. 13. *a*, sand-dollar egg rolled as it was cut in two. The egg membrane between the two pieces was twisted into a thread joining the two. *b*, egg shortly after fertilization showing fertilization membrane about each connected by a filament. *c*, the two pieces in an early segmentation stage.

The difference in reaction of sperm to an egg which has been denuded of its membrane as well as of its jelly, and to one which has not is very striking. An egg within its membrane is quickly surrounded by spermatozoa as they are trapped in the jelly surrounding the membrane. In a membraneless egg no crowding of spermatozoa is noticeable and heavy insemination is necessary

to bring about fertilization. When a cloud of sperm has been blown upon a naked egg, one may frequently observe a spermatozoön swim toward it, wander over its surface, and then swim away. On the other hand, the empty membrane with its investing jelly immediately becomes covered with a halo of spermatozoa. This observation accords with the interpretation of Buller ('02), that the investing jelly determines the direction of the sperm which are captured by it, and that there is no apparent chemotactic substance excreted by the egg to attract the sperm.

The difference in position of the polar bodies in the starfish egg with respect to the fertilization membrane as shown by Gemmill ('12) (see also Chambers and Mossop, '18, and Garrey, '19) may be explained as follows: When the polar bodies form prior to fertilization they rise off the surface of the egg, carrying with them the closely adherent membrane. When they are pinched off the egg membrane remains continuous about the egg and subsequent insemination results in the formation of a fertilization membrane with the polar bodies lying outside. If, however, the eggs are inseminated before extrusion of the polar bodies, the egg membrane lifts off as the fertilization membrane and, when the polar bodies are formed, they lie within the membrane.

In the sea-urchin egg the identity of the egg membrane with the fertilization membrane is more difficult to demonstrate. In Fig. 14 is shown the effect of locally injuring the surface of the sea-urchin egg. In *a* is a disintegrated mass produced by tearing a spot on the surface with a needle. In *b* this area is shown as a bulge which may be explained as being produced by the interior pressure of the egg on a surface weakened by the loss of an investing membrane. In *c* the egg has been fertilized. The fertilization membrane is formed over all the surface except at the injured place. In *d* segmentation has occurred and a blastomere protrudes through the gap in the fertilization membrane.

A better demonstration is the case shown in Fig. 15. At 4:26 the tip of a needle was punched through the cortex. Within a few seconds the cytoplasm distal to the needle flowed away, leaving the needle lying under a delicate membrane (Fig. 15-*a*). At 4:27 the egg was inseminated with the needle still in place. At 4:29

the fertilization membrane was formed, showing its continuity with the delicate membrane previously noticeable (Fig. 15-*b*).

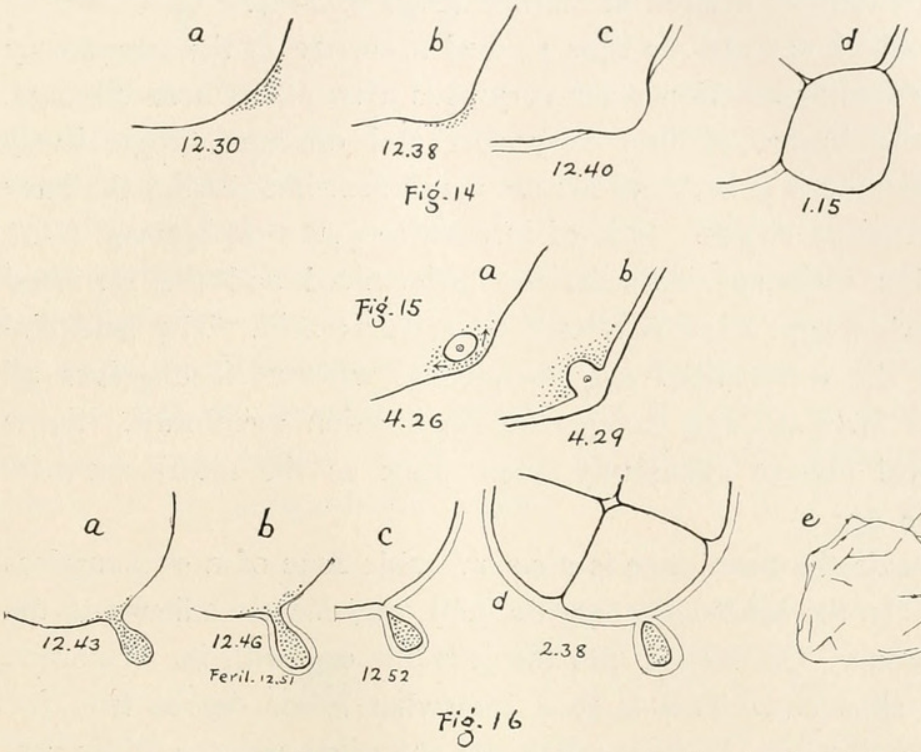

FIG. 14. Sea-urchin egg with surface torn producing local cytolysis. *a*, a new surface film has formed under the cytolyzed area which is being extruded. *b*, a bulge appears in the region of the new surface showing this region to be weaker than elsewhere on the egg surface. *c*, egg after fertilization exhibiting a fertilization membrane over the egg except at the place previously torn. *d*, the same egg 35 minutes later with a blastomere protruding through the tear.

FIG. 15. *a*, needle piercing sea-urchin egg near its periphery. The cytoplasmic granules are flowing in the direction of the arrows. One minute later the egg was inseminated. *c*, an intact fertilization membrane forms, inclosing both egg and needle tip.

FIG. 16. *a*, protrusion on surface of egg produced by pulling at cortex with needle. *b*, three minutes later the investing membrane lifted off surface of protrusion. *c*, one minute after fertilization. The protrusion has been pinched off from the egg and its investing membrane can be seen to be continuous with the fertilization membrane. *d*, empty and collapsed fertilization membrane.

In the sea-urchin egg the membrane often rises off a protrusion caused by pulling at the cortex with the needle. Such a case is shown in Fig. 16. The protrusion was formed at 12:43. At 12:46 a membrane had lifted off the protrusion. At 12:51 the egg was inseminated, and one minute later the membrane was

found continuous with the fertilization membrane. The protrusion subsequently pinched itself off and persisted in a sac-like protuberance of the fertilization membrane (Fig. 16-*d*–*e*).

In all of the various eggs studied a change in the consistency of the membrane takes place very soon after it has been elevated. The membrane, at first very soft and delicate, progressively toughens until it becomes almost parchment-like during the later segmentation stages. It is of interest to note that Harvey ('10) found a difference between the unfertilized and the fertilized sea-urchin egg when subjected to sulfuric acid. The acid dissolves the unfertilized egg completely, whereas it dissolves all of the fertilized egg except the fertilization membrane. Some chemical change apparently takes place as the membrane lifts off the egg.

Outside the membrane is a considerable zone of a structureless jelly. In the sand-dollar egg the jelly very loosely adheres to the membrane. On cutting into the jelly the egg with its membrane easily slips out. This is to a somewhat lesser degree true for the starfish egg. In the starfish egg one often sees the under surface of the jelly pushed away from the surface of the unfertilized egg by the protruding polar body.

The question as to whether the membrane lifts off the surface of the egg or whether the egg shrinks leaving the membrane behind has been raised by Glaser ('14) in spite of McClendon's ('10) statement to the contrary. Glaser, by making a large series of measurements, claims that the egg shrinks upon fertilization, and that the initial diameter of the completed fertilization membrane is equal to that of the unfertilized egg. Glaser's measurements were made on the assumption that the eggs always maintain a spherical shape. This is not true. The mature unfertilized egg is very soft and if allowed to lie on the bottom of a glass dish tends to flatten into the shape of a disc. Upon fertilization the egg rounds up as the fertilization membrane leaves its surface. One can readily see if the observations are taken of eggs in one plane only that erroneous conclusions may be arrived at.

I used two methods to ascertain the diameter of starfish eggs before and after fertilization. One method was to place a drop

containing a few eggs on a gelatin-coated slide. The eggs were rolled over by means of a micro-needle and only those which maintained their spherical shape were measured. With a micro-pipette sperm were introduced into the drop without disturbing the relative positions of the eggs. A second method was to place several eggs in a hanging drop in a Barber moist chamber. By piercing the surrounding jelly with a needle the egg to be measured could be held suspended in the middle of the drop. Numerous measurements of the starfish egg were made at different times through several summers and in every case the egg maintained its original size as the fertilization membrane rose off its surface. Not only does the egg not decrease in volume, but it slightly *increases* in size until segmentation occurs. The accompanying table is one sample of the measurements made:

	Un-fertil.	Minutes after Fertilization.					
		1″	2″	7″	10″	20″	70″
Egg diameter	3.4	3.4	3.4	3.4	3.5 x 3.55	3.5 x 3.6	3.5 x 3.6
Fertilization membrane diameter		3.5	3.6	3.65 x 3.7	3.65 x 3.7	3.75 x 3.75	3.9 x 3.9

The conclusions from this table apply both to starfish and sea-urchin eggs. They may not necessarily be true for other species.

Fig. 17 shows successive steps in pulling a starfish egg out of its fertilization membrane. No second membrane is ever formed even with superimposed insemination. Occasionally the hyaline plasma layer in such an extruded egg swells up and simulates a second membrane, and it is probably this that has been described by certain investigators as a second fertilization membrane. The hyaline plasma layer will be discussed under heading 5.

An unfertilized mature sea-urchin egg may be rolled about and its contents churned to the extent of producing "fountain currents" within the egg (Chambers, '17-*b*). This is done by pushing an egg in a drop shallow enough to compress the egg. Currents are produced which flow backward immediately under the surface of the egg and forward along its central axis (Fig. 18). By careful manipulation it is possible to do this without rupturing

the investing membrane. Such an egg is capable of forming a normal fertilization membrane when inseminated. If the pushing process be carried too far, a distinctive quiver can be recognized, as of something giving way. On subsequent insemination such

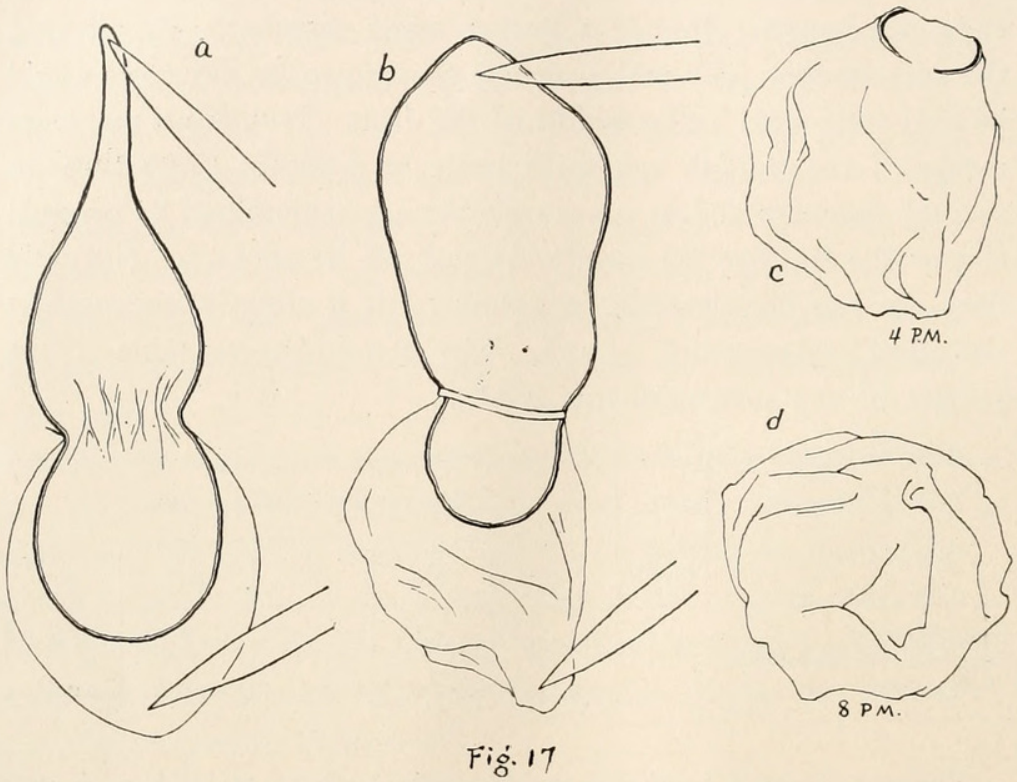

Fig. 17

Fig. 17. *a* and *b*, successive steps in pulling a starfish egg out of its fertilization membrane. *c*, empty membrane at 4:00 P.M. *d*, ditto four hours later at 8:00 P.M. The membrane persists as a collapsed remnant for a long time.

eggs produce a collapsed fertilization membrane. The quiver undoubtedly was due to a rupture of the egg membrane. On account of this rupture the fluid, which presumably collects under the membrane, leaks out and the membrane is not lifted uniformly.

4. The Cortex and Interior of the Unfertilized Egg.

The cytoplasm of the immature starfish egg is uniformly semi-solid. A gash made in it with a needle is maintained for some minutes before closing up. When the germinal vesicle breaks down naturally, the egg protoplasm becomes more fluid so that a gash

through such an egg quickly closes up. The cortex—*i.e.*, the surface of the egg immediately beneath the egg membrane—tends always to remain more solid (Chambers, '17-*a*). Because of this difference in consistency the cortex and medulla of the egg can be separated from one another as follows ('21[a]): If the surface of the mature starfish egg be torn with a needle and the egg then be caught at the opposite side and pulled to the edge of the

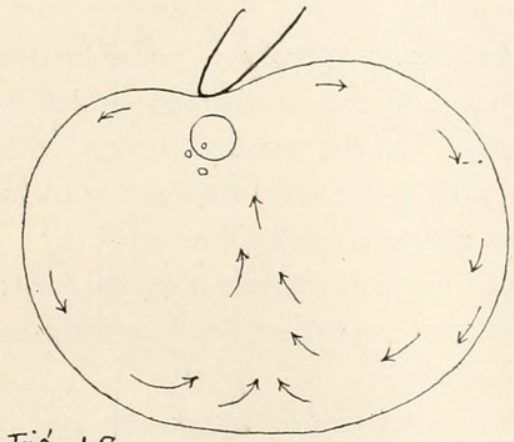

Fig. 18

Fig. 19

FIG. 18. Currents produced within a sea-urchin egg by pushing a sea-urchin egg held against a coverslip by a shallow film of water. The direction of the currents is shown by the arrows. The nucleus, after being carried about with the current, tends to come to rest in the location shown in the figure.

FIG. 19. Part of the cortex of a fertilized egg after the appearance of the hyaline plasma layer. The cortex was ruptured in one place and cytoplasmic granules can be seen issuing through the rupture in the hyaline plasma layer and the investing fertilization membrane.

hanging drop, the compression on the egg produced by the shallow water at the edge of the drop will cause the fluid interior to ooze out through the tear to form a spherical exovate (see Fig. 25, page 344). One may so manipulate the process as to cause the egg nucleus either to remain behind in the cortex (the cortical remnant) or to pass into the extruded sphere of endoplasmic material.

The cortical remnant is relatively solid and remains more or less inclosed within the egg membrane and its jelly. If left long enough it will eventually round up so as to present the appearance of a diminutive egg surrounded by a collapsed and wrinkled egg membrane.

The endoplasmic material which has escaped from the egg into the sea water is fluid and tends immediately to round up. On tearing with a needle its surface behaves like that of a highly viscous oil drop, adheres tenaciously to glass. As long as it possesses an intact surface it looks exactly like an egg fragment and will undergo disintegrative changes similar to those of entire eggs on being torn with the needle (cf. Chambers, '17-*a*).

The ability to produce endoplasmic spheres is possibly due to the relatively tough egg membrane in the starfish egg which helps to keep back the adherent cortex. In the sea-urchin egg, with an extremely delicate egg membrane, it has been impossible to cause the interior to flow out, as the cortex tends to flow with it.

The sand-dollar egg behaves very much like the starfish egg. The egg membrane is appreciable in the unfertilized egg and endoplasmic spheres are readily produced.

A difference in the functional activities of the cortex and interior of the starfish egg is discussed under the headings 6 and 7.

5. The Hyaline Plasma Layer.

Prior to fertilization the cytoplasmic granules in the sea-urchin and sand-dollar egg lie close to the surface. Within ten minutes after fertilization the granules have undergone a centripetal migration, leaving an appreciable peripheral zone of a hyaline appearance which has been called the hyaline plasma layer (Loeb's gelatinous film, '13, p. 19).

The microdissection needle indicates that this layer is relatively firm and gelatinous. The very fluid internal cytoplasm may be made to flow out through a rupture in this layer if the egg be torn. This is shown in Fig. 19. The cytoplasmic granules lie against the inner boundary of this layer and may be seen oozing out through the small tear in this layer and through a tear in the fertilization membrane to the exterior.

The hyaline plasma layer adheres very tenaciously to the needle and when an egg has been deprived of its fertilization membrane the egg sticks to everything it touches.

Loeb has called attention to the fact that the hyaline plasma

layer in a segmented egg bridges the segmentation furrow. When the furrow is first formed, however, the hyaline plasma layer does not bridge the furrow, but is carried in on the walls of the cleavage furrow (Fig. 20-*a*, *b*, *c*). The layer is thicker in the floor of the

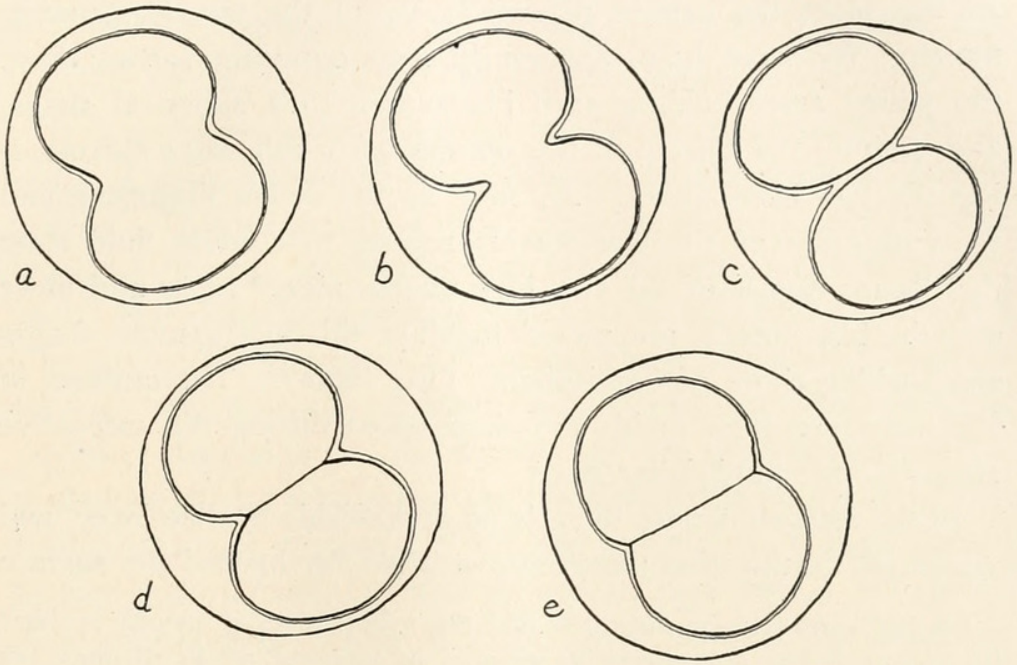

Fig. 20

FIG. 20. Contour of a sand-dollar egg at various stages of its cleavage into two blastomeres. In *a* and *b* the hyaline plasma layer is seen carried in on the walls of the deepening furrow. In *c* the egg has segmented in two with the hyaline plasma layer on opposite sides of the furrow tending to merge into each other. In *d* this process is carried further. In *e* the two blastomeres are tending to assume the shape of hemispheres with the hyaline plasma layer bridging the furrow.

furrow, but it is only later when the furrow has cut through the egg that the hyaline plasma layers on the opposite surfaces of the furrow run together. Each half of the segmenting egg tends to assume the shape of a sphere owing to the separation of the two asters of the amphiaster (Chambers, '17-*b*, '19). If there were no other forces at play, the two blastomeres, when formed, should be spheres. In the sea-urchin egg the adhesiveness of the hyaline plasma layer tends to draw the two blastomeres together; also the fertilization membrane, not rising to any great extent off the surface of the egg, must exert some pressure on the two blastomeres. In the sand-dollar the fertilization membrane is well

lifted, so that there is plenty of room within the membrane, permitting the two blastomeres to assume almost spherical shapes (Fig. 20-*c*). When the cleavage furrow is completed the two blastomeres are contiguous only where the two spheres touch. At this place the hyaline plasma layers of the two blastomeres merge. We have here, apparently, two opposing forces; first, the jellied aster holding each blastomere to a spherical shape, and, second, the affinity of the plasma layer substance surrounding the two blastomeres. As soon as the asters disappear and the cytoplasm of the blastomeres reverts to a more fluid state the plasma layers of the two blastomeres merge more and more and the blastomeres are pulled together till they assume shapes approaching those of hemispheres (Fig. 20-*e*). The outlines in Fig. 20 are camera lucida drawings taken during the successive stages of one sand-dollar egg.[1]

In the starfish, where there is no appreciable hyaline layer, and where the fertilization membrane is lifted far beyond the surface

[1] It has recently been intimated that the microdissection method is unreliable as a means of ascertaining changes in viscosity in the dividing egg because of supposed discrepancies in the results obtained by Seifriz ('20) and myself ('17[b] and '19). As a matter of fact the results of Seifriz harmonize perfectly with mine. Seifriz states "there is a pronounced decrease in viscosity of the central region of the cell with the first appearance of the amphiasters." This statement has been interpreted as running counter to mine. This is not true for although my results indicate that the astral portion of the amphiaster is jellied, I definitely state (p. 494, '17) that the central region and the zone between the two halves of the egg are fluid where "a distinct flow of granules medianward can be observed."

Again, on completion of cleavage Seifriz notes that the two blastomeres become liquid. This statement also fits in with my results. I state (p. 51, '19) that, immediately after cleavage and while the two blastomeres *are still spherical,* the firmness of the cytoplasm persists. Later, when the asters disappear the cytoplasm liquefies and the two blastomeres crowd up against one another. Seifriz noted this last liquid state of the two blastomeres without considering the state prior to it.

I may mention here a possible criticism of the centrifuge method in ascertaining viscosity variations. There are critical stages in the developing asters during which agitation causes their disappearance. This was noted long ago by Wilson. On bringing the eggs to rest the asters reappear and development proceeds normally. I have already discussed this matter fully ('19). The centrifuge and miscrodissection methods of studying the physical state of protoplasm should serve as valuable checks on one another, if only the investigators in these fields would agree on cooperation.

of the egg, the blastomeres are practically non-adhesive, and they maintain more or less spherical shapes till well on into the later segmentation stages.

6. The Localization of a Material Which Affects the Life of the Unfertilized Starfish Egg.

It is well known that immature starfish eggs can be kept in sea water at room temperature for 36 hours or more without disintegrating. That the germinal vesicle or nucleus is responsible for this length of life can be demonstrated by cutting an immature egg in two. The nucleated fragment lasts fully as long as the entire egg. The non-nucleated portion, on the other hand, disintegrates within three to four hours. In mature unfertilized eggs the conditions are quite different. In the mature egg the germinal vesicle has broken down and the nuclear sap has diffused throughout the egg. Loeb ('02) and Mathews ('07) showed that such eggs have a higher rate of oxidation than immature eggs and if left unfertilized disintegrate within 8 to 10 hours whereas the immature eggs last for days.

The non-nucleated fragment of the mature egg lasts as long as the whole egg, evidently owing to the dispersed nuclear sap of the dissolved germinal vesicle. What is significant is that the nucleated fragment lives no longer than the non-nucleated fragment. Both contain the dispersed nuclear sap, while the nucleated fragment possesses also the definitive mature egg nucleus which is ultimately to become the female pronucleus. Apparently it is the dispersed nuclear sap and not the definitive mature egg nucleus which is chiefly concerned. In the formation of the nucleus of the mature egg we have possibly something analogous to the state of affairs in many Protozoa where the nuclear apparatus consists of a tropho- or macro-nucleus concerned chiefly in the metabolic activities of the cell, and the kineto- or micro-nucleus which has only to do with the reproductive activities. In the starfish egg we may consider the germinal vesicle as a combined tropho- and kineto-nucleus. On the approach of maturation the tropho-nuclear material (nuclear sap) diffuses throughout the egg, leaving behind the kineto-nuclear part, the mature egg nucleus, which gives off the polar bodies to become ultimately the female pronucleus.

The fluid interior of the mature unfertilized egg, if isolated by being made to escape through a tear or the cortex, withstands disintegration for 24 to 36 hours. The presence of even a small part of the original cortex in organic continuity with it causes it to disintegrate in about the same time as an entire mature egg. This would indicate that the reactions which make for disintegration reside chiefly in the cortex. This, together with the fact that the cortex of the egg is necessary for fertilization, would indicate that the cortex is the seat of the initial activation processes of the egg. The relatively inactive central material of the starfish and sand-dollar egg somewhat resembles that of the *Linerges,* the Scyphomedusan, which Conklin ('08) has described. Conklin speaks of "the large cavity in the line of the first cleavage furrow filled with gelatinous or fluid substance, which forms the ground substance of the central area of the unsegmented egg." He found that most of the ground substance escapes into the cleavage cavity and suggested that it is the fluid yoke which is gradually used up in the nourishment of the embryo. The central substance of the *Linerges* egg is probably not strictly analogous with that of the starfish or sand-dollar egg. In *Linerges* cleavage is of a type peculiar to yolk-laden eggs and the central substance escapes during the first cleavage. On the other hand, in the echinoderm egg the nucleus lies well within the central substance of the egg and, upon fertilization, all of the endoplasm is used up in the formation of the cleavage asters and nothing apparently escapes into the early cleavage cavity. We can not, therefore, conclude that the interior of the Echinoderm egg consists of entirely inert material. It lacks certain essential features, but when coexistent with the cortex it plays a full part in the cleavage of the egg.

7. The Localization of a Substance Which Renders a Starfish Egg Fertilizable.

Wilson ('03[ab]) in *Cerebratulus* and *Renilla* and Yatsu ('04 and '08) in *Cerebratulus* have shown that non-nucleated fragments of the egg are capable of fertilization only after the germinal vesicle has broken down. With more delicate methods

rendered possible by the microdissection instrument it has been possible to work out this problem in detail and to ascertain to some extent the distribution of the material which renders fertilization possible.

A number of fully grown immature starfish eggs were enucleated by carefully dissecting out their germinal vesicles. None became fertilized when inseminated. In another lot of immature eggs the germinal vesicle was torn while in the egg (Fig. 21). Immediate

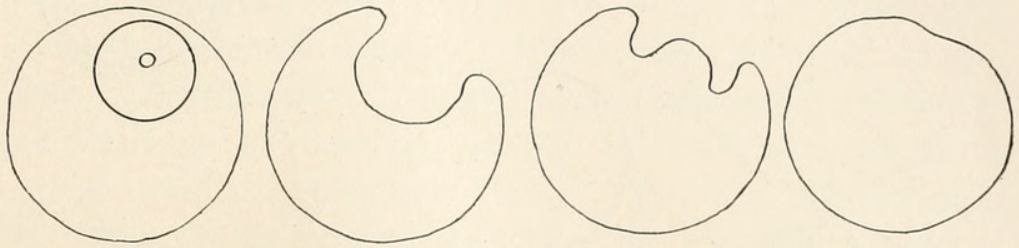

FIG. 21. A starfish egg whose germinal vesicle is eliminated by puncturing it (cf. Fig. 9). The cytoplasm surrounding this nucleus was also destroyed. This enucleated remnant is nonfertilizable.

dissolution of the nuclear membrane took place with a disintegration of the cytoplasm around the nuclear area. Those eggs which succeeded in forming a protective surface film to prevent spread of the disintegration process subsequently rounded up. Upon insemination none of the eggs showed any sign of being fertilized.

Eggs were then taken with the germinal vesicle in various stages of normal dissolution and cut into nucleated and non-nucleated portions. The eggs may be grouped into stages *b*, *c* and *d*, according to the stage of dissolution of their germinal vesicles, as shown in Fig. 6 (page 323). Whenever the cut passed through the nuclear area during the nuclear stages *b*, *c* and *d*, disintegration always took place, involving all of the nucleated portion and a small part of the non-nucleated piece (Fig. 23 *a*, *b* and *c*). When the cut did not pass through the nuclear area all persisting nucleated portions matured normally and upon insemination formed fertilization membranes and segmented. Of the non-nucleated portions those from eggs in stage *b* are non-fertilizable (Fig. 22). Those from eggs in stage *c* form fertilization membranes upon insemination. Nuclear division also takes place, so that the egg

fragment becomes multi-nucleated but remains unsegmented (Fig. 23-*c*). Non-nucleated fragments of eggs in a later stage (stage *d*) proceed somewhat farther (Fig. 24). The multi-nucleated masses arising from them make several periodic attempts at segmentation (Fig. 24-*c*). Small furrows appear over the surface of the egg, cutting in between the peripherally arranged nuclei.

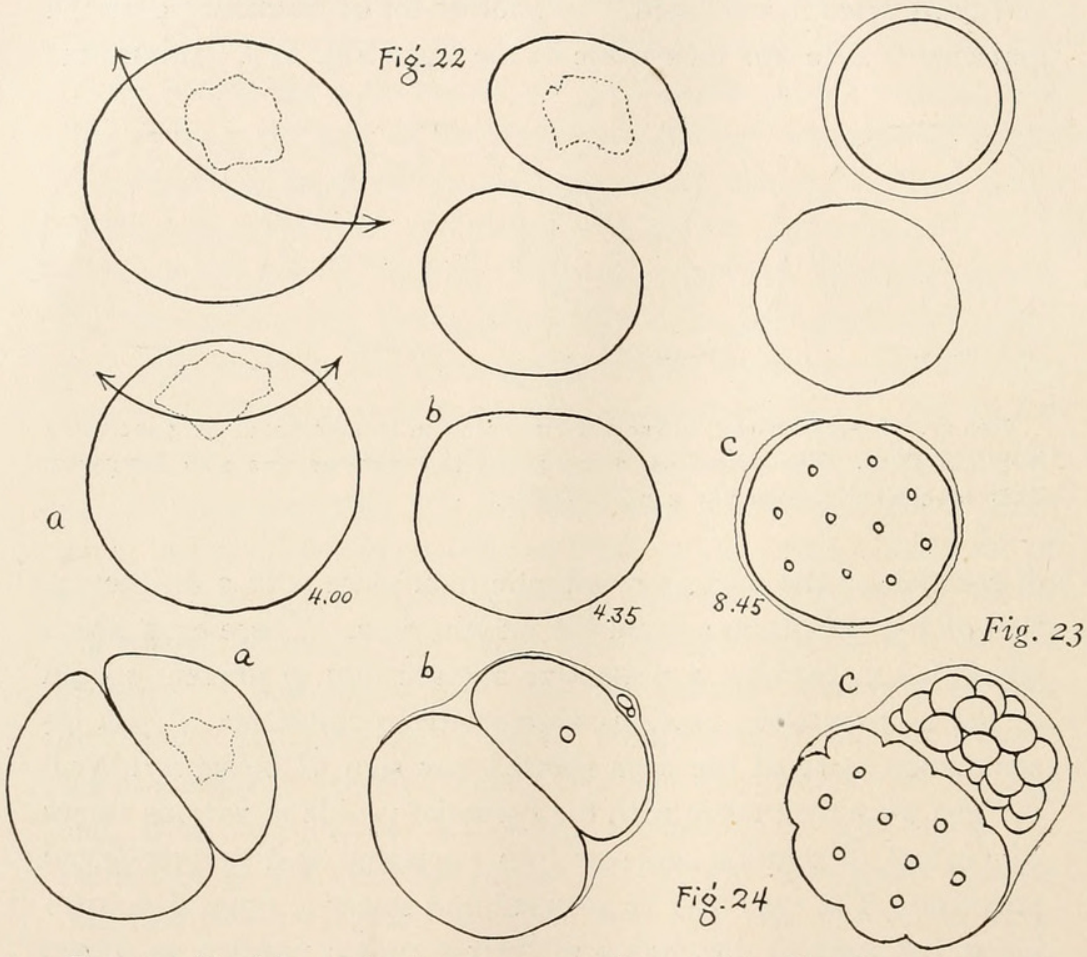

FIG. 22. Starfish egg in stage corresponding to *b* in Fig. 6 cut into two fragments. The non-nucleated fragment contains no material from the germinal vesicle and is nonfertilizable.

FIG. 23. Starfish egg in a later stage corresponding to *c* in Fig. 6 cut through the nuclear area. The cytoplasm in the injured nuclear area disintegrated leaving a non-nucleated fragment, *b*. That the fragment is fertilizable is shown in *c* by the formation of a fertilization membrane and the repeated division of the sperm nucleus. The fragment, however, is unable to segment.

FIG. 24. *a*, starfish egg in stage *d* of Fig. 7 cut into a nucleated and non-nucleated fragment. *b*, both fragments fertilized. The nucleated fragment segmented in the normal way with a number of blastomeres. The non-nucleated fragment became multinucleated and furrows appeared over its surface in an attempt at segmentation.

These furrows then disappear, to reappear again after a short interval. This may occur several times until the egg finally reverts to a spherical shape and remains so. In stage *f* the germinal vesicle has disappeared except for the definitive egg nucleus. Of such eggs any non-nucleated portion down to a certain size is capable of being fertilized and undergoing cleavage.

The above experiments lead one to infer the existence of a substance in the germinal vesicle which, on dissolution of the nuclear membrane, diffuses throughout the cytoplasm. The fertilizability of any egg fragment apparently depends upon the extent of diffusion of this substance. An egg fragment taken when a minimum amount of this substance has diffused into it will allow the sperm nucleus which has entered into it to divide. The presence of a little more of this substance will allow the fragment to undergo abortive segmentation. It is not until a sufficient amount is distributed throughout the egg that any fragment can develop properly.

Mature eggs were now studied, and it was found that any egg fragment in order to be capable of fertilization must contain a portion of the original cortex. The cortex and interior of mature unfertilized eggs were separated according to the method described under heading 4 (Fig. 25 *a* and *b*). The endoplasmic sphere and the cortical remnant were then inseminated. The fragment consisting of the cortical remnant is readily fertilizable and undergoes segmentation (Fig. 25 *b* and *c*). The endoplasmic sphere is non-fertilizable, no matter whether it contains the egg nucleus or not.

That the protoplasm of the endoplasmic spheres has not been irreparably injured in the process of flowing through a small tear in the cortex is shown in the following experiment. Eggs were squashed until the endoplasm protruded as lobate processes, whereupon the pressure on the eggs was lifted and the extrusion allowed to flow back into the egg. Such eggs are fertilizable and are capable of undergoing cleavage. One such case is illustrated in Fig. 26 where the cortex was torn in two places on squashing the egg and two exovates were formed. The nucleated exovate was allowed to pinch itself off. The other exovate flowed back into the remainder of the egg upon insemination (Fig. 26 *b* and *c*). A fairly com-

plete fertilization membrane formed around the egg except at the two torn spots and cleavage followed.

Endoplasmic exovates were also produced which remain connected by a bridge of protoplasm to the collapsed cortical portion

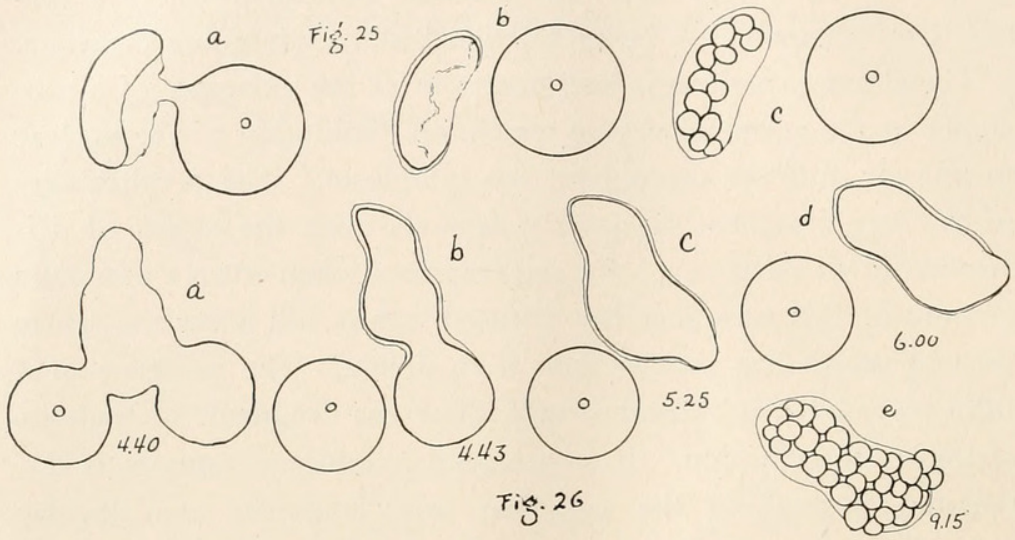

FIG. 25. *a*, nucleated exovate of internal cytoplasm produced by squashing a starfish egg. *b*, fragments inseminated after the endoplasmic sphere was pinched off. Only the ectoplasmic remnant forms a fertilization membrane. *c*, the endoplasmic sphere remains inert and nonfertilizable (cf. Fig. 12).

FIG. 26. *a*, starfish egg squashed producing two endoplasmic exovates. *b*, the nucleated exovate was pinched off. Upon insemination the other exovate drew back into the ectoplasmic remnant which formed a fertilization membrane. *c*, *d* and *e*, the ectoplasmic remnant underwent segmentation showing that the disturbance due to the squashing does not prevent segmentation. The endoplasmic sphere remains inert (*d*).

of the egg. On being inseminated the exovate either is drawn back into the cortical portion as the latter rounds up with the formation of a fertilization membrane or is pinched off, after which it remains as an inert body.

The possibility suggested itself that the substance which renders an egg fertilizable has a tendency to collect in the surface film of an egg and that, if an exovate remained in organic continuity with the egg, this substance might spread to the surface film of the exovate, thus rendering it fertilizable. Endoplasmic exovates were, therefore, produced which remained connected for varying lengths of time with the cortical portion of the egg. Some of the exovates remained connected for as long as fifteen minutes. Before insemi-

nation they were pinched off from the cortical portion of the eggs. None developed of those which were separated in such a way that there was no question as to their lacking any of the original cortex of the egg.

An endoplasmic sphere, in order to develop at all, apparently must incorporate in its substance at least a part of the original cortex of the egg. This is shown in Fig. 27. An exovate was

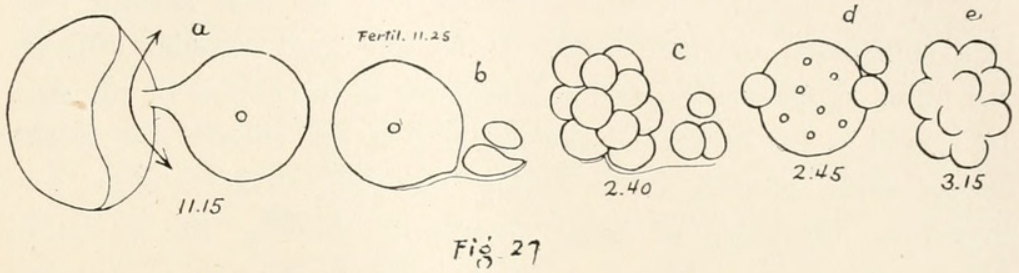

Fig 27

FIG. 27. *a*, an exovate is produced by squashing and most of the ectoplasmic part is cut away along line of arrow. *b*, the endoplasmic sphere formed itself incorporating a small part of the cortex. Upon fertilization the small cortical region formed a partial fertilization membrane. *c*, many furrows form simultaneously over the surface of the egg showing that it has been fertilized. (Note that the small cortical piece to one side of the egg has segmented in two.) *d*, the egg has reverted into a multinucleated nonsegmented mass except for three blastomere-like bodies which were pinched off. *e*, the fragment is again attempting to segment.

produced by crushing an egg (Fig. 27-*a*). However, before the exovate was set free most of the cortical remnant was cut away, leaving a very small piece which was drawn into the circumference of the endoplasmic sphere. On being inseminated a small shred of the egg membrane lifted off from this remnant, and this was all that constituted the fertilization membrane (Fig. 27-*b*). A sperm on entering this sphere underwent nuclear division several times. This was followed by cleavage furrows which formed on the surface of the egg between the peripheral nuclei and gave to the egg the appearance of a mulberry (Fig. 27-*c*). Some of the furrows deepened sufficiently to pinch off nucleated bodies. A few minutes later the furrows became obliterated and the main body of the egg appeared again as a non-segmented but multinucleated mass (Fig. 27-*d*). This process may occur several times (Fig. 27-*e*). The ability of an exovate to approximate normal segmentation is a function of the amount of the original egg cortex which it incorporates.

The inability of the endoplasmic sphere to develop is not due to the lack of successful sperm entry. Sections show that the sperm enter with ease but they remain unchanged and no asters form about them. In this regard the sperm react exactly as they do when they have entered immature eggs.

There must be something localized in the cortex which is necessary for successful fertilization and development (cf. Lillie, '14, '18). On the evidence presented here we may assume that this substance, originally within the germinal vesicle, diffuses out upon its dissolution and accumulates in the cortex of the egg. It is held in the cortex of the egg and is not carried out in the endoplasmic spheres on crushing the egg. The spheres are, therefore, incapable of being fertilized. Finally, the variation in the ability to segment among exovates containing varying amounts of cortical material indicates that there must also be a definite minimum amount of this substance present in order that an egg fragment may develop.

Conclusions.

1. The nucleus possesses a morphologically definite membrane.

2. Tearing the nucleus results in an immediate change of the nuclear membrane, followed by a disintegration of the cytoplasm surrounding it. This is most striking in the relatively large nucleus (germinal vesicle) of the starfish egg.

3. Injection of the germinal vesicle sap of one egg into the cytoplasm of another egg starts up disintegration processes in the injected area.

4. The mature egg nucleus can be pinched into two fragments. The fragments behave like fluid droplets and will run together when contiguous. Eggs whose nuclei have been operated upon in this manner are capable of normal segmentation.

5. A membrane can be demonstrated adhering to the surface of the unfertilized starfish, sea-urchin and sand-dollar eggs. This egg membrane is most pronounced in the starfish and least of all in the sea-urchin. In the starfish and sand-dollar the membrane can be stripped off without injuring the egg. In the starfish a very delicate egg membrane can be demonstrated investing half-sized

immature eggs. This membrane becomes more pronounced as the eggs reach their full growth and still more so as the egg matures. In the sea-urchin the immature eggs exhibit no trace of a membrane until the eggs begin maturation. In the mature unfertilized sea-urchin egg the membrane has reached a development comparable to that of the half-grown immature egg of the starfish.

6. The egg membrane rises off the surface of the egg upon fertilization and constitutes the fertilization membrane. No appreciable diminution in volume of the egg occurs during this process.

7. An egg, whose membrane has been removed, is fertilizable and segments without a fertilization membrane.

8. The hyaline plasma layer, which forms on the surface of the sea-urchin and sand-dollar egg within ten minutes after fertilization, binds the blastomeres together. In the starfish egg no such layer is formed, and, if the fertilization membrane be removed, the blastomeres tend to fall apart.

9. The fertilizability and approach to normal development of an egg fragment is directly proportional to the amount of a substance which emanates from the germinal vesicle during maturation.

10. The unfertilized mature egg possesses a more solid cortex of appreciable thickness inclosing a highly fluid interior. The fluid interior of the starfish and sand-dollar eggs can be made to ooze out through a tear in the cortex, whereupon it forms a surface film on coming into contact with sea water. In this way the internal and cortical material of the egg can be isolated from one another. Both round up, the internal material immediately and the cortical after some time.

11. Endoplasmic material, possessing a small part of the original cortex, is fertilizable and the approach to normal development is in direct proportion to the amount of cortical material present. The presence of even a small amount of cortical material causes disintegrative changes to set in at about the same time as in a whole egg.

12. The following table gives, for the various kinds of fragments of immature and mature starfish eggs, the length of time that they withstand disintegration when left standing in seawater and also whether they are or are not capable of being fertilized:

	Immature		Mature			
					Nucl. or Non-nucl.	
	Nucl. fragm. or entire egg	Non-nucl. fragm.	Nucl. fragm. or entire egg	Non-nucl. fragm.	Ectoplasmic remnant	Endoplasmic sphere
Longevity in hours...	24–36	2–3	8–10	8–10	8–10	24–36
Fertilizability...	+ (when mature)	−	+	+	+	−

As regards longevity it will be seen that the immature egg depends upon its nucleus (germinal vesicle) to prevent disintegration, for a fragment lacking the nucleus disintegrates very quickly. On the other hand, the mature egg, which has become permeated with the nuclear sap of the germinal vesicle, behaves quite differently. The non-nucleated fragment of a mature egg lasts longer than that of an immature egg and it is significant that the presence of the nucleus of the mature egg, which consists of not much more than the chromosomal constituents, has no effect in preventing disintegration.

The long period that the endoplasmic sphere withstands disintegration indicates that the factors which make for disintegration reside chiefly in the original cortex of the mature egg.

In regard to fertilizability it is evident that the substance which renders cytoplasm fertilizable emanates from the germinal vesicle and finally becomes localized in the cortex of the mature egg.

We can, therefore, distinguish three factors in the starfish egg; one affecting longevity, the second affecting disintegration and the third affecting fertilizability. The first and third have been traced to the germinal vesicle of the immature egg. The second is a function of the egg cortex.

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NOTE CONCERNING THE ORIGIN OF POLARITY IN THE FROG'S EGG. A CORRECTION.

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In connection with work published in 1919 (Biol. Bull., Vol. 37: 312–361) on the modification and control of development in the frog egg, it seemed desirable to determine if possible the origin of the polarity of the egg. The position taken was that polarity must be either a matter of inheritance or of determination by factors external to the egg. If the former possibility is true the problem is, of course, simply made more remote. The second possibility, since it is known or believed that polarity arises in a number of plant and animal eggs, in response to external factors, seemed the logical one to test, especially since it is the one most readily investigated experimentally. The first question was to determine the relation, if any, between the polarity of the egg and its mode of attachment to the ovarian membrane. Here it was found and it has since been confirmed, that in 75–80 *per cent.* of the cases, the pedicle which attaches the follicle to the ovarian membrane, is located on or within 20° of the equatorial region of the egg. Since a band 40° wide over the equatorial region of a sphere involves only about 34 *per cent.* of the total area it would seem that the pedicle is not located at random over the surface of the egg but with reference to some other factor, or factors.

Since the polar axes of the ovarian eggs bear every relation to gravity this factor is made highly improbable as having any influence on the origin of polarity.

The next question to arise was the relation of the polarity of the egg to its food and oxygen supply—the blood flowing through the follicular vessels. From observations made at that time on both injected and living specimens I believed and stated, p. 321, of the above mentioned paper, that " . . . in every case observed, the greater part of the arterial blood supply was restricted to the pigment hemisphere " and that the blood supply of the unpigmented hemisphere was largely venous. It was further sug-

gested that "the data indicate that polarity in the egg arises . . . in response to external conditions, viz., to the blood supply of the egg: that region of the oögonium chancing to be most richly supplied with arterial blood being destined to become, by virtue of this respiratory and nutritive relation, the animal pole of the egg."

It may be stated here that the problem was by no means considered solved and in 1919 plans for its further and more complete investigation were fairly well worked out. The investigation has continued with numerous interruptions and is still incomplete, but pending its outcome it has seemed desirable to make this statement.

It now appears that the previous observations were not sufficiently extensive to warrant the general statements indicated above. Certainly there is a considerable range of variation from what I thought was the typical situation and illustrated in Fig. 3 of the 1919 paper. And, it may be added, the figure is correct. But, on the other hand, cases have been observed more recently where the vegetative hemisphere was largely supplied by arterial blood, as well as various intermediate conditions. Furthermore one occasionally finds in the vessels that run to the follicle in the *mature* or nearly mature egg, a direct shunt between the small artery and vein. As far as the existence of any definite relation between the polarity of the *mature* or *nearly mature* egg and arterial or venous blood supply is concerned, I am obliged to withdraw the suggestion as it first appeared. It seems evident enough that polarity must be established early in the history of the egg, possibly in relation to the vascular supply. Supposing, as a working hypothesis, that such is the case it is conceivable that the vascularization in the follicle may change considerably especially as the egg approaches maturity—the only stage previously examined. It is along these lines that the investigation is being continued with the hope of throwing further light on the question.