STUDIES OF FERTILIZATION

VI. THE MECHANISM OF FERTILIZATION IN ARBACIA

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ONE FIGURE

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I. INTRODUCTION

The fifth study of this series on "The behavior of the spermatozoa of *Nereis* and *Arbacia* with special reference to egg extractives" forms the prelude to the present study. In that paper I dealt with the phenomena of activation and of aggregation (chemotaxis) of spermatozoa and with their agglutination by egg secretions of the same species. Regarding fertilization as involving chemical interaction of the ovum and the spermatozoon, I concluded from some of the modes of behavior of the spermatozoa, especially the agglutination phenomena, that "these small motile cells may prove better indicators of some of the reactions involved in fertilization than the slowly reacting egg" and that we might "confidently expect that study of the reactions of spermatozoa will break a new path into the field of fertilization."

The results described in the present paper have been obtained largely by the use of sperm-suspensions as indicators, but also by the study of an inhibitor of fertilization contained in the blood of certain individuals of the same species. These methods reveal a striking series of facts which appear to me to justify a somewhat new point of view in regard to some of the problems of fertilization. I have already briefly presented this point of view in a rather special form with an outline of the main facts in a preliminary paper (Lillie '13).

The essential conclusion is that fertilization is a reaction between three bodies of which one is born by the sperm and one by the egg; the third body, which is secreted by the egg, reacts with both the others. The spermatozoon functions essentially as an activator of the third body which I propose to name 'fertilizin';¹ the latter when activated enters into certain reactions in the cortex of the egg which lead to membrane formation. In order to give a concrete working conception I have pictured the fertilizin as possessing two side chains active in fertilization, viz: one

¹ This is the same substance which I called the 'sperm isoagglutinin' in the preceding paper of this series, because it causes agglutination in sperm suspensions of the same species.

reacting with the sperm which I call the 'spermophile side-chain' or group and the other reacting with the egg which I call the 'ovophile side-chain' or group. The chemical group of the sperm which reacts with the fertilizin is named the sperm receptor and that of the egg the egg receptor.

We are thus furnished with a concrete conception which answers very well for purposes of description; it need not however, be taken too literally. The theoretical aspect of the results is discussed in section IV.

The present paper presents in some detail the evidences on which this general conception of fertilization rests; it deals exclusively with *Arbacia*; in a later paper I shall present some confirmatory evidence from the study of *Nereis*. We shall consider first the action of the spermophile side-chain of the fertilizin and the evidence that the latter is a necessary link in the fertilization process. Second, we shall consider the nature of the ovophile side-chain of the fertilizin, the means of inhibiting its action and of neutralizing such inhibition.

The terminology has been largely adopted from immunology, because it seemed best suited to express the facts. If it seems rather bizarre to the zoölogical reader I must ask him not to conceive prejudice for this reason against the facts themselves, which stand on their own feet quite apart from any terminology or theory. The terminology and the theory are significant only to the extent that they give a brief description of the facts, and serve as a working hypothesis.

II. THE SPERMOPHILE SIDE-CHAIN

I. A QUANTITATIVE METHOD OF STUDYING THE PRODUCTION OF FERTILIZIN BY OVA

The fundamental fact which forms our point of departure is that fertilizable eggs of *Arbacia* suspended in sea-water secrete a substance (fertilizin) for which only the spermatozoa of the same species constitute an efficient indicator. Similarly the only cells that produce the substance are the egg-cells of the same species; it is not found in the blood or in extracts of other tissues.

The presence of the substance is shown by the agglutination of sperm-suspensions of the same species. If a few drops of sea-water containing this substance in sufficient concentration be added to a 1 per cent sperm-suspension in a test-tube a flocculent condition becomes apparent to the naked eye in a few moments. The qualitative aspects of the reaction were described quite fully in the preceding paper of this series, and need not therefore be repeated here.

In my previous papers ('12 and '13) no attempt was made to establish a quantitative method of study. But in order to carry the subject farther it was necessary to determine how much of the sperm agglutinating substance was produced by the ova under various conditions. As noted in previous papers the reaction is reversible. With high concentrations of the agglutinating substance it may be several minutes before the agglutinated masses break up and reversal is complete; with low concentrations on the other hand the agglutinated masses are smaller and their disintegration is correspondingly more rapid. It was therefore possible to establish as a unit concentration of the agglutinating substance the greatest dilution at which an unmistakable reaction is given. Such a reaction lasts only a few seconds. It would be obviously very tedious to establish the *exact* point at which the reaction ceases in a long series of experiments, and in practice I prepared a series of dilutions each member of which was one-half of the preceding member, and the last one to give the reaction was regarded as the unit concentration. The method is as follows: Taking a given solution of the fertilizin a series of dilutions is made. If these begin with (a) 1/100 (i.e., 99 parts of sea-water plus 1 part of the solution) a half dilution of (a) is made, (b) (i.e., 1/200); a half dilution of (b) follows (c) 1/400, (d) 1/800, (e) 1/1600, (f) 1/3200, etc. If 1/1600 is negative, 1/800 may give a 4 to 6-second reaction; if 1/800 is negative 1/400 may give a 4 to 6-second reaction, etc; when the solution was known to be strong it was usual to begin with a 1/100 dilution; otherwise we might begin with a 1/10 dilution, followed by a 1/20, 1/40, etc., or 1/2 followed by 1/4, 1/8, 1/16, etc., the last member of a series to give a positive reaction was accepted as

of unit concentration. Thus any given solution could be rated as of 10, 100, or 6400, etc., agglutinating strength depending on the dilution test.

The most delicate method of determining the reaction is the raised cover-slip method described in a previous paper ('13). The sperm-suspension used as indicator should be about 1 per cent (i.e., one drop dry sperm to about 99 drops sea-water) and should be not more than 10 minutes old. A few drops of this are mounted on a glass slide beneath a long cover-slip supported by glass rods about 1 to 1.5 mm. in diameter. The slide is placed on the stage of a microscope in focus under a magnification of about 40 diameters and a drop of the solution to be tested is blown into the sperm suspension with a capillary pipette attached to a flexible rubber tube. The agglutination reaction begins within less than a second if the solution be of considerable agglutinating power, or within 2 or 3 seconds if the solution is diluted nearly to the unit reaction. In the latter case it lasts only 4 to 6 seconds. The masses formed are relatively small in the latter case, and larger and denser proportionately with the higher concentrations.

The reaction is accompanied by a strong stimulation of the spermatozoa, and the reversal of the reaction by slackening of movement, which is more or less proportional to the strength of the solution, so that after agglutination with a strong solution the spermatozoa are practically paralyzed for a time. For this reason the agglutination masses formed by a strong solution do not break up readily and it is difficult to fix the exact cessation of the reaction; but with concentrations up to about 20 units the beginning and end of the reaction are both clearly marked. For a more detailed account of the reaction with higher concentrations, see Study V (Lillie '13, p. 550).

As an important detail of technique, I would say that for the determination of the more delicate reactions it is desirable to have the sperm suspensions free from blood corpuscles or other foreign particles. I therefore proceed as follows: the sea-urchin is dried slightly with a towel and is opened by a circular cut on the oral side of the equator. If it be a ripe male the thick sperm will begin to exude from the genital pores. The animal is then

laid aboral side down in a Syracuse watch crystal and the sperm allowed to collect in the crystal. In this way, as much as 2 cc. of thick perfectly clean sperm may be obtained from a large ripe male. If a few drops of sea-water have dripped off with the sperm it may be removed with a pipette, but if the sperm is abundant it is so dense that the fluid lies on it without intermingling. Such a mass of sperm will keep perfectly fresh for several hours, and suspensions may be made from it as needed for the tests. But the suspension used in testing must be freshly prepared if delicate reactions are desired.

The fertilizin is actively secreted by the eggs in sea-water as noted beyond; the concentration in the fluid of a given suspension of eggs in sea-water is a function of the quantity of eggs in the suspension. It will be convenient, therefore, in referring to any given agglutination solution, to have a method of expressing the relative quantity of eggs in proportion to the sea-water. The most convenient way of expressing this relation is to express the bulk of eggs as a percentage of the entire suspension. Thus 1 cc. of eggs added to 9 cc. of sea-water will be denominated a 10 per cent egg suspension, etc. This form of expression was not adopted until after the experiments were done; the figures are, therefore, not always even numbers, and are not intended in any case to be absolutely exact, but this is a matter of little importance as the density of a given egg suspension is only one factor in the concentration of the fertilizin in it; the condition of the eggs, whether entirely ripe or immature, fresh or stale, with or without jelly, is more important.

2. THE QUANTITY OF FERTILIZIN PRODUCED BY OVA OF ARBACIA

a. By a single washing

July 6, 1913: For this experiment I used the eggs of a single female shed in a watch crystal in the same manner as described above for the collection of clean sperm. The eggs were thus free from blood or fragments of the ovaries, and contained only a trace of the sea-water. Graded quantities of eggs were put in a series of six test-tubes containing 5 cc. of sea-water each. The eggs were placed in the tubes at 9:45 A.M.; each tube was turned upside down six times and the eggs then allowed to

settle; there was no shaking or rough handling of the eggs, so that they were absolutely uninjured. At 10:20 A.M., after the eggs had settled to the bottoms of the tubes, samples of the sea-water in the tubes 1 to 5 were removed and tested by a series of dilutions; at 11:45 A.M. a sample of 6 was taken and tested. The concentration of the sperm agglutinating substance in each is shown in table 1.

TABLE 1

TUBE	EGG SUSPENSIONS PER CENT	AGGLUTINATING STRENGTH OF SUPER- NATANT SEA-WATER	TESTS	
			Dilutions	Reactions
1	0.5	50	1/50	4-5 seconds
2	1.0	100	1/100	4-5 seconds
3	2.0	100 +	1/100	10 seconds
4	4.0	400	1/400	6 seconds
5	8.0	800	1/800	6 seconds
6	16.6	1600	1/1600	7-8 seconds

It is interesting to note that the quantity of fertilizin produced is so nearly proportional to the quantity of eggs. (No. 3 would probably have tested to 1/200 with perfectly fresh sperm.) We might expect in general that the quantity of fertilizin produced would continue to be proportional to the quantity of eggs. This however, may not be the case, as there are indications that in the presence of a certain concentration in the sea-water more is not secreted by the eggs.

It would appear that after the eggs had settled in the tubes into a dense layer on the bottom they ceased to produce more agglutinating substance, because the concentration in tube 6 in which the sea-water had stood over the eggs much longer than in tubes 1 to 5 was proportional to the quantity of eggs and not to the time.

Higher concentrations of the fertilizin were often obtained: August 9: A 25 per cent egg suspension stood for 12 minutes. The supernatant fluid then gave the following sperm-agglutination tests: diluted to 1/6400, 7-second reaction; 1/3200, 15-second reaction. August 18: egg suspension (density not given) was shaken. After settling of the eggs the supernatant fluid

gave the following sperm-agglutination tests: diluted to 1/12800, 5-second reaction; 1/6400, 10 to 12-second reaction.

Enough has been said to indicate the large amounts of this substance produced by the eggs in sea-water. No attempt has been made to produce the highest attainable concentration. The standardization of solutions for experiments was a daily procedure and in the experiments themselves hundreds of tests were made.

Hitherto, I think, the unfertilized egg has been generally regarded as a very inert cell, almost in a state of suspended animation. But it is necessary to modify such a point of view very greatly. The egg of *Arbacia*, at least, is a very actively secreting cell. Although the method which I have used enables us to calculate only the relative concentration of the agglutinating substance produced, yet the quantity must be very considerable when we consider that in tube 1, of the preceding experiment the egg suspension was only 0.5 per cent and that the supernatant fluid was charged with 50 units of the agglutinating substance in 35 minutes.

b. Comparison of ripe and unripe eggs

The tests already given are from fresh, ripe eggs. Ovaries containing a high proportion of ovocytes produce less fertilizin proportionately; but such ovaries could be obtained only at the beginning of the season so that only a few tests were made. On June 16 I compared equal amounts of ripe ovaries with practically all eggs mature, and unripe ovaries with only 50 per cent of the eggs mature. One part finely cut up ovary was added in each case to 3 parts sea-water. The first tested to 1/612, the second to 1/64. In a second determination the same day, in which *a* had only 5 per cent unripe eggs (ovocytes with germinal vesicle) and *b* had 50 per cent, one part ovary to 2 parts sea-water in each case, the tests were *a* 1/1000, *b* 1/600. In the latter case the strength of the solutions was about proportional to the percentage of mature eggs. In the former case the unripe ovary did not give as large a production of the fertilizin as would be expected

from the relative proportions of ripe eggs. Presumably, the ovarian tissue was relatively more bulky in this case.

The ovocytes are surrounded with the same layer of jelly in *Arbacia* as the mature eggs; hence we must conclude that the jelly is not the source of the agglutinating substance to whatever extent it may become charged with it after maturation. An important point is involved in this conclusion discussed farther on.

I think we are justified in concluding that ovocytes do not secrete the agglutinating substance. The conditions in *Nereis* fortify this conclusion, inasmuch as the ovocytes with intact germinal vesicle do not secrete the fertilizin, but as soon as insemination takes place it is poured out abundantly and the germinal vesicle breaks down.

In this connection it is important to note that in some animals ovocytes with intact germinal vesicle do not respond to the spermatozoon, even if cut in two so that the protoplasm is laid bare. Wilson ('03) has shown this for the nemertean, *Cerebratulus*, and Delage ('01) for the eggs of *Strongylocentrotus* and *Asterias*. As I show farther on that the fertilizin is necessary for fertilization, we may find in these facts evidence that the fertilizin is not only not secreted but is not even preformed in ovocytes with intact germinal vesicle, at any rate not in the forms mentioned. Its absence would furnish sufficient explanation of the failure of such egg-fragments to fertilize, and the consequent impossibility of producing merogony until the germinal vesicle breaks down.

Delage ('01) also shows that, as soon as the germinal vesicle begins to fade, in *Asterias*, and the nuclear sap diffuses into the cytoplasm, the egg becomes fertilizable and merogony can be induced. According to my interpretation the formation of fertilizin must begin at this time. At any rate the non-fertilizable condition of the cytoplasm before the germinal vesicle begins to fade is associated with the absence of fertilizin.

It is not necessary to assume that the fertilizin is contained in the nuclear sap; on the contrary, the long-continued production of this substance, as described in the next section, runs counter to this idea. It is more probable that it exists in a state comparable to zymogen ready to be converted into active form, and

that the escape of nuclear sap furnishes the necessary conditions. In some eggs, therefore, we might anticipate that sufficient substance might exude from the germinal vesicle without rupture to bring about formation of active fertilizin. Theoretically this should occur in such eggs as those of *Nereis* in which fertilization occurs before rupture of the germinal vesicle.

c. Fertilizin produced in a series of washings; rôle of the jelly

The production of this substance by the egg, in sea-water must be regarded as an active process of secretion, and, as will be seen immediately, it goes on continuously, in the case of *Arbacia*, as long as the eggs remain alive.

The last fact was ascertained in several attempts to remove all the fertilizin from the eggs by repeated washings. In these experiments it was also ascertained that while the jelly layer surrounding the eggs is saturated with fertilizin, eggs deprived of jelly still continue to produce it in considerable quantities. The method of making such tests is to suspend a measured amount of eggs in a given amount of sea-water and allow them to settle; as much of the supernatant fluid is then poured off as is possible without losing any eggs and kept for testing, and an equivalent amount of fresh sea-water replaced; the eggs are mixed up thoroughly, and allowed to settle, and the process is continued. In one series running three days in which the quantity of eggs was originally 2 cc. and the total volume of sea-water and eggs in the tube 10 cc., 6 to 8 cc. being poured off at each settling, thirty-four changes were made, and the agglutinating strength of the supernatant fluid diminished from 100 at first to 20 at the end. The details are given in table 2: Under the heading 'washings' the denominator gives the quantity of eggs and sea-water left after pouring off the supernatant fluid, and the numerator the total quantity after addition of the fresh sea-water.

This long series is amazing in its revelation of the extraordinary energy of the eggs in producing the fertilizin. In this case the eggs were allowed to retain their jelly, which, however, slowly dissolved until it was nearly all gone in washing 24.

TABLE 2

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 14			
1 10/3.0 cc.	1/100—10—12 sec.	2.0 cc.	11:01 A.M.
2 10/2.5 cc.	1/100— 8 sec.		
3 10/2.5 cc.	1/50 —10—11 sec.	1.15 cc.	11:12 A.M.
4 10/2.0 cc.	1/50 — 10 sec.	1.1 cc.	11:22 A.M.
5 10/2.0 cc.	1/100— 6 sec.		11:32 A.M.
6 10/2.0 cc.	1/50 — 9 sec.	1.2 cc.	1:55 P.M.
7 10/1.7 cc.	1/100— 12 sec.	0.9 cc.	2:20 P.M.
8 10/1.7 cc.	1/100— 7 sec.		2:35 P.M.
9 10/1.7 cc.	1/100— 7 sec.		2:45 P.M.
10 10/1.7 cc.	1/100—11—13 sec.	0.8 cc.	2:53 P.M.
11 10/2.0 cc.	1/100— 11 sec.	0.8 cc.	3:03 P.M.
12 10/1.7 cc.	1/100— 8 sec.	0.8 cc.	3:13 P.M.
13 10/1.5 cc. ¹	1/100— 12 sec.		3:23 P.M.
14 Fluid not changed ²	1/100— 11 sec.		4:12 P.M.
August 15			
14a 10/2.8 cc.	1/50 slow atypical reaction		8:18 A.M.
15 10/1.9 cc.	1/100— 12 sec.	0.7 cc.	9:20 A.M.
16 10/2.0 cc.	1/100— 13 sec.	0.7 cc.	9:55 A.M.
17 10/2.0 cc.	1/100— 7 sec.	0.65 cc.	10:22 A.M.
18 10/2.0 cc.	1/25 — 7 sec.		10:40 A.M.
	(Note sudden fall 17-18)		
19 10/2.0 cc.	1/50 — 5 sec.		11:04 A.M.
20 10/2.0 cc.	1/25 — 5 sec.	0.6 cc.	11:34 A.M.
21 10/2.0 cc.	1/20 — 9 sec.	0.5 cc.	2:15 P.M.
22 10/4.0 cc.	1/20 — 7 sec.		2:45 P.M.
23 10/ .0 cc.	1/20 — 7 sec.	0.55 cc.	3:45 P.M.
24 10/2.0 cc.	1/20 — 7 sec.		4:25 P.M.
No jelly left on the eggs, except a thin layer on a few			
August 16	Continued the above series	to 33 washings from	9:08 A.M. to
4:05 P.M.			
25			
26			
27	1/20 — 7 sec.		
28			
29			
30 ³			
31	1/20 — 5 sec.		
32	1/10 — 10 sec.		
33	1/20 — 18 sec.		

¹ The jelly is now dissolved until only a thin layer is left. Washings 3-14 were absolutely colorless; and the following ones remained so until 35.

² The eggs were precipitated by slight centrifuging in the first 13 washings, except 6; in the others they were allowed to settle. The gradual reduction in bulk of the eggs is due to loss of jelly, not to loss of eggs.

³ A drop from No. 30 was fertilized with excess of sperm at 11:36 A.M.; 5 per cent only segmented, irregularly; no membranes formed.

TABLE 2—Continued

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913 August 17			
34 12/1.5 cc.	1/20 — 5 sec.	0.325 cc.	9:15 A.M.
35 On stirring up, the eggs disintegrate, and the fluid becomes highly colored; the agglutinating substance is <i>absolutely neutralized</i> . Test 1/1 negative, but the fluid is highly chemotactic and activating.			

In order to determine the rôle of the jelly in charging the sea-water with fertilizin, I killed some eggs by heating them for 5 minutes to 60° C. (August 15, 1913,) and submitted them to a series of 22 washings in 3 days, by which time the jelly was nearly all dissolved away: the agglutinating power decreased from 800 at first to 1 at the end. The agglutinating substance could be detected as long as any jelly remained.

On the other hand, eggs may be entirely deprived of jelly by shaking, and such eggs will continue to produce fertilizin as long as eggs with jelly, though in lesser quantities at first. This matter is so fundamental that I include another long table giving a comparison of eggs deprived of jelly by shaking and those with jelly (table 3).

August 21, 1913: In this experiment ripe eggs of *Arbacia* were washed once and divided in three equal lots, *A*, *B*, and *C*, in 10 cc. sea-water in graduated cylinders. They were allowed to settle, and at 9:20 A.M. the bulk of eggs was 1.8 cc. in *A*, 2 cc. in *B*, and 2 cc. in *C*. Thus there were about 10 per cent fewer eggs in *A* than in *B* and *C*. At 9:23 *A* was transferred to a test-tube and given 6 vigorous shakes to get rid of the jelly. A sample was then examined in India ink and it was found that 90 per cent of the eggs were entirely freed from the jelly; in the remainder it was usually reduced to a thin layer. *A* was then returned to its cylinder in 10 cc. sea-water and allowed to settle. As the eggs settled in *A* it could be seen that the fluid was only slightly colored; so that it was certain that not many eggs were injured in the shaking.² At 9:40 A.M. the bulk of eggs was as follows: *A*, 0.6 cc.; *B*, 1.6 cc.; *C*, 1.6 cc. As care was taken to avoid loss of eggs in *A* the

² It should be noted that in such an experiment, it is extremely important not to shake too much, for if the eggs themselves are injured so that the color escapes, a complicating factor comes in considered beyond (see section 6, p. 544).

decrease in bulk is due to loss of jelly, in *B* and *C* simply to further settling. *C* was kept for control. *A* and *B* were submitted to repeated washings as in table 3, p. 536.

This experiment positively decides the question as to production of fertilizin. It continues to be produced by the unfertilized eggs without jelly as long as they live. The jelly is, naturally, saturated with fertilizin, and it seems probable that it may act to prevent too rapid exhaustion of the secreting mechanism of the egg. It is of course possible that all that the egg gives off is pre-formed, but it is more probable that loss of this substance by the egg acts as stimulus to regeneration of more.

Apparently the eggs do not secrete the fertilizin in such a series of washings after they have settled in a mass at the bottom of the tube; for it will be noticed that the concentration of the substance in the successive washings is not a factor of the time intervals. On the contrary, a long time interval between washings is often associated with a temporary decrease in concentration, as may be noted, for instance, in the interval of 15 hours between 14 and 14a in table 2. In this case 14 and 14a are actually the same body of supernatant fluid and the concentration is much decreased in the second test 15 hours after the first, as though the eggs were gradually destroying the fertilizin. This subject is fully treated in section 6 (anti-fertilizin).

3. THE BINDING OF FERTILIZIN BY SPERM

In my previous paper (Lillie '13) I showed that the spermatozoa fix the agglutinating substance, and assumed as a working hypothesis that the fixation was due to chemical union. The fact on which the statement was based was the disappearance of the agglutinating substance from a sperm suspension when not present in excess. No attempt was made, however, to make quantitative determinations, so the matter was taken up again in 1913 in a more exact manner. The amount of the agglutinating substance actually fixed by the sperm is surprisingly small when the great avidity of the sperm for it is taken into account.

TABLE 3

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 21			
1 A 10/2.0		0.6 cc.	9:42 A.M.
B 10/3.0		1.6 cc.	9:42 A.M.
2 A 10/2.0		0.6 cc.	10:18 A.M.
B 10/2.4		1.4 cc.	10:18 A.M.
3 A 10/2.6		0.55 cc.	10:40 A.M.
B 10/3.0		1.4 cc.	10:40 A.M.
4 A 10/2.4		0.55 cc.	11:04 A.M.
B 10/2.7		1.3 cc.	11:04 A.M.
5 A 10/3.0		0.55 cc.	11:27 A.M.
B 10/2.6		1.2 cc.	11:27 A.M.
6 A 10/2.0		0.55 cc.	11:47 A.M.
B 10/2.7		1.2 cc.	11:47 A.M.
7 A 10/2.0		0.5 cc. plus	2:03 P.M.
B 10/1.6		0.8 cc.	2:03 P.M.
8 A 10/2.5	1/10 neg.; 1/1—50 sec.		2:30 P.M.
B 10/2.5	1/20—13 sec.		
9 A 10/2.0			3:15 P.M.
B 10/2.0			3:15 P.M.
10 A 10/2.0			3:45 P.M.
B 10/2.3			3:45 P.M.
11 A 10/2.0			4:06 P.M.
B 10/2.4			4:06 P.M.
Fertilization 1			
12 A 10/2.0			4:40 P.M.
B 10/2.5			4:40 P.M.
13 A 10/1.5			7:32 P.M.
B 10/1.2			7:32 P.M.
August 22			
14 A 10.4/3.4		0.4 cc.	8:18 A.M.
B 10/2.8		0.6 cc.	8:18 A.M.
15 A 10/1.4			8:38 A.M.
B 10/2.0			8:38 A.M.
16 A 11/1.8	1/10 trace; 1/1—60 sec.		9:13 A.M.
B 11/2.2	1/10—20 sec.		9:13 A.M.
Fertilization 2			
17 A 10/1.7			9:40 A.M.
B 10/2.5			9:40 A.M.
18 A 10/1.0			10:05 A.M.
B 10.5/2.4			10:05 A.M.
19 A 10/1.0			10:23 A.M.
B 10/3.0			10:23 A.M.

TABLE 3—Continued

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 22			
20 A 10/1.0	1/10 trace; 1/1 trace and strong chemo- taxis		10:48 A.M.
B 10/2.4	1/10—9 sec. going to pieces in A		
Note: Some eggs			
21 A 10/1.5			11:10 A.M.
B 10/3.0			
22 A 10/1.0			11:30 A.M.
B 10/2.2			
23 A 10/1.4			2:12 P.M.
B 10/2.0			
24 A 10/1.4	1/1 trace and strong chemotaxis		2:33 A.M.
B 10/1.8	1/1—25 sec., slight chemotaxis		
25 A 10/1.0			2:55 P.M.
B 10/1.2			
26 A 11/1.0			4:03 P.M.
B 11/2.0			
27 A 10/1.0			4:19 P.M.
B 10/2.0			
Fertilization 3			
28 A 10/2.0			4:36 P.M.
B 10/1.0			
29 A 10/1.0			7:45 P.M.
B 11/2.0			
August 23			
30 A 10/1.0	1/1—20 sec., chemo- taxis ¹		8:37 A.M.
B 10/2.5	1/1—30 sec., no chemotaxis		
31 A 10/1.5	1/1—14 sec. strong chemotaxis		9:13 A.M.
B 10/2.0	1/1—20 sec. strong chemotaxis		
Fertilization 4			
32 A 10/1.0			10:10 A.M.
B 10/2.0			
33 A 10/1.0	1/1—11 sec. strong chemotaxis		11:30 A.M.
B 10/1.6	1/1—11 sec. strong chemotaxis		

¹ The chemotaxis noted in this series is due to injured eggs which secrete anti-fertilizin and conceal the fertilizin. See discussion.

TABLE 3—Continued

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913 August 23			
34 A 10/0.5 B 10/1.0			2:36 P.M.
35 A; fluid not changed	1/1—11 sec.		
B fluid not changed	1/1—10 sec.		
August 24			
36 A; fluid not changed	1/1—8 second reac- tion		9:35 A.M.
B fluid not changed	1/1—7 second reac- tion		
37 Eggs shaken	and broken; fluid not	changed.	9:42 A.M.
A	1/1—negative		
B	1/1—4 sec.; 2d test n	egative	

Thus on August 13, I standardized an agglutination solution in sea-water to about 128 agglutinating power and placed 1 cc. in a tube (A). To this was added 1 cc. of a 3.3 per cent sperm suspension; 1 cc. of the agglutinated mixture A was then added to tube (B) containing 1 cc. of the same sperm suspension; 1 cc. of B was then added to 1 cc. of the same sperm suspension in tube (C). Thus the 128 power agglutinating solution was diluted with 3.3 per cent sperm twice in A, four times in B, and eight times in C.

- (A) 1 cc. 128 agglutinating power solution plus 1 cc. 3.3 per cent sperm
- (B) 1 cc. A plus 1 cc. 3.3 per cent sperm
- (C) 1 cc. B plus 1 cc. 3.3 per cent sperm

The sperm was then centrifuged off in the three tubes and on test the supernatant fluid was negative in C, faintly positive in B and gave a 60 to 80-second reaction in A. On renewed test, B was found almost negative. The result is then that 1 cc. of a 128 power agglutinating solution may be fixed by 3 cc. of 3.3 per cent sperm.

Other tests of the binding power of sperm gave results of the same order of magnitude. Thus on August 9, I used a 64 power agglutinating solution as follows:

- (A) 1 cc. 64 agglutinating solution plus 1 cc. (approx.) 3 per cent sperm
- (B) 1 cc. A plus 1 cc. 3 per cent sperm
- (C) 1 cc. B plus 1 cc. 3 per cent sperm
- (D) 1 cc. C plus 1 cc. 3 per cent sperm

In this case after centrifuging off the sperm, *B*, *C* and *D* were negative; *A* was faintly positive. That is, 1 cc. of approximately 3 per cent sperm fixed 1 cc. of a 64 power agglutinating solution. This shows a greater binding power than in the previous experiment, but easily within the limits of error arising from difficulty of standardizing the sperm suspension, owing to the unavoidable inclusion of some fluid in the sperm. Other tests gave comparable results. Reduced to common terms:

August 9: 1 cc. 3 per cent sperm fixes 1 cc. 64 power agglutinating solution.

August 18: 1 cc. 3.3 per cent sperm fixes 0.66 cc. 64 power agglutinating solution

The difference is considerable, but when one considers first the difficulty of obtaining equivalent sperm suspensions and second the 2 to 1 method of standardizing the agglutinating solutions, and third, possible variations in the delicacy of the sperm suspensions used as indicators, the results lie well within the limit of error and indicate that there is a definite quantitative relation involved.

The possibility remains that the disappearance of the agglutinating substance in sperm suspensions is a phenomenon of adsorption rather than of chemical union. Experiments planned for an adequate test of this possibility were crowded out by other problems.

If sperm suspensions are allowed to stand for some hours they reach a condition when they will agglutinate only with strong solutions. If the binding power is then tested it is found that they fix as much of the agglutinating substance as before. The fixing power of the sperm is thus entirely independent of its capacity for being agglutinated, which is of course to be expected. But it is important, nevertheless, to emphasize it; the capacity for being agglutinated depends upon the motility of the spermatozoa, the binding capacity depends merely upon the presence of

chemical side-chains (receptors) capable of uniting with the agglutinating substance. When such a union takes place the heads of the spermatozoa must be supposed to become adhesive; if they move about rapidly, there are more collisions and hence more agglutination. With stale spermatozoa, either the movements are too slow to produce much agglutination, or the receptors are cast off and lie free in the medium, so that their binding does not affect the physical properties of the spermatozoa themselves. The latter possibility should be investigated, but was not this summer owing to the multitude of other problems.

A little light may be thrown on this problem by the results of June 26: In that case 4 drops of a strong, but not definitely standardized, solution of the agglutinating substance was added to 4 cc. of a 3 per cent sperm suspension 3 hours old. The tube was then centrifuged strongly and most of the spermatozoa precipitated in a mass at the bottom; however the supernatant fluid remained opalescent. Tested immediately with a fresh sperm suspension the fluid gave a 17 to 25-second agglutinating reaction. The tube was allowed to stand five minutes longer and was then tested again; the precipitated sperm remained in a solid mass, but the supernatant fluid had become negative; it contained no more free agglutinating substance. The quantity of sperm in suspension seemed much too small to account for the neutralization, which I am inclined to attribute to free receptors. But no really adequate test was made.

As to the interpretation to be given to the reversal of agglutination of the sperm it may be supposed either, (1) that the adhesive condition on which the agglutination depends exists only during an early union of the agglutinating substance with the sperm receptors and is lost as the union becomes firmer; or (2) that the bound receptors are cast off by the sperm. In view of the nature of the mechanism of the fertilization process, discussed beyond, I am inclined to lean to the second hypothesis.

4. SEPARATENESS OF HETERO-AGGLUTINATING SUBSTANCES IN EGG-SECRETIONS

I stated in my previous paper that in strong agglutinating solutions from *Arbacia* eggs and ovaries there is present also a substance that agglutinates the sperm of *Nereis*, and I argued that this was probably distinct from the iso-agglutinating substance because (1) it is contained in the blood of *Arbacia* which is normally entirely free from the iso-agglutinating substance, and (2) it may disappear from agglutinating solutions on standing while the iso-agglutinating substance remains apparently intact.

Some farther experiments made this year demonstrate the fact that the substance in agglutinating solutions from *Arbacia* eggs, that attacks *Nereis* sperm is a separate substance. We may note in the first place that the effect of this substance on the *Nereis* sperm is distinctly toxic. A drop containing it injected into a *Nereis* sperm suspension beneath a raised cover slip causes the formation of a permanent coagulated ring at the margin of the drop. In this respect the action differs from the iso-agglutination which is without toxic effects.

In the second place, the quantity present in strong iso-agglutinating solutions of *Arbacia* is extraordinarily variable; thus on June 18, 1913, an agglutinating solution was made by addition of 3 parts sea-water to 2 parts cut-up ovaries and eggs of *Arbacia*. The test on *Nereis* sperm showed: (1) undiluted, formation of a solid ring-coagulum; (2) diluted one-half with sea-water, solid ring-coagulum; (3) diluted to one-fourth with sea-water, slight permanent coagulum; (4) diluted to one-eighth, small coagulated masses; (5) diluted to one-sixteenth, nearly neutral; (6) diluted to one-twenty-fifth, absolutely neutral. Acting on *Arbacia* sperm the same solution gave a 5-second reaction at 1/200 dilution. Other samples equally strong in the iso-agglutinating substance were nearly neutral for *Nereis* sperm undiluted.

In the third place it is possible to precipitate out all of the *Nereis*-active substance with *Nereis* sperm and leave the full complement of iso-agglutinating substance. Thus on June 19 an agglutinating solution was made by addition of 3 parts sea-

water to 2 parts ovaries and eggs of *Arbacia*; the fluid was filtered off. Undiluted it had a powerful effect on *Nereis* sperm but was negative at 1/16 dilution; 1 cc. was then placed in each of three tubes *A*, *B*, *C*. To *A* was added 2 drops of an aggregative *Nereis* sperm-suspension, to *B* 4 drops of the same, to *C* 8 drops of the same. Precipitates formed in all. On testing the supernatant fluids all were found to be negative to *Nereis* sperm. The hetero-agglutinating substance was completely fixed even in *A*. Tested for the iso-agglutinating substance, *A* was shown to be equal in power to the control. The tests gave:

- (A) 1/800—faintest reaction
- Control 1/800—faintest reaction
- (A) 1/400—12 second reaction
- Control—1/400—8 second reaction

Thus while all the *Nereis*-active substance was destroyed the iso-active substance remained in full amount.

This is of course conclusive; but I was also able to show that the sperm of *Ctenolabrus* (teleost) may precipitate the hetero-active substance leaving the iso-agglutinating substance intact. The hetero-active substance would therefore appear to be rather generally toxic to foreign sperm. It is present in greater quantity in the blood than in egg solutions as shown by definite experiments which need not be quoted in detail. It is rather probable, indeed, that its presence in the egg solutions is due to contamination by the blood. But I did not test this out fully, though it could readily be done by successive washings of the eggs.

A rather embarrassing fact that appeared in the course of the above tests is that *Nereis* sperm may destroy considerable quantities of the iso-agglutinating substance if present in excess of the amount required to fix the hetero-active substance. This came out first in the test of *C* (under June 19 above), which contained at least 4 times the amount of *Nereis* sperm needed to fix the hetero-active substance, for the supernatant fluid in this tube turned out to be several times less active in its iso-agglutinating properties than the untreated control. It looked as though the *Nereis* sperm would combine more actively with the iso-agglutinating substance than the *Arbacia* sperm itself.

There are, however, strong reasons against accepting this conclusion: (1) there is no visible effect of the iso-agglutinating substance on the Nereis sperm as should be the case if there were actual combination. (2) The action of the Nereis sperm is ferment-like, inasmuch as the iso-agglutinating substance continues to disappear progressively for a long period of time in the presence of excess of Nereis sperm, whereas this is not the case in the combination with Arbacia sperm. I shall therefore omit the details of the experiments under this head, being convinced that we are dealing here with a case of actual destruction of the iso-agglutinating substance and not with chemical combination.

5. SOME SPECIAL PROPERTIES OF THE FERTILIZIN

It would be interesting to know something concerning the chemical nature of this substance which is secreted by the eggs in such considerable quantities. This subject was, however, left for another occasion, as the investigation of its biological properties offered more problems than could be investigated at the time. Apart from these biological properties, all that I am now able to say about it is of a negative nature.

(a) It is colorless: Thus in table 2 all the washings after the first two were colorless though the concentration of the substance was considerable; similarly in table 3. (b) It must possess very considerable molecular size for it can be completely removed from any solution by filtering through a Berkefeld filter. Whether the solution be fresh or not, or of whatever concentration makes no difference; the filtrate obtained through a Berkefeld filter was invariably devoid of sperm agglutinating properties. On the other hand, it readily passes specially hardened filter paper. (c) Correspondingly it is non-dialyzable, not passing through the walls of celloidin tubes even in 24 hours. (d) It is extremely heat resistant being destroyed only slowly at the boiling point (see Study V, 1913). (e) As stated in my preliminary paper, I am indebted to Dr. Otto Glaser for the determination that it does not give the usual protein tests, even in the most concentrated solutions obtainable (see Glaser, 1914a) It is

thus colorless, non-filterable, non-dialyzable, very thermo-stable and apparently non-protein in its character.

The stability of the substance is also evidenced in its lasting qualities. Filtrates from egg-suspensions were kept and tested after various intervals of time. Thus the filtrate from a 33 per cent egg-suspension of June 21, was of 1600 agglutinating power; on August 26 it was of 100 agglutinating power. Similarly, the filtrate from a 33 per cent egg-suspension of June 20 was of 1600 agglutinating power; on August 26 it was of 100 agglutinating power. On the other hand, an agglutinating solution of June 30, of 800 agglutinating power was practically negative on August 26. In this tube there was more evidence of bacterial action than in the other two. Again, a distilled water extract kept from September, 1912, was of 16 agglutinating power on July 7, 1913.

In Study V, I also noted that its production is confined entirely to the eggs. It is not contained in the blood, and could not be extracted from other tissues.

6. ANTI-FERTILIZIN

We have noted the extreme avidity of the spermatozoa for the fertilizin produced by the eggs. Neither the blood nor tissues appears to contain any substance capable of uniting with the spermophile side-chain which causes agglutination of the spermatozoa, or of neutralizing its action. However, such a substance is contained within the egg itself, and its discovery is one of the most interesting points in the mechanism of fertilization, because it would appear from the facts considered in section 7 that it is the occupancy of the spermophile side-chain of the fertilizin by this substance, which I propose to call anti-fertilizin, that prevents polyspermy, or refertilization of eggs.

Its presence can be demonstrated in the test-tube by methods which extract the internal substance of the eggs. But as the jelly surrounding the eggs contains an immense excess of fertilizin, complete neutralization of sperm agglutinating power can be obtained only after the jelly is removed, and still better if the eggs have been repeatedly washed until their fertilizin production is greatly reduced.

To consider the latter method first: The matter was first brought clearly to my attention in the experiment listed in table 2. In the 34th washing the supernatant fluid tested to 1/20 dilution; it was poured off, leaving 1.5 cc. in the tube, and 10.5 cc. of sea-water was added. When the eggs were stirred up they at once disintegrated, liberating the red pigment so that the fluid which in previous washings had been colorless became bright red. When this fluid was tested with a fresh sperm suspension it was absolutely without agglutinating effect. The dilution of the 20 power fluid of the 34th washing was 8 times, so that even if the eggs had suddenly ceased the production of the agglutinating substance the fluid would have contained 2.5 agglutinating units and should have given at least a 10-second agglutination reaction; but it was absolutely negative. The agglutinating substance had therefore been neutralized by some substance escaping from the disintegrating eggs.

In the experiment listed in table 3, the repetition of this result was of course looked for. On the morning of August 24, after 35 washings on previous days, I noticed that pigment was beginning to diffuse from the eggs in tubes *A* and *B*, but it had not extended above the 1.5 cc. mark. The eggs were beginning to break down. Without disturbing them, I tested the colorless supernatant fluid and got undiluted an 8-second reaction in *A* and a 7-second reaction in *B*. Both tubes were then vigorously shaken until the eggs were disintegrated and the fluid colored with the escaping pigment. Tested in 4 minutes *A* was negative for agglutination, *B* gave a 4-second reaction. Tube *B* was again shaken and in 11 minutes more its agglutinating action had also disappeared.

There can, therefore, be no doubt that the egg contains in its interior a substance that unites with the spermophile group of the fertilizin.

In these experiments it was interesting to note that after the agglutinating action had disappeared, the solution was very strongly chemotactic, so that the drop injected into the sperm suspensions formed a ring of active, entirely unagglutinated spermatozoa. The chemotactic substance is certainly distinct

from the fertilizin. The same observation was made also on other occasions.

The presence of the anti-fertilizin in the eggs could also be demonstrated in extracts of fresh eggs. These were made (1) by extracting in distilled water, (2) by drying eggs and grinding them in sea-water, (3) by grinding fresh eggs with sand in a mortar. I had previously often noticed that such extracts gave decidedly atypical agglutination of the spermatozoa, as compared with the action of the secretion of the eggs into sea-water. Distilled water extracts were then examined to find the cause for such atypical behavior. After it became clear from the experiments quoted above that the eggs contained the anti-fertilizin, the method of making the distilled water extracts was modified by first shaking the eggs as free from jelly as possible, so as to avoid the great excess of fertilizin contained in the jelly. These experiments yielded the following results:

August 22, 1913: 10:35 A.M. washed eggs were shaken six times in a test tube to get rid of the jelly. The eggs were washed twice again and shaken six times more; 60 per cent lost jelly; let stand. 2:15 P.M. Poured off sea-water to 2 cc. and added fresh sea-water to 8 cc. and divided equally in two test tubes, *A* and *B*; allowed to settle. 2:27 P.M. Poured off sea-water from settled eggs to 1 cc. in both *A* and *B*. To *A* added 7 cc. sea-water. To *B* added 7 cc. distilled water. The eggs were allowed to stand until they were completely plasmolyzed in *B* and the supernatant fluid was bright red. Tests were then made within an hour, or less, of the supernatant fluid in *A* and *B*, for the fertilizin, with somewhat surprising results:

(*A*) sea-water secretions
Dilution tests

1/160—negative
1/80 —negative
1/40 —negative
1/20 —6 sec. reaction

1/1 —90 sec. reaction

(*B*) distilled water extract
Dilution tests

1/1280—faint trace
1/640 —7 sec. reaction
1/320 —12 sec. reaction
1/160 —12 sec. reaction
1/80 —12 sec. reaction
1/40 —10 sec. reaction
1/20 —14 sec. reaction
(strong chemotaxis)
1/1 —12 sec. reaction
(strong chemotaxis)

Thus if only extreme dilutions were compared the conclusion would be that *B* is 32 times stronger than *A*, whereas comparison of the undi-

luted fluid would lead to the conclusion that *A* was $7\frac{1}{2}$ times stronger than *B*. The fact that the time of the reaction of the *B* extract is about the same at all concentrations is in most pronounced contrast to the sea-water secretions in a very large number of tests, which had shown that the duration of the reaction is about proportional to the concentration of the agglutinating substance. The inference from the fact would be that the extract (*B*) contained an inhibitor for the agglutinating reaction which is diluted equally with the agglutinating substance. This might be a substance entering into combination with the agglutinating substance, but slowly and loosely at first.

4:07 P.M. Tube *B* was shaken vigorously to complete the plasmolysis. Tube *A* was stirred up. Both were then left until the next morning.

August 23, 1913: Tests of agglutinating power on fresh sperm:

<i>Tube A</i> <i>Dilution tests</i>	<i>Tube B</i> <i>Dilution tests</i>
1/20— 6 sec.	Negative at all dilutions 1/1280,
1/10—12 sec.	1/640, 1/320, 1/160, 1/80, 1/40, 1/20,
1/1 —190 sec.	1/10, 1/1, but showing decided chemotaxis.

Thus the neutralization of the fertilizin had become complete in the intervening time.

The disappearance of the agglutinating substance is not due to any destructive effect of distilled water on it, for control experiments showed that this was not the case.

A similar experiment on August 20 gave even more clear cut results. In this case (1) the eggs were deprived of jelly by 6 shakes, and examination of the eggs in ink showed that jelly was almost completely removed. (2) The eggs were allowed to settle and the supernatant fluid poured off. (3) The eggs were then divided into two equal parts in test-tubes *A* and *B*. (4) To *A* added 9 cc. fresh sea-water, to *B* added 9 cc. distilled water. (5) Each was stirred up twice and stood 30 minutes.

Tested with fresh 1 per cent sperm suspension, *A* gave a 10 second reaction at 1/40 dilution. *B* was negative at 1/2 and gave a faint reaction only, undiluted. Thus as *B* must have contained as much fertilizin as *A*, its disappearance must have been due to escape of anti-fertilizin by plasmolysis, inasmuch as we know that distilled water does not destroy it.

It would follow from this experiment in which the jelly was practically entirely removed, that the eggs contained an amount of anti-fertilizin sufficient to neutralize all the fertilizin present in them.

A third experiment yielded results similar to the first:

August 23, 1913: Fine lot of fresh eggs; shaken 6 times, 2:15 P.M. in test tube. Examination in ink showed about half have entirely lost

jelly, and that it is much reduced in nearly all. About 2 per cent of the eggs broken.

2:33 P.M. washed: Divided in two equal lots in test-tubes *A* and *B*.
3:08 P.M.: After settling of eggs poured off sea-water down to 2 cc. in each tube. To *A* added 6 cc. sea-water. To *B* added 6 cc. distilled water. Stirred several times until *B* was thoroughly laked.

Tests with fresh 1 per cent sperm

A. 3.40 P.M.	B. 4.05 TO 4.15 P.M.	B. 4.35 P.M.
	1/640— 6 sec. reaction	1/640— 6 sec. reaction
	1/320— 7 sec. reaction	1/320— 8 sec. reaction
	1/160— 10 sec. reaction	1/160—12 sec. reaction
1/80— 4 sec. reaction	1/80 — 36 sec. reaction	1/80 —25 sec. reaction (chemotaxis)
1/40— 8 sec. reaction	1/40 — 60 sec. reaction	1/40 —22 sec. reaction
1/20— 13 sec. reaction	1/20 —110 sec. reaction	1/20 —25 sec. reaction
1/10— 17 sec. reaction (1)	1/10 — 22 sec. reaction	1/10 —50 sec. reaction
1/1 —130 sec. reaction (1)	1/1 — 75 sec. reaction	1/1 —90 (?) sec. reaction

The second test of *B* indicated some progress in the neutralization of the fertilizin (cf. 1/20, 1/40, 1/80). The *B* dilution series is extremely irregular. Reading back from 1/1 (first test) the big jump from 1/10 to 1/20 indicates a breaking of the fertilizin X anti-fertilizin combination at this dilution.

August 24: Test showed some fertilizin present in *B*. Having poor sperm for indicator, left until next day. Shook up *B* vigorously; stirred *A*.

August 25: *B* now negative; *A* strongly agglutinative.

The distilled water extracts thus show the presence of a substance which combines quickly with the spermophile group of the fertilizin. In the experiment where the jelly was all removed by the preliminary shaking, there was practically immediate neutralization of the fertilizin. In the first and third, where the fertilizin was present in excess owing to the presence of some jelly, the excess was only slowly combined. Experiments with extracts of dried eggs (August 26) and with destruction of eggs by shaking or grinding also showed the presence of anti-fertilizin.

7. THE NECESSITY OF FERTILIZIN FOR FERTILIZATION

It will be apparent from the preceding facts and considerations that the fertilizin answers some of the requirements of a binding link between ovum and spermatozoon. When we consider the extraordinary activity of the unfertilized egg in its secretion, and the equally extraordinary avidity of the spermatozoa for it, one cannot escape the conviction that it must be a link in the normal fertilization process. When, moreover, one finds that the egg contains a more centrally located substance that can occupy the same combining group as the sperm, one seems to have before one, a view of a mechanism for preventing polyspermy.

I adopted, then, the working hypothesis that this substance is necessary for fertilization and there followed immediately three corollaries, viz: (1) If it were possible to extract this substance from eggs, they should no longer be capable of fertilization; (2) fertilized eggs are incapable of uniting again with spermatozoa, hence, if the hypothesis is correct, they could no longer contain free fertilizin; (3) eggs in which membranes have been formed by methods of artificial parthenogenesis become incapable of fertilization; such eggs must also, therefore, be devoid of free fertilizin after they have reached the non-fertilizable condition if the hypothesis is correct. These consequences of the theory were actually found to be true.

a. Fertilization of washed eggs

The only way of which I could think of extracting the fertilizin from the eggs without injury, is the method of repeated washings. But we have seen (tables 2 and 3) that it is actually impossible to remove all the fertilizin from a mass of eggs by any number of washings, for the eggs continue to produce it until they go to pieces. However, the quantity produced in successive washings slowly diminishes. Therefore, we might expect on the basis of the theory, that there would be a gradual reduction of the percentages of eggs fertilized after many repeated washings. This actually occurs, as table 4 shows. It might also be anticipated that as

the quantity of fertilizin decreases, the developmental energy of the fertilized eggs might decrease, owing to relative incompleteness of fertilization. This also occurs. In table 3 (p. 536) it will be seen that fertilizations were made at the times of the 11th, 16th, 27th and 31st washings. In each case about three drops of both egg-suspensions *A* and *B* were taken, including several hundred eggs and fertilized with considerable excess of fresh sperm. It will be recalled that the eggs of lot *A* had been deprived of their jelly by shaking, whereas those of lot *B* retained the jelly. *A* lost its fertilizin relatively rapidly therefore, at first, but later the repeated washings caused removal of all the jelly in *B* also.

Table 4 shows the fertilization results, as regards the percentage of eggs segmenting (200 eggs were counted in each case). There is thus the anticipated decrease in percentage of fertilizations. Moreover, the time between insemination and the appearance of cleavage increases very much, and the eggs cease after a while to produce fertilization membranes and the cells tend to fall apart.

TABLE 4

DATE		NUMBER OF WASHINGS	
August 21	4:06 P.M. Fert. 1.....	11	A 76.0% segmented B 97.5% segmented
August 22	9:13 A.M. Fert. 2.....	16	A 76.0% segmented B 92.0% segmented
August 22	4:19 P.M. Fert. 3.....	27	A 83.5% segmented B 97.5% segmented
August 23	9:13 A.M. Fert. 4.....	31	A 39.0% segmented B 30.0% segmented
August 23	11:30 A.M. Fert. 5.....	33	A 25.0% segmented B 24.0% segmented

Concomitantly, with these effects of the series of washings, the developmental energy becomes greatly reduced. This was very obvious from the second fertilization. On August 24 (48 hours after fertilization) a large quantity of living material was contained in the second *A* fertilization, but not one had even approximately pluteus structure. The most common form was a

stereoblastula. In the second *B* fertilization there were a few abnormal prismatic plutei, while the majority were gastrulae; some stereoblastulae. The third fertilization resulted in extremely abnormal ciliated types. The fourth and fifth did not proceed beyond abnormal cleavage stages.

The eggs have evidently lost something which affects their power of fertilization. Table 3 shows the measure of loss of the sperm-agglutinating substance, and justifies the general conclusion that this is a factor in the result. The loss of other substances may also combine in the decrease of fertilizing power, but of this we know nothing definite. As a matter of fact, fertilizing power is gradually lost with decrease of fertilizin content of the egg.

A considerable number of eggs go to pieces during the washings. This was especially noticeable in the *A* series of the above experiment. Thus after the 19th washing my notes record that eggs were stuck to the tube to the 3.5 cc. mark, whereas in *B* where the eggs were protected by jelly, only a very few stuck and these did not extend above the 1 cc. mark. Such eggs liberate antifertilizin, and if this is present in sufficient amount it may conceal the fertilizin production of the intact eggs. This phenomenon appeared a number of times in my earlier experiments when eggs were shaken too strongly in the effort to get rid of the jelly, resulting in injury to a large proportion of eggs, which soon broke down and neutralized the fertilizin produced by intact eggs.

One other experiment with apparently much more decisive results may be given (Aug. 18, 1913). In this experiment the shaking lasted 45 seconds and over 99 per cent of the eggs were stripped of jelly. The eggs were then precipitated by the centrifuge using just enough force to ensure complete precipitation, and the supernatant fluid poured off. It was very highly colored, showing that many eggs had been broken. It tested to a dilution of 1/12800, giving a 5 second reaction. The eggs were then repeatedly washed about 10/1.5 each time. The time of change and tests of supernatant fluids are shown in table 5, p. 552.

3:45 P.M. The fluid in 15 now gives a 6 second reaction, showing that some eggs are still producing fertilizin, but that it is rapidly neutralized.

After the 8th washing these eggs had extremely little capacity for fertilization, although only 24 hours old: Fertilization 1. 9:08 A.M. August 19: A few drops of stirred up egg suspension put in 9 cc. seawater and 3 drops 1 per cent sperm added. This is an enormous excess

TABLE 5

WASH- ING	TIME	TEST
2	2:48 P.M.	1/6400
3	3:15 P.M.	1/800
4	3:47 P.M.	1/100 (bulk of eggs 0.2 cc.)
5	4:05 P.M.	1/20
6	5:25 P.M.	1/10
7	Aug. 19: eggs had stood in tubes over night	
	8:37 A.M.	1/10 negative. 1/1—13 seconds
8	9:10 A.M.	1/10—11 seconds
9	9:42 A.M.	1/10—10 seconds
10	10:00 A.M.	1/10—(faint)
11	10:20 A.M.	1/1 — 11 sec. (strong chemotaxis)
12	10:47 A.M.	1/1 — 9 sec. (strong chemotaxis)
13	11:08 A.M.	1/1 — 5 sec.
14	11:53 A.M.	1/1 — 4 sec.
15	2:00 A.M.	1/1 — (negative)

of sperm. The eggs were rapidly literally covered with a single layer of adherent spermatozoa, but in spite of this only about 1 per cent divided (out of 200 eggs counted 3 were divided irregularly at 11:15). Some fresh eggs fertilized for control of sperm at same time showed practically all eggs divided at this time. Fertilization 2. 10:02 A.M. Same as 1, with control: 2:03 P.M. About 1 per cent divided; irregular, delayed; 80 per cent of control. Fertilization 3. 11:02. Same as 1, with control: 2:15 P.M. About 2 per cent divided; control 99 per cent divided.

Thus these eggs had almost entirely lost the capacity for fertilization with their loss of fertilizin, although they were only 24 hours in the sea-water. However, I regard the result given in the first experiment under this head as more typical for mere loss of fertilizin. In the experiment just quoted, the eggs had been injured to such an extent that they were liberating anti-fertilizin also, and I believe that this combined with the fertilizin in the cortex of the egg so as to block fertilization in this way. The extraordinarily rapid decrease of agglutinating power of successive washings (1, 12,800; 2, 6400; 3, 800; 4, 100; 5, 20; 6, 10; 7, 2 (?); 8, 10; 9, 10; 10, 10; 11, 1; 12, 1; 13, 1; 14, 1; 15, 0) is such as occurs only with injured eggs.

Other experiments give similar results. In one of these, the fertilizing power was reduced to about 1/8 in the third washing

after violent shaking which removed the jelly and in which the agglutinating power had been reduced from 400 to 10. It would appear from this that it makes no difference how rapidly we dispose of the fertilizin; the eggs are no longer capable of fertilization when it is gone.

b. Cessation of fertilizin production after fertilization

In a previous paper (Lillie '13) I noted that eggs of *Nereis* cease to produce sperm-agglutinating substance soon after fertilization, but that apparently this was not the case in *Arbacia*. I, however, ventured the suggestion that the apparent continued production of the sperm-agglutinating substance in *Arbacia* might be due to the presence of unfertilized eggs, of which there was a considerable percentage in the cultures. This may have had something to do with the results there described, but the factor that leads to an apparent continuation of fertilizin production in fertilized eggs is its presence in high concentration in the jelly, a condition that I, by no means, realized at first. When this factor was eliminated it was easy to demonstrate that fertilized eggs no longer produce the substance. The crucial experiments were as follows:

Experiment 1: August 23, 1913: A large quantity of unusually fine eggs were washed three times and fertilized at 2:30 P.M. with considerable excess of sperm. At 2:40 P.M. microscopical observation showed that nearly all had beautifully formed fertilization membranes. About 10 per cent of the eggs were without jelly (observation in ink). These usually had as fine membranes as the others.

The eggs were divided in two equal quantities in test tubes *A* and *B*. 2:50 P.M.: *B* was shaken 6 times to get rid of the jelly. Examination in ink showed that the jelly was removed completely from practically all the eggs. *A* (with jelly left on eggs) and *B* were then submitted to a series of washings with tests for fertilizin as shown in table 6, p. 554.

Thus in five washings the *B* fertilized eggs without jelly no longer gave the fertilizin test. This number of washings would be only barely sufficient to remove the agglutinating substance present before fertilization and the loose jelly. Two successive negative tests were given, showing that the production of the agglutinating substance has ceased absolutely. The *A* eggs were

TABLE 6

3:10 P.M.	A 1	Washed 8/2.0	
	B 1	Washed 8/1.0	
3:25 P.M.	A 2	Washed 8/1.5	
	B 2	Washed 8/1.0	
3:43 P.M.	A 3	Washed 8/2.5	
	B 3	Washed 8/1.0	Test 1/1—22 seconds
4:08 P.M.	A 4	Washed 8/2.0	
	B 4	Washed 8/1.0	Test 1/1— 9 seconds
4:53 P.M.	A 5	Washed 8/1.5	Test 1/1 over 75 seconds
	B 5	Washed 8/1.0	Test 1/1—negative
4.58 P.M.	A 5		Second test 1/1—60 seconds
	B 5		Second test 1/1—negative

apparently still producing it vigorously, but that is only because they carried the fertilizin-saturated jelly with them. Ninety-eight per cent of the *B* eggs segmented perfectly.

The result is in accord with the assumption that all free fertilizin is fixed *at the moment of fertilization*, or membrane formation. But I know of no way of absolutely demonstrating that the fixation takes place thus rapidly. But if such fixation is the mechanism for prevention of polyspermy, as I believe, the fixation of all free fertilizin must take place in a few seconds at most.

A duplicate experiment was made on August 25. The methods of Experiment 1 were repeated exactly. The eggs were not, however, so good a lot and the membranes did not stand put so far from the eggs. The shaking of lot *B* removed the jelly from most, but not from all. In this experiment tube *B* came negative on the sixth and seventh washings; whereas tube *A* gave a 90-second reaction, undiluted, on the sixth washing. Over 98 per cent of the eggs segmented and the unsegmented eggs had spindles.

Both experiments were clear-cut; there can be no question that after fertilization the eggs cease to produce fertilizin. Two effects are involved; the disappearance of fertilizin, and failure to produce more. The first effect might be due to extrusion of all fertilizin from the eggs; but it seems improbable that this could take place with sufficient rapidity to prove effective as a mechanism for preventing polyspermy, and inasmuch as we know that the eggs contain anti-fertilizin capable of occupying the spermo-

phile group of the fertilizin, it is more reasonable to conclude that the disappearance of sperm-agglutinating power from the washings of fertilized eggs deprived of jelly is due to neutralization of the substance remaining in the egg. In this connection it is instructive to note that eggs commonly lose pigment on insemination; I always have found the anti-fertilizin accompanied by pigment; it is, therefore, natural to suppose that anti-fertilizin is carried to the surface of the egg on insemination, thus affording the opportunity for combination with the fertilizin and its neutralization.

The other possibility that the sperm combines with all the free fertilizin in the egg appears to me inadmissible, because (1) the surface of the egg is relatively so enormous, and (2) the amount of fertilizin which the sperm is capable of binding is very small relative to the amount produced by the eggs even at a single washing (cf. sec. 3, p. 538). The bulk of the sperm actually used for insemination is insignificant compared to the bulk of the eggs, and only a small proportion of the sperm used actually fertilizes so that the amount fixed by the sperm itself is excessively minute compared to the quantity contained in the egg.

We have seen (p. 529) that 1 cc. of eggs charges 5 cc. of seawater to 1600 units. We have also seen that such eggs contain or produce many times this quantity in successive washings; and that it takes 1 cc. of 3 per cent sperm to fix 1 cc. of 64 power agglutinating solution. Thus it would require $1 \text{ cc.} \times 1600/64 \times 5$ or 125 cc. of 3 per cent sperm to neutralize the agglutinating substance produced by one washing of 1 cc. of eggs in 5 cc. of seawater. But a single drop of 3 per cent sperm would be much more than sufficient to fertilize the same amount of eggs. The disproportion is even greater than indicated in this rough calculation, which is, however, sufficient to show the absurdity of explaining neutralization of the agglutinating substance of the fertilized eggs by the sperm used in fertilization.

Theories of prevention of polyspermy, or of the non-fertilizable character of eggs already fertilized, proceed along two lines: (1) that the membrane formed on fertilization is impermeable to spermatozoa, (2) that the protoplasm of the fertilized eggs has

undergone some physiological' change which prevents union with the sperm. The first theory is an old one based on conditions in the sea-urchins; it is inapplicable to the ova of most animals, which do not form special membranes after fertilization; whatever value the fertilization membrane may have in special cases as guarding against polyspermy, it has been shown for some cases at least that the real cause of failure of refertilization is more deeply seated. Thus Wilson ('03) shows for *Cerebratulus* that after the germinal vesicle has faded, cytoplasmic fragments devoid of nucleus may be fertilized and undergo typical merogonic development; but enucleated fragments of fertilized eggs are incapable of refertilization: "Even when such fragments are placed in water containing sperm immediately after the section before they have assumed a spherical form, they fail to fertilize, though spermatozoa may be observed adhering to their periphery." Wilson rightly attributes the sterility of such fragments to a physiological change of the cytoplasm.

The theory of a physiological change of the cytoplasm following fertilization has been generally accepted, but there has been no idea of the nature of the change involved. Wilson directs attention to the similar sterility of egg fragments prior to fading of the germinal vesicle and suggests that the cytoplasmic change following fertilization is physiologically the opposite of that following the fading of the germinal vesicle. Now, as I have shown the probability that the agglutinating substance is not present in an active form in *Arbacia* prior to fading of the germinal vesicle, and that it becomes neutralized by union with anti-fertilizin following fertilization, it is clear that this mechanism answers the requirements of the problem. The fundamental mechanism for the prevention of polyspermy is the neutralization of the fertilizin by the anti-fertilizin present in the egg; i.e., the occupancy of the spermophile side-chain of the fertilizin by the anti-fertilizin.³

³ Note: Delage ('01) determined for *Asterias glacialis* that fragments of the ovocyte with intact germinal vesicle are sterile, so that merogony cannot be produced. As soon as the germinal vesicle fades fragments are fertilizable and merogonic development may follow. Wilson's observations are in accord with

The question may be raised why such neutralization of the fertilizin is delayed until the moment of fertilization? The answer to this difficulty is fairly clear. The fertilizin is located in the cortex of the egg, and the anti-fertilizin is more deeply situated; they therefore do not interact so long as the cell body as a whole is quiescent. But as soon as the cortical fertilizin becomes activated by union with the sperm it at once begins to attack certain substances in the egg, as demonstrated in the third part of this paper; this sets up diffusion evidenced by escape of pigment, and by cytoplasmic flowing, and the two substances are brought together and interact. While this explanation is partly hypothetical, the spatial separation of the fertilizin and anti-fertilizin and the quiescent character of the cell-body in the unfertilized egg are facts; so also are the movements of diffusion and the cytoplasmic currents set up on fertilization. However it is possible that some other factor is operative in the interaction of fertilizin and anti-fertilizin following fertilization, which has entirely escaped attention.

c. The cessation of fertilizin production after formation of membranes by butyric acid

Some methods of artificial parthenogenesis cause eggs to become non-fertilizable; others apparently do not. In the sea-urchins it would seem that those methods that cause membrane formation result in the non-fertilizable condition while those which do not, need not have such an effect. Thus the use of hypertonic sea-water causes a certain amount of parthenogenesis

this as noted. But Delage farther asserts that a second change occurs after the formation of the second polar globule so that non-nucleated fragments again become sterile, although entire eggs can still be fertilized. This result is unsupported so far as I know, and would be difficult to explain on the basis of the mechanism described in this paper except on the assumption that the original contribution of nuclear sap necessary for the production of fertilizin has become exhausted, and that presence of the nucleus had become necessary for continued production of fertilizin. Delage shows that during the fertilizable period of the cytoplasm when merogony can be effected parthenogenetic agents are especially effective, a result that agrees particularly well with my point of view.

in sea-urchin eggs without membrane formation but the development tends to be quite abnormal. Loeb has observed that membrane formation may be induced in such eggs after they have reached even the 8 or 16-celled stage by addition of sperm; they are still fertilizable, so far at least as the cortical changes are concerned. It is fair to assume in such a case that the lack of membrane formation is evidence of persistence of free fertilizin in the cortex, for the only cases in which I found this substance absent from mature eggs are eggs with membranes formed. This case, therefore, need offer no difficulty for the contention that the fertilizin is necessary for fertilization.

On the other hand, Loeb's method of treatment with fatty acids leads to membrane formation and such eggs are non-fertilizable, as Loeb states and I have myself observed. Loeb, however, goes on to assert that if such eggs are shaken so as to destroy the membrane and if sperm is then added, a new membrane is formed and the typical development of sperm-fertilized eggs follows. This would seem at first sight to run counter to my theory unless it could be shown that membrane-formation by butyric acid differed from membrane formation by sperm in not causing cessation of fertilizin production. However, as I shall show immediately, the production of fertilizin does cease after the formation of good membranes by butyric acid.

It is necessary, therefore, to examine Loeb's statement rather carefully. It is as follows:

Dr. Kupelwieser und ich stellten diesen Versuch an, indem wir die Membranbildung bei unbefruchteten Eiern mittels Buttersäure hervorriefen, diese Membran dann *unmittelbar nach Bildung derselben*, durch Schütteln der Eier zum Platzen brachten und sofort Samen zufügten. Die Eier bildeten eine neue *dem Zytoplasma dicht anliegende Membran* und furchten sich mit einer der Samenbefruchtung entsprechenden Geschwindigkeit, und Zwar zunächst ausnahmslos in zwei Zellen. (Loeb '09, p. 210; italics mine).

It is implied that such superposition of fertilization on parthenogenesis would not take place unless the experiment was performed immediately after membrane formation. Presumably some fertilizin remained unbound for a short time and on account

of its relatively small amount the new membrane was formed close to the egg. The implied fact that the experiment failed after a short time supports my theory of the function of fertilizin, and the stated result is not at all inconsistent with my views. According to my point of view the possibility of superimposing fertilization on parthenogenesis would persist so long as a sufficient quantity of fertilizin remained unbound, i.e., so long as the reaction was incomplete.⁴

The non-fertilizable character of such eggs implies on my theory an absence of free fertilizin. This condition obtains, as the following experiments show:

Experiment August 26, 1913: A quantity of fresh *Arbacia* eggs were taken and some were set aside in a test tube as control (0.14 cc. when settled). The balance were concentrated in 3.4 cc. sea-water.

3:47 P.M. The latter were then added to 25 cc. butyric acid in sea-water made by adding 2.8 cc. $\frac{N}{10}$ butyric acid to 50 cc. sea-water.

3:47 1/2 P.M. Half of the eggs with acid were poured into 1500 cc. sea-water in crystallization dish *A* and stirred up to stop the action of the acid.

3:48 P.M. The remainder were poured into 1500 cc. sea-water in crystallization dish *B* and stirred up. *B* was over-exposed and the eggs agglutinated heavily. In *A* there was no agglutination and nearly all of the eggs formed fine membranes. The jelly was absolutely all gone owing to the action of the butyric acid, so there was no need to shake them.

The eggs in *A* and *B* were then concentrated, and placed in graduated tubes similar to the control. When settled there was 0.3 cc. eggs in each, estimated to be at least 4 times as many as in the control tube when allowance is made for the bulk of the jelly in the control. All were then submitted to successive washings with tests as shown in table 7, p. 560.

Thus the *A* eggs, with membranes formed, came negative in four washings, at least as rapidly as fertilized eggs, whereas the unfertilized control with fewer eggs was still showing an agglutination strength of about 10 (control 4, 1/1—50 seconds). There

⁴ The essential problem is whether fertilization is of the nature of an irreversible chemical reaction, as I claim, or is merely a physical surface effect. Although this question has been investigated a good deal, it is still far from being definitively settled. The students of artificial parthenogenesis take the latter point of view generally.

TABLE 7

	WASHINGS	TESTS FOR FERTILIZIN
4:13 P.M.		
Control 1	10/1.2	
4:30 P.M.	A 1 12/2.4	Test 1/10—11 seconds
	B 1 12/1.5	
Control 2	11/2.0	Test 1/1 —2 plus min.
4:42 P.M.	A 2 13/2.0	Test 1/1 —32 seconds
	B 2 13/1.5	Test 1/1 —45 seconds
4:50 P.M.		
Control 3	10/1.0	No test
4:55 P.M.	A 3 13/2.0	Test 1/1 — 7 seconds
	B 3 13/2.0	Test 1/1 —11 seconds
5:05 P.M.		
Control 4	10/1.0	Test 1/1 —50 seconds
5:08 P.M.	A 4 12/2.0	Test 1/1 negative
5:13 P.M.	B 4 12/1.5	Test 1/1 — 9 seconds
Later	A 5 about the same	Test 1/1 —negative
	B 5 as above	Test 1/1 — 8 seconds

is no doubt, therefore, that in eggs with membranes formed by butyric acid the agglutinating substance is fixed, as it is in fertilized eggs. Presumably, the same would hold true for other parthenogenetic methods which render the eggs incapable of fertilization.

The next day the washings were continued. On the sixth, washing *A* again showed a trace of the agglutinating substance, but came negative again on the remaining three washings. The reappearance is to be attributed to the few eggs without membranes which had been active all night, accumulating in this time sufficient agglutinating substance to show in the test though not producing sufficient to show in repeated washings. The *B* eggs came negative in the ninth washing, while the control eggs still gave a 40-second reaction undiluted.

The *B* eggs although exposed longer to the butyric acid did not form membranes, and they continued to produce fertilizin longer than the *A* eggs but in much less quantity than the control eggs. It might therefore, be expected that they would retain capacity for fertilization longer. The fertilization capacities were not

worked out in this experiment; however, Herbst ('00) found in his experiments on superposition of fertilization on parthenogenesis that eggs overexposed to butyric acid could be fertilized, though incompletely.

Ninety-five per cent of the *A* eggs showed no evidence of cytolysis on the next day, beyond the membrane formation. They were intact and healthy looking. The remaining 5 per cent were fragmented, or segmented, into about 16 to 32 cells, within the membrane and appeared cytolized. In the *B* eggs the agglutination had disappeared, and there was about the same percentage of dividing eggs as in *A*, but in absence of a membrane the cells were breaking apart.

A similar experiment on August 25 gave the same result, viz., disappearance of the agglutinating substance after membrane formation by butyric acid.

Summarizing this section, then, we may say that eggs are rendered incapable of fertilization by methods that eliminate the sperm-agglutinating substance whether (a) the substance is lost, as in some eggs after repeated washings, or (b) fixed by previous fertilization, or (c) fixed by parthenogenetic methods. The conclusion is therefore justified that the agglutinating substance is necessary for fertilization.

8. SUMMARY OF PART II: SPERMOPHILE GROUP OF THE FERTILIZIN

After considering a method for determining the quantities of fertilizin produced by ova of *Arbacia*, we demonstrated (1) that unfertilized eggs in sea-water produce this substance in extremely large quantities. The secretion begins presumably in the ovary with breaking of the germinal vesicle. Although the jelly is saturated with it and liberates it by solution in the sea-water, eggs without jelly continue to secrete if for three days, at least, during repeated washings, or as long as they remain alive. (2) The spermatozoa of *Arbacia* possess extreme avidity for the fertilizin and bind it in definite amounts. In the case of fresh active sperm the binding is evidenced by reversible agglutination of the spermatozoa. But a stale sperm suspension may also bind it, although

the agglutination does not take place, except in high concentrations. (3) The fertilizin of *Arbacia* eggs does not combine with *Nereis* sperm. (4) A substance, which I have named anti-fertilizin, may be extracted from *Arbacia* eggs, by breaking them up or by extracting them in distilled water, which has the property of combining with the spermophile group of the fertilizin. It is present in sufficient amount to neutralize all the fertilizin contained in the eggs, excluding the jelly. (5) When the fertilizin is extracted from the eggs by repeated washings, they are no longer capable of fertilization. (6) Fertilized eggs produce no more fertilizin; that present at the moment of fertilization is neutralized by anti-fertilizin. (7) Eggs with membranes formed by butyric acid become incapable of fertilization, and they contain no free fertilizin.

III. THE OVOPHILE SIDE-CHAIN

INTRODUCTION

Having shown then, that the union of the agglutinating substance with the spermatozoon enters in some significant way into the process of fertilization, the problem was to ascertain in what way. The simplest idea (*viz.*, that the union is itself the fertilization process) was soon shown to be untenable, for the reason that the perivisceral fluid (blood) of the sea-urchin especially of ripe males and females, often contains a substance which absolutely inhibits fertilization in the presence of any quantity of sperm, but which has no inhibiting effect at all upon the sperm-agglutinating reaction. It does not enter into combination with the spermophile side-chain. In other words, the binding of the agglutinating substance by the sperm may be complete, but in the presence of an inhibitor contained in the blood none of the usual effects of insemination, no matter how heavy, follow.

The next suggestion was fairly obvious, *viz.*, that the substance which we have been calling the agglutinating substance on account of its effect on the spermatozoa, in reality possesses two side-chains active in fertilization, *viz.*, a spermophile and an ovophile side-chain; the binding of the sperm activates the ovo-

phile combining group of the fertilizin which then seizes upon egg receptors, and it is the latter union which results in membrane formation. If this were so, it is obvious that the spermatozoon is only secondarily a fertilizing agent, in the sense of initiating development, and that the egg is in reality self-fertilizing, an idea that agrees well with the facts of parthenogenesis and with the amazing multiplicity of means by which parthenogenesis may be effected. For the agents need only facilitate the union of the fertilizin and egg receptor.

The inhibiting action of the blood, from this point of view, is a deviation effect due to occupancy of the ovophile group of the fertilizin, either because the inhibitor in the blood is an antibody to the fertilizin or because it possesses the same combining group as the egg receptor. In such a case, the ovophile group of the fertilizin being already occupied by the inhibitor, fertilization could not take place.

This theory transfers the fertilizing power from some hypothetical substance contained in the spermatozoon to a definite substance contained in the egg itself, in relation to which the sperm acts merely as an activator. It does not necessarily predicate the precise mode of action of the fertilizing substance although the assumption is here made that chemical union with certain molecules in the egg, called egg receptors, is a necessary part of its action.

In the present section we shall proceed to examine the evidence for the existence of an ovophile as well as a spermophile combining group of the fertilizin.

1. THE INHIBITING EFFECT OF BLOOD OF THE SEA-URCHIN UPON FERTILIZATION OF EGGS OF THE SAME SPECIES

It is a common practice of embryologists to wash the eggs of marine invertebrates, which they propose to fertilize, twice or several times with sea-water, because it is a matter of experience that this procedure very greatly increases the proportion of eggs that fertilize. However, I know of no study of this really very striking phenomenon. The eggs are usually obtained for fertili-

zation by opening the animal and removing the ovaries so that the eggs are contaminated by blood or tissue secretions, and it is evident that the latter in some way inhibit the fertilization reaction; though they could hardly be supposed to have any direct injurious effect upon either sex element.

The necessity of washing the eggs carefully before fertilization, had been forced upon my attention by the poor results of certain experiments; and after some of the results recorded in the first part of the paper had been obtained, the phenomenon appeared to possess great significance, because the failure to fertilize in the case of unwashed eggs was clearly due to the presence of blood of the species. Some element of the blood must block the mechanism of fertilization, and it was clear that the analysis of the phenomenon must aid in understanding the mechanism.

The first experiments were undertaken to ascertain the extent of the inhibiting action of the blood of the sea-urchin. The nature of the effect may be seen from the protocol of the following experiment:

July 7, 1913: The blood of several males and females was obtained by cutting open the shell near the oral membrane and pouring out the perivisceral fluid (blood) in a finger-bowl. After the usual loose clot was formed the plasma was filtered through a soft filter-paper. The eggs used in the experiment came from one female and were washed in sea-water. The experiment was then set up as follows:

- 1 Quantity of eggs x plus 25 cc. sea-water plus 4 drops 1 per cent sperm
- 2 Quantity of eggs x plus 12.5 cc. filtered blood plus 12.5 cc. sea-water plus 4 drops 1 per cent sperm
- 3 Quantity of eggs $2/5$ X plus 10 cc. filtered blood plus 4 drops 1 per cent sperm

The fertilizations were made about the same time and the result was that in 1 practically all divided, in 2 only a fraction of 1 per cent divided, in 3 none divided. Moreover in 2 and 3 no fertilization membranes were formed in the undivided eggs and the egg nucleus remained intact; no spindles were formed. And this in spite of the fact that the spermatozoa were very active, so abundant that they formed 'halos' around the eggs by penetrating the jelly, and many reached the surfaces of the eggs.

This experiment was followed by a considerable number carried out in more detail. Four of these experiments are tabulated in table 8. The left-hand column gives the percentage of blood in

the sea-water in which the fertilizations were made. The other vertical columns give the percentages of eggs segmented for each concentration of blood on the same horizontal level. The last reading in each column gives the control, i.e., fertilizations made in sea-water alone with the same amounts of the same eggs and sperm used in the blood solutions. The eggs in the blood solutions were washed in the same way as the controls prior to insemination.

TABLE 8

	JULY 8: MIXED BLOOD OF MALES AND FEMALES	JULY 15: BLOOD OF MALES ALONE	JULY 22: BLOOD OF MALES ALONE	
Blood serum in sea-water	Segmented eggs			
		Exp. 1	Exp. 2	
1 per cent	75 per cent	90 per cent	90 per cent	89 per cent
3 per cent		25 per cent	60 per cent	21 per cent
5 per cent	10 per cent	10 per cent	30 per cent	0
7 per cent		1 per cent	30 per cent	6 per. cent
9 per cent		25 per cent	45 per cent	19 per cent
10 per cent	about 0.2 per cent	50 per cent		
11 per cent			20 per cent	0
13 per cent			20 per cent	0.1 per cent
15 per cent			1.5 per cent	0
20 per cent	about 0.2 per cent			1.75 per cent
40 per cent	about 0.2 per cent			
100 per cent				0
Control: same eggs in sea- water.....	75 per cent	99 per cent	95 per cent	99 per cent

It will be noticed that in all these cases there is a marked reduction in the percentage of fertilizations when 5 per cent blood is present in the sea-water, greater in some cases than in others, which leads to the conclusion that the inhibitor is present in higher concentrations in some samples of blood than in others. This relation is dealt with below. In Experiments 1 and 2 of July 15, and in the experiment of July 22, there will be noticed after the first fall with increasing blood-concentration a rise and second fall in the percentages of fertilizations as measured by cleavage of the eggs. This is unquestionably of some significance, but I have been unable to ascertain what its meaning may be. The same phenomenon occurred in later experiments.

While the experiments cited give effects only of mixed blood from the two sexes, and the blood of the males alone, other experiments, not so complete in all details, showed that females contain the inhibitor in their blood at least to the same degree as the males. If there is any sexual difference it is in favor of the females. The blood of the males was used in many tests alone because there was danger, in opening the females, of wounding the ovaries and so getting fertilizin into the blood, which as shown beyond, greatly modifies the results. There was of course the danger in using blood from males of getting spermatozoa from wounded testes, but as the eggs were in any case to be fertilized the result could hardly be affected by this; some control experiments showed that this was a rare occurrence in any event.

Individual variability in the amount of the inhibitor present in the blood being indicated in these experiments, a special study was made of this factor in order to test its extent, and if possible to correlate it with the condition of the animals. The individual tests were as follows (table 9): the columns give the percentage of eggs segmented after 2 to 3 hours. All the fertilizations of July 28 were made with a single sperm suspension and a single lot of eggs; similarly for each succeeding date. The various numbers therefore act as controls on one another. In all, a great excess of sperm was used. The counts were carefully made and checked by my assistant, Miss Brockett.

These tests of bloods of individuals bring out very extreme individual differences in the content of fertilization inhibitor in the blood, ranging in fact all the way from no inhibition to absolute inhibition of fertilization. But it is not easy to correlate the variations with the conditions of the individuals. The general hypothesis that I have been led to adopt, provisionally at least, is that the quantity of the inhibitor in any individual blood is related to the size and degree of maturity of the gonads. It would seem to be a very simple matter to determine whether this is or is not the case. However, in the first place, after the beginning of the season there are practically no animals with immature gonads to test, and in the second place, if the hypothesis were correct, it would be impossible to know in advance how long the

TABLE 9

MALES—20% BLOOD		FEMALES—20% BLOOD		FEMALES—100% BLOOD
July 28				
1	65 per cent	1	75 per cent	11 per cent
2	69 per cent	2	93 per cent	84 per cent
3	71 per cent	3	72 per cent	11 per cent
4	72 per cent	4	45 per cent	1 per cent
5	85 per cent	5	85 per cent	84 per cent
6	82 per cent	6	87 per cent	63 per cent
7	27 per cent	7	89 per cent	81 per cent
8	84 per cent	8	52 per cent	0
		9	80 per cent	24 per cent
		10	92 per cent	93 per cent
		11	6 per cent	2 per cent
		12		55 per cent
		13		0
		14		most
		15		over 90 per cent (rough estimate)
MALES—100% BLOOD		FEMALES—100% BLOOD		
July 29				
9	100 per cent	16		77 per cent
10	85 per cent	17		39 per cent
11	56 per cent	18		100 per cent
12	87 per cent	19		0
13	43 per cent	20		100 per cent
Control 97 % (fertilized in sea-water)				
July 30				
14	2 per cent	21		35 per cent
15	25 per cent	22		100 per cent
16	29 per cent	23		15 per cent
17	93 per cent	24		65 per cent
18	93 per cent	25		19 per cent
19	100 per cent	26		99 per cent
		27		100 per cent
Control 100 per cent (fertilized in sea-water)				
July 31				
20	96 per cent	28		0
21	2 per cent	29		1 per cent
22	74 per cent	30		0
		31		38 per cent
		32		1 per cent
		33		41 per cent
		34		41 per cent
Control 94.5% (fertilized in sea-water)				
		35		89 per cent
		36		34 per cent

inhibitor might remain in the blood after the gonads were emptied. Thus the mere fact that an individual with small gonads had much inhibitor in its blood would not prove that the gonads had little to do with the formation of the inhibitor, for there would be no way of telling whether the reduction of the gonads was recent or not.

The considerations that led to the adoption of the above hypothesis are (1) that the condition of the gonads is the most variable thing in the summer sea-urchins, which coincides well with the variability of the amount of the inhibitor; (2) in general, the fluctuation through the season of periods of maturity of the sea-urchins coincides very well with the fluctuations in the amount of the inhibitor. Thus the animals were in very fine breeding condition during the first three weeks of July when the first tests showing abundance of the inhibitor in the blood, were made. They were in very poor condition then for about 10 days up to about August 2, when the individual tests were made showing such surprising decrease of the inhibitor. On August 6 when the material became better again, the inhibiting effect of the blood was stronger once more. (3) Although it was difficult, as said, in the individual tests to correlate the amount of the inhibitor with the condition of the animals, yet especially in the case of the females, those with large gonads appeared to have a large amount of inhibitor present. Thus females 28, 29, 30 and 32 were all ripe females that shed eggs when inverted after opening, in a watch crystal, whereas females 31, 33, 34, 35 and 36 were all individuals with medium size ovaries that shed no eggs when similarly inverted. Female 8, on the other hand, had small ovaries, shed no eggs, and yet possessed much inhibitor in its blood.

The conditions that determine the amount of inhibitor in the blood are thus a matter for more careful investigation. It may be possible to regulate the quantity experimentally by injection of fertilizin or of entire eggs. This merely indicates a possible method of approaching the problem.

I spent a great deal of time in endeavoring to meet the possible objection that the inhibiting action of the blood might be due to

contamination with the poisonous secretions of the epidermis, especially of pedicellariae. It seemed at first that this objection might be valid, for the period of my tests coincided with the season of poor material when the blood was relatively free from inhibitor. During this period, instead of following my earlier practice of opening the animal rapidly and pouring out the blood, I very carefully cut within the leathery peristome, avoiding all possible contamination with epidermal secretions and removed the blood with pipettes. This blood showed as a matter of fact little inhibiting effect. However, with the return of animals in good breeding condition, it was easy to show that the inhibitor was contained in the blood itself; and not only this, but also that epidermal secretions so far from increasing the inhibiting action of the blood, actually decreased it, a matter to which we return beyond.

The next question was whether the inhibitor acted on the egg alone, on the sperm alone, or merely by intervening in the reaction between the two? In the experiments undertaken to answer this question it was possible to show that the inhibitor certainly does not act strongly on either the egg or the spermatozoa alone. It is possible to collect eggs or sperm in filtered blood known to have a strong inhibiting action, and, after considerable exposure, to restore by washing nearly full fertilizing power, at least, to both kinds of sexual elements.

July 8: The blood used in this experiment allowed only 1 per cent fertilization when diluted to 5 per cent with sea-water; it therefore contained a great deal of the inhibitor.

1. As regards the effect on eggs alone: 9:41 A.M. The eggs of one female were placed direct from the ovary in some of this filtered blood. Transfers of two drops of eggs to 10 cc. of sea-water plus one drop 1 per cent sperm were made as follows: a. 9:42, b. 9:50, c. 9:55, d. 10:03, e. 10:23. About 60 per cent of each lot segmented. The blood carried over would be about 0.625 per cent. Thus the inhibiting effect was removed to a very great extent by washing. 2. In the case of sperm collected in the same blood and used for fertilization at 1 minute, 12 minutes, 22 minutes, and 37 minutes, no indication of inhibition of fertilizing power as compared with an identical series of controls was observed.

July 17: After 5 minutes' exposure to male blood sperm fertilized 20 per cent of the eggs; control sperm 90 per cent. (sperm = 0.004 per cent).

July 18: Sperm suspensions in two lots of blood, (a) from males, (b) from females. (a) Fertilized 40 per cent in 5 minutes and 25 per cent in 18 minutes, (b) fertilized 30 per cent in 5 minutes and 20 per cent in 18 minutes. Control sperm fertilized 70 per cent each time. (Sperm = 0.0026 per cent)

July 19: Sperm suspensions in two lots of blood, (a) from males, (b) from females. (a) Fertilized 14 per cent in 10 minutes and 18 per cent in 30 minutes; (b) fertilized 23 per cent in 10 minutes and 20 per cent in 30 minutes. Control sperm fertilized 86 per cent in 10 minutes and 76 per cent in 30 minutes. (Sperm = 0.012 per cent).

July 21: Sperm suspensions in blood of males. In this case the sperm fertilized perfectly in two fertilizations. Sperm = 0.007 per cent in first and 0.0068 per cent in second. The blood contained a large amount of inhibitor as only 0.5 per cent segmented in it with 0.6 per cent sperm suspension.

August 1: A very detailed experiment showed that a specimen of blood very powerful in inhibitor had but little effect on the fertilizing power either of eggs or of sperm after washing.

On the whole we must conclude that though exposure to blood containing inhibitor may decrease the fertilizing power of sperm or of eggs, its effect is secondary and can probably be entirely removed by sufficient washing or overcome by sufficient concentration of sperm.

2. MODE OF ACTION OF THE INHIBITOR

It was natural to suppose that the inhibitor acted by occupying the side-chain of the fertilizin with which the sperm unites normally. If this were the case it must be possible to neutralize the agglutinating action of the fertilizin by a sufficient quantity of blood containing the inhibitor. Therefore, if we took two equal amounts of an agglutinating solution and diluted the one with sea-water and the other with blood containing the inhibitor, the agglutinating action ought to disappear more rapidly in the latter than in the former. This, however, is not the case, as the following experiment will show.

July 11, 1913: A given agglutinating solution was tested in the usual way by diluting with sea-water and found to be of 400 agglutinating power. Part of it was then diluted to 1/100 with blood; on test with sea-water sperm suspension it gave two tests of 22 seconds each. Diluted to 1/400 with blood, it gave a 10 second reaction with a sea-water sperm suspension, and an 11 second reaction with a blood sperm suspension.

The same result was obtained in other experiments and I could find no evidence that blood decreased the delicacy of the agglutinating reaction.

If then the fertilizin is essential for fertilization, and if the inhibitor in the blood does not operate by preventing union of this substance with the sperm, it follows that another side-chain of the fertilizin capable of being occupied by the inhibitor is also operative in fertilization. There must be action of the fertilizin on the egg as well as on the sperm, and hence we may assume an ovophile as well as a spermophile side-chain of the fertilizin, and the inhibitor must act by occupying the former.

Now Robertson ('12) has stated that various proteins when added to the sea-water can be shown to inhibit membrane formation by sperm, though as a matter of fact his experiments demonstrate such a result only for ovomucoid in concentrations above 0.25 per cent. It might therefore be supposed that the inhibiting effect of blood of the same species was only a special case of protein inhibition. There are, however, considerations that show such a view to be untenable. (1) As already shown the degree of inhibition by undiluted blood ranges all the way from zero to 100 per cent; and we cannot suppose that the amount of protein in the blood of different individuals varies to the extent required to explain such an extraordinary difference. (2) In the second place the mode of neutralizing the inhibitor, as shown below, involves an enormous increase of colloid content of the blood, for it consists in saturating the blood with fertilizin and dissolved jelly; the blood may thus become actually syrupy in consistency and yet have no inhibiting effect on fertilization. (3) I have tested the effect of egg-albumen in the sea-water up to 0.15 per cent without finding any inhibition of fertilization. Of course in higher concentrations a certain degree of inhibition may occur. However the reasons given under 1 and 2 above, certainly exclude the idea that we are dealing with an effect common to colloids in general, which must be assumed to operate through changes in the membrane. It is necessary to assume some specific substance acting as inhibitor.

Fortunately the idea that the inhibitor operates by occupying an ovophile side-chain of the fertilizin is capable of a ready test, for then it should be possible to neutralize the inhibiting effect of the blood, which now becomes ex. hyp. a deviation effect, by treating the inhibiting blood with free fertilizin. This substance should bind all molecules of the inhibitor so that they could no longer exert any deviating effect, their bonds for fertilizin being already occupied. This experiment succeeds perfectly as a matter of fact.

Before citing the experiments in detail, I would like to dwell a little more on their significance. In the first place a negative result would go far to overthrow the entire theory, for then it would become necessary to assume, if the theory were to be retained, that the combination of fertilizin and inhibitor could take place in the egg but not in the test tube, an exceedingly improbable assumption. The positive result, actually obtained, is in accord with the theory, and it is exceedingly difficult to frame any other interpretation of such a result. The alternative point of view would be to postulate a neutralization of a hypothetical membrane effect of the inhibitor, a conception exceedingly difficult to support as we have seen, and now rendered doubly difficult through the neutralization of such effect by excess of a substance which by itself in high concentrations actually reduces the percentage of fertilizations. Such an alternative interpretation appears to me impossible.

The experiments

July 21, 1913: The following substances were prepared: (1) Fresh filtered blood of male sea-urchins. (2) Filtrate from 15 cc. sea-water plus 7.5 cc. ripe *Arbacia* eggs, i.e., an agglutinating solution in sea-water, which gave an 8 second reaction with fresh sperm at 1/1600 dilution. (3) Filtrate from 15 cc. of the same male blood as in 1, plus 7.5 cc. eggs; i.e., part of the blood (1) saturated with the egg secretions. It gave a 10 second agglutinating test at 1/1600 dilution.

Two drops of fresh sperm (50 per cent) were then stirred into 5 cc. of each of the above and into (4) 5 cc. sea-water for control. These sperm suspensions prepared at 10:50 A.M. were thus about 0.6 per cent. One drop of double washed eggs was then added to each sperm suspension 1-4 at 11:45.

TABLE 10

		SEGMENTED EGGS
(1)	0.6 per cent sperm suspension in blood.....	0.5 per cent
(2)	0.6 per cent sperm suspension in 1600 agglut. power sea-water.....	99.0 + per cent
(3)	0.6 per cent sperm suspension in 1600 agglut. power blood.....	100.0 per cent
(4)	0.6 per cent sperm suspension in sea-water (control)...	100.0 per cent

This experiment was unusually successful; the eggs and sperm were perfect, giving 100 per cent segmentations (4). The blood contained so much inhibitor that only 0.5 per cent of the eggs segmented (1). But its action was entirely neutralized by the addition of the agglutinating substance (3). Fertilization 2 shows that in the presence of such an excess of sperm, the agglutinating substance itself has but little inhibiting power.

In a similar experiment of July 19, 0.9 per cent sperm suspensions were made in solutions 1 to 6 below, and a drop of eggs added to each with results noted in table 11.

TABLE 11

	FERTILIZATIONS MADE IN	PERCENTAGE OF SEGMENTED EGGS
(1)	Filtered blood of male sea-urchins.....	0
(2)	Filtered blood of female sea-urchins.....	0
(3)	800 power agglutinating solution in sea-water.....	93%
(4)	1600 power agglutinating solution in mixed blood of males and females.....	73%
(5)	Mixture of equal parts of 1 and 3.....	0
(6)	50 % dilution of 1 with sea-water.....	0
(7)	Sea-water control.....	97%

1, 2 and 6 show the inhibiting power of the blood on fertilization even with such enormous excess of sperm; 4 shows neutralization of the inhibiting power by a large quantity of the agglutinating substance although the neutralization was apparently not complete (cf. control); 5 shows that the agglutinating substance contained in an equal part of 800 power sea-water solution was inadequate to neutralize the inhibitor in this sample of blood; a considerable part of the inhibitor must have remained free; it must be present therefore, in high concentration.

Experiments were then made to test the neutralization of the inhibiting power of the blood at various dilutions.

July 22: Used filtered male blood which permitted only 0.5 per cent fertilization. Part of this was saturated with agglutinating substance by addition of 50 per cent by bulk of eggs, and filtered. Fertilizations were then made in a series of sea-water dilutions of each, using a single lot of eggs and of sperm (table 12).

TABLE 12

A SERIES—BLOOD ALONE			B SERIES—BLOOD SATURATED WITH AGGLUTINATING SUBSTANCE ABOUT 6400 STRONG		
Fertilizations in 0.1% sperm suspensions in		Per cent segmented	Fertilizations in 0.1% sperm suspensions in		Per cent segmented
(1)	1% blood	89.0%	(1)	1%	99.0%
(2)	3%	21.0%	(2)	3%	89.5%
(3)	5%	0	(3)	5%	97.0%
(4)	7%	6.0%	(4)	7%	94.5%
(5)	9%	19.0%	(5)	9%	93.5%
(6)	11%	0	(6)	11%	94.0%
(7)	13%	0.1%	(7)	13%	87.5%
(8)	15%	0	(8)	15%	91.0%
(9)	20%	1.75%	(9)	20%	71.0%
(10)	100%	0	(10)	100%	3.0%
Control in sea-water 99%					

It will be seen that this sample of blood is very powerful in inhibitor (A series), and that the agglutinating substance protects from its action at every concentration. The decreases in percentage of segmented eggs in B9 and B10 are to be attributed probably to excess of the agglutinating substance, which was exceedingly concentrated, and which always has some diminishing effect on percentage of fertilizations when the sperm suspension is not too concentrated. In this experiment it will be noted that the agglutinating substance was 6400 strong as contrasted with 1600 on July 21, and the sperm suspension was six times more dilute.

July 25: (A) Used filtered blood of females; (B) part of A saturated with agglutinating substance by addition of eggs; tested 3200 strong. Fertilizations were made in 0.2 per cent sperm suspensions in the following dilutions of each, with per cent of segmented eggs as noted, (table 13):

TABLE 13

A SERIES			B SERIES		
	DILUTIONS	PERCENTAGE OF SEGMENTED EGGS		DILUTIONS	PERCENTAGE OF SEGMENTED EGGS
(1)	10%	17%	(1)	10%	73.5%
(2)	40%	5%	(2)	40%	58%
(3)	70%	0	(3)	70%	40%
(4)	100%	0	(4)	100%	6%
(5)	Control in sea-water 58.5%				

The same protective action of the agglutinating substance is shown, with some decrease at the higher concentrations due, presumably, to excess of the agglutinating substance. The fertilization B-1 actually is better than the control.

August 6: This experiment was made up of a series of combinations of blood *A* plus fertilizin-saturated blood *B*. Fertilizations made in 0.2 per cent sperm suspensions in each combination.

TABLE 14

(A) Filtered serum of males and females
 (B) 23 cc. *A* plus 11.5 cc. ripe eggs. Centrifuged after 10 min. and filtered; sperm-agglutination test, $1/3200=9$ sec.

	FERTILIZATIONS IN	PERCENTAGES OF SEGMENTATION; 400 OF EACH COUNTED, 200 BY MISS BROCKET, 200 BY SELF
	5.0 cc. <i>A</i> + 0 <i>B</i>	
(1)	4.9 cc. <i>A</i> + 0.1 cc. <i>B</i>	0.5%
(2)	4.8 cc. <i>A</i> + 0.2 cc. <i>B</i>	0.5%
(3)	4.7 cc. <i>A</i> + 0.3 cc. <i>B</i>	1.75%
(4)	4.6 cc. <i>A</i> + 0.4 cc. <i>B</i>	8.5%
(5)	4.5 cc. <i>A</i> + 0.5 cc. <i>B</i>	25.0%
(6)	4.4 cc. <i>A</i> + 0.6 cc. <i>B</i>	32.75%
(7)	4.3 cc. <i>A</i> + 0.7 cc. <i>B</i>	31.75%
(8)	4.2 cc. <i>A</i> + 0.8 cc. <i>B</i>	72.0%
(9)	4.1 cc. <i>A</i> + 0.9 cc. <i>B</i>	62.25%
(10)	4.0 cc. <i>A</i> + 1.0 cc. <i>B</i>	85.0%
(11)	3.0 cc. <i>A</i> + 2.0 cc. <i>B</i>	82.75%
(12)	2.0 cc. <i>A</i> + 3.0 cc. <i>B</i>	80.5%
(13)	0 <i>A</i> + 5.0 cc. <i>B</i>	50.0%
(14)		30.25%
Control	in sea-water	89.5%

The protective action of the agglutinating substance gradually rises up to number 10 and then falls off, owing, presumably, to excess of agglutinating substance.

I have given a considerable number of the experiments because the matter under consideration is of great significance. There can be no question that some substance derived from the eggs themselves protects against the inhibiting substance in the blood. Until some method is devised for obtaining the agglutinating substance pure, it is impossible to meet entirely the objection that it may be some substance other than the agglutinating substance that protects against the inhibitor. The agglutinating substance can, however, be demonstrated in high concentrations, as the protocols of the experiments show. Until some reason for a contrary assumption is shown, the neutralization of the inhibitor

must be attributed to this, which is indeed the only assumption consistent with the other known facts.

Now if the agglutinating substance protects from the inhibitor by occupying its combining group, it would be logical to expect to find in some tissue of the species another kind of molecule possessing an identical side-chain and hence equally capable of protecting against the inhibitor. Such a substance was found in the shell but not in the intestine. A sample experiment may be cited.

August 2, 1913: A large quantity of blood was collected from females, and the serum filtered. (A) One part set aside. (B) A second part was saturated with the agglutinating substance by addition of eggs; *B* tested to 1/3200; (C) To a third part was added a large quantity of the shell and peristomes of the sea-urchins used: *B* and *C* filtered again.

Fertilizations were then made in 0.2 per cent sperm suspensions in identical concentrations of each in sea-water as shown in table 15.

TABLE 15

	DILUTIONS OF SERA	PERCENTAGES OF EGGS SEGMENTED		
		A	B	C
(1)	1%	98.0%	98%	97%
(2)	10%	94.0%	99+%	96%
(3)	50%	58.5%	96%	99%
(4)	100%	15.0%	94%	97%
Control fertilized in		sea-water: 99%		

The conclusion would seem to be that shell secretions (poison from pedicellariae?) protect against the inhibitor in the blood. The inhibitor was not very strong in this case, and test of *C* showed that it contained a small quantity of agglutinating substance owing, as sometimes happened, to injuries to the ovaries in collecting the blood, but there did not seem to be enough (7 second reaction undiluted) to influence the result.

The above result was obtained in an attempt to meet the possible objection that the inhibiting action of the blood might be due to the inclusion of poison from pedicellariae. It appears, on the contrary, that shell-secretions decrease the inhibiting action of the blood, an entirely unexpected result.

As the result met the anticipated objection and did not appear to lie in the main line of the research, the experiment was performed only three times in all, with essentially similar results.

To explain such a result one of two hypotheses is possible: (1) that shell secretions destroy the inhibitor; (2) that they unite with its inhibiting side-chain, and thus possess a combining group identical with the ovophile group of the fertilizin; though they do not possess the spermophile group. The reaction takes place rapidly; hence the second assumption is more likely.

3. SUMMARY OF PART III

In this section we have shown:

1. That the blood of male and female sea-urchins contains a varying amount of a substance which inhibits fertilization without exerting any injurious effect upon either the sperm or the ova. This substance is probably more abundant in the blood of mature individuals than in others.

2. The inhibitor does not act by blocking the combination of the sperm receptors with the fertilizin, for it has no inhibiting effect upon the agglutinating action of the fertilizin.

3. It acts by occupying the ovophile group of the fertilizin, thus preventing action of the latter upon the egg by union with egg receptors.

4. This conclusion is demonstrated by the fact that the inhibitor may be entirely neutralized by a sufficient quantity of the agglutinating substance.

IV. GENERAL DISCUSSION

The essential conception that results from the experiments is that the formation of the fertilization membrane is due to activation of an ovogenous substance, which I have named fertilizin to express this idea. Following insemination any excess of the fertilizin is bound, or neutralized, by another ovogenous substance, which I have named anti-fertilizin, and polyspermy is thereby prevented. The experiments demonstrate that reactions in fertilization occur as described in sections II and III, but the

intimate nature of the reactions themselves is unknown. To represent them in terms of the Ehrlich hypothesis as definite lock-and-key chemical combinations is of course to go beyond the facts. But it is necessary in some way to gain definiteness of formulation not only for purposes of description, but also to obtain a definite working hypothesis. The method of formulation which I have adopted has both of these advantages. Indeed some of the experiments could hardly have been suggested by any other hypothesis.

1. THE MECHANISM OF FERTILIZATION

In terms of such an hypothesis the mechanism of fertilization may be summed up in a diagram (fig. 1, sector 1). The cortex of the egg contains the fertilizin (see explanation of symbols) of which three molecules are represented each with a spermophile

Fig. 1 In successive sectors of the egg there are represented the mechanism of fertilization and the blocks to the mechanism, as follows:

Sector 1 The arrangement of substances in the unfertilized egg and in the spermatozoon that are active in fertilization. See explanation of symbols.

Sector 2 The mechanism of normal fertilization. The sperm receptor unites with the spermophile group of the fertilizin and the egg-receptors with the ovophile group of the fertilizin owing to activation of the latter by the sperm (a). Molecules of the anti-fertilizin combine with the spermophile group of the adjacent fertilizin (b and c) and thus block the way for supernumerary spermatozoa. This is the postulated mechanism for prevention of polyspermy. At the same time molecules b and c of the fertilizin have also united with the egg-receptors.

Sector 3 Inhibition of fertilization by loss of the active body, fertilizin.

Sector 4 Theory of antagonistic action of spermatozoa of different phyla. The sperm receptors are occupied by combining groups cast off by the antagonistic spermatozoa.

Sector 5 Fertilization is blocked by occupancy of the egg-receptors. Purely hypothetical.

Sector 6 Theory of inhibitory action of blood of the same species. The ovophile group of the fertilizin is occupied by molecules in the blood (inhibitor) possessing the same combining group as the egg-receptors. Molecules of the blood inhibitor also shown in the medium.

The fertilizin is represented in the diagram as occurring only in the cortex of the egg. But it also occurs in high concentration in the jelly surrounding the egg. The spermatozoon must thus normally arrive at the egg-membrane loaded with combined fertilizin. This fact, however, makes no essential difference in the theory, and its representation would complicate the diagram.

Figure 1

and an ovophile combining group. The spermatozoon is represented with a receptor capable of fitting the spermophile group of the fertilizin; the egg similarly contains receptors capable of fitting the ovophile group of the fertilizin. Within the egg represented as alternating with the egg-receptors, are also found certain molecules (anti-fertilizin) capable of uniting with the spermophile group of the fertilizin.

Union of a sperm receptor with a spermophile group of the fertilizin molecule activates the latter so that the ovophile group forms a union with an egg-receptor (fig. 1, sector 2, *a*); and I have postulated that the fertilizing action is the result of this union. But the activation of the fertilizin can by no means be regarded as confined to those molecules bound by the single penetrating spermatozoon. On the contrary, as we have seen that all the spermatozoa used in insemination bind only a minute proportion of the fertilizin contained in the eggs, it is necessary to assume that the activation of the fertilizin spreads beyond the point of attack of the successful spermatozoon; this is represented in figure 1, sector 2, *b* and *c*. Inasmuch as membrane formation is practically instantaneous all around the cortex, the activation of the fertilizin must spread with extreme rapidity; an activated molecule of the fertilizin must transmit the activated condition to its neighbor, in a fashion that may be compared by analogy to the spread of a stimulus. The assumption here is no different in principle from that which must be made in any theory of the fertilizing effect of the spermatozoon, and therefore, no new difficulty is introduced.

The postulated mechanism admits of five blocks in the process of fertilization viz.: 1. Absence of the fertilizin (fig. 1, sector 3); 2. Occupancy of the sperm receptors (fig. 1, sector 4); 3. Occupancy of the egg receptors (fig. 1, sector 5); 4. Occupancy of the ovophile side-chain of the fertilizin (fig. 1, sector 6); 5. Occupancy of the spermophile side-chain (fig. 1, sector 2, *b* and *c*). Of these we have examined and discussed in the body of this paper the first, fourth, and fifth, the first in the case of the long washed eggs, (p. 549) the fourth in the case of the inhibitor contained in the blood (p. 563), and the fifth in the case of the non-fertilizable

condition of the already fertilized eggs or those with membranes formed by other methods (p. 556). These cases have been sufficiently discussed already.

But there are two methods of blocking the mechanism according to the hypothesis, which we have not found, viz.: 2 (supra) the occupancy of the sperm receptors in such a way that these side-chains are no longer free to unite with the spermophile group of the fertilizin, which would result in loss of fertilizing power of the spermatozoon (fig. 1, sector 4); and 3 (supra), viz.: occupancy of the egg receptors, or molecules of the protoplasm, with which the fertilizin reacts (fig. 1, sector 5). The discovery of these missing links in the theory would serve as strong confirmatory evidence of its essential correctness.

The latter of these two missing links remains purely hypothetical, but I think it is possible that the former may be represented by the phenomenon of the antagonism of sperm suspensions of different animal phyla as described first by Godlewski ('10 and '11) and subsequently by Herlant ('12, *a* and *b*).

These two authors give quite different interpretations of the results. While Godlewski, relying on the analogy of the antagonistic action of heterogenous hemolytic sera, regards the phenomenon as proof of Loeb's 'lysin theory' of fertilization, Herlant is more conservative, and believes that the explanation cannot be sought in any fundamental alteration of either sexual element; but is rather to be found in a purely 'humoral mechanism,' (i.e., in the effect of substance accompanying the spermatozoa) which he supposes to modify the physical state of the surface of the eggs in such way that the spermatozoa cannot penetrate. Herlant apparently overlooks the fact that penetration of the spermatozoon is not necessary for the cortical changes, as has been shown both by Loeb ('09 and '13) and myself ('12). There is nothing in the results of either author inconsistent with the idea, that the mechanism of inhibition in this case may be due to what I have called, "occupancy of the sperm-receptors" as indicated in sector 4 of the diagram. This may be taken to mean merely such mutual action of the spermatozoa (or extractives thereof) on each other as to inhibit the union with the fertilizin.

This could readily be tested experimentally, for, in such a case, the mixed sperm suspension should no longer agglutinate on exposure to the fertilizin of the eggs used in the experiment. If, for instance, we were to find that sperm suspensions of *Arbacia* and *Nereis* were antagonistic in the sense of Godlewsky, so that mixtures of the two would not fertilize *Arbacia* eggs, then according to my hypothesis the *Arbacia* sperm in the inhibited mixture would not agglutinate with *Arbacia* fertilizin. The experiment yet remains to be performed.

2. THE TWO PHASES OF FERTILIZATION

In the second study of this series (Lillie '11) I showed that fertilization involves two phases in the case of the egg of *Nereis*; the first phase is accomplished before the penetration of the spermatozoon, and leads to changes in the cortex of the egg analogous to membrane formation in the sea-urchins. If the spermatozoon be then prevented from entering, the egg completes the maturation process only and does not segment. The cleavage of the egg is caused by some action of the spermatozoon after penetration. Loeb had also shown by entirely different methods the existence of two similar phases in the fertilization of the ovum of the sea-urchin.

The question, therefore, arises, does the mechanism of fertilization discussed in this paper deal only with the first, or with both phases of fertilization? To this it must be answered that the mechanism in question is primarily the mechanism of membrane formation. The described results demonstrate this so positively that farther discussion is superfluous on this point.

But the question remains whether there is any relation of such cortical changes to the internal phase of fertilization? Whether, for instance, if the spermatozoon could enter the egg without first forming the combination with the cortical fertilizin, it would then be in condition to carry out the second phase of fertilization, and cause segmentation of the egg? It has been shown, as is well known, that the egg can be induced to develop apparently without the cortical changes involved in membrane formation,

by the use of hypertonic sea-water for instance. It therefore seems possible that the two phases of fertilization may be disconnected in the case of the spermatozoon also, activated perhaps by different parts of it. On the other hand there is the possibility that fertilization is a continuous process and that the second step cannot proceed if the first be skipped; that the spermatozoon in other words receives some necessary modification, by union with the fertilizin or other substances experienced in the cortex of the egg.

The latter point of view seems more probable to me for the following reasons. In the first place I have made some yet unpublished observations in the case of *Nereis* which show that if the cortical changes be induced by artificial means there is a brief period in which insemination of the eggs may be followed by penetration of the spermatozoon, but without causing cleavage of the egg. Such a result might conceivably be due to a lack of synchrony between the movements of the egg-nucleus and the sperm-nucleus owing to the start given the egg-nucleus by the treatment prior to insemination. This explanation does not seem to me to be entirely reasonable, because, in *Nereis*, the sperm enters during maturation and has therefore abundance of time to prepare for union with the egg-nucleus. It seems more probable that the egg has lost something which renders the introduced sperm inefficacious. As a matter of fact, all the fertilizin is thrown off or neutralized in *Nereis* during the cortical changes, as I have determined by numerous experiments. Miss Allyn ('12) also determined for *Chaetopterus* that potassium chloride, which starts parthenogenetic development in this form, "initiates changes which increase with time and which are inimical to normal fertilization. They do not prevent the entrance of the sperm into the egg, but they prevent the normal behavior of the sperm in the egg." The cortical changes are not very obvious in *Chaetopterus*, but it is reasonable to suppose by analogy that in initiating parthenogenesis the fertilizin is bound; the sperm entering after this event are relatively inefficacious.

Dr. G. L. Kite kindly allows me to refer to some yet unpublished experiments performed last summer in which he injected,

by means of the Barber apparatus, from two or three to over twenty spermatozoa of the star-fish into eggs of the same species. There was never any indication of cleavage. The experiments were limited owing to interruption of Dr. Kite's work, so that the evidence in this case is not yet wholly conclusive; but the results indicate the necessity for normal penetration, if the spermatozoon is to be effective.

We therefore see a second function of the fertilizin, viz.: to prepare the spermatozoon for exercising fertilization effect in the interior of the egg. *The spermatozoon needs itself to be fertilized.*

De Meyer ('11) has observed swelling of the head of the spermatozoon under the action of egg-extractives, and I have described the same thing for *Nereis* ('13). But how such changes intervene in the internal fertilization reaction remains problematical. It is improbable that the swelling itself is significant; it is rather to be taken as indication of a more deeply seated physiological change which is essential for the completion of subsequent reactions.

3. THE LYSIN THEORY OF FERTILIZATION

Loeb's lysin theory of fertilization may be taken as the type of all theories which postulate that the spermatozoon produces and bears a fertilizing substance. Loeb holds that the spermatozoon causes membrane formation and thus initiates development by virtue of a lysin, and he holds this idea not because he, or some other, has isolated this substance and tested its action, but because the action of the spermatozoon in initiating development resembles the action of certain cytolytic substances, more especially, fatty acids, certain glucosides and the blood of some foreign species. Indeed he holds that all cytolytic agents induce membrane formation, and the action of the spermatozoon is regarded as merely a special instance. The spermatozoon, according to Loeb, introduces a second substance into the egg whose function it is to inhibit the harmful effects of membrane formation. However the effect of fertilization in increasing the rate of oxidation within the egg is due not to a catalyzer introduced by the spermatozoon but to the activation of catalyzers

already in the egg as shown by the fact that the rate of development is not increased by superposing fertilization on parthenogenesis or by polyspermic fertilization.

The evidence for the existence of a fertilizing lysin in the sperm is thus entirely analogical and would apply equally well to the conception that the sperm causes membrane formation by activating a substance in the egg, which would make the first part of Loeb's theory of fertilization consistent with the last part. Direct evidence is lacking for the lysin theory, but the whole series of facts described in this paper is direct evidence for the existence of the fertilizing substance in the ovum. We have obtained it in sea-water solution and we have studied its effects both on the spermatozoon and on the ovum and have found it to answer to all the necessary requirements. If the conclusions are accepted, we can hardly continue to believe in the existence of the hypothetical sperm-carried lysin.

But apart from this consideration, the lysin theory exhibits certain features of inadequacy. In the first place the failure of attempts to isolate such a substance from spermatozoa and obtain fertilization can be explained, as Loeb has pointed out, on the assumption that, though presumably present in sperm extracts, it lacks power of penetration in the isolated condition and needs the penetrative power of the living spermatozoon to carry it through the egg membrane. A similar assumption would have to be made with reference to the sperm receptors in my theory; so that this lacuna is not peculiar to the lysin theory.

In the second place the lysin theory affords no explanation whatever of the non-fertilizable condition of fertilized eggs. If membrane formation is the result of a superficial cytolysis produced directly by a lysin carried by the sperm and checked by operation of a second spermatogenic substance effective after penetration of the spermatozoon, what is to prevent a second cytolysis by a second insemination? There is nothing in the lysin theory that affords the least explanation of this universal phenomenon; even concentrated sperm suspensions do not refertilize or exert a 'cytolytic' effect on fertilized eggs.

Loeb has noted that it is impossible to ascribe the increase of rate of oxidation after fertilization to the introduction of a cata-

lyzer by the sperm because polyspermy does not increase the rate of development over the normal. But he has neglected to note that the same argument applies to his lysin theory; if this theory were correct we should expect that polyspermy would increase the cortical cytolysis above the normal, and result in an unusually distended fertilization membrane; but this is not the case; membrane formation is not excessive, so far as observed, in polyspermic eggs. Yet the lysin theory requires that this should be the case.

It is also inconceivable that a lysin could act effectively in such high dilutions as the respective bulks of spermatozoon and ovum render necessary. Wilson estimates the bulk of the spermatozoon at about 1/400,000 that of the egg in the sea-urchin. The sperm head is not visibly diminished after entrance so it would be unreasonable to suppose that more than 10 per cent of its substance has been used in membrane formation. The lysin theory supposes that a spermatozoon cytolyzes a bulk of 4,000,000 times its lysin content! Not only so, but inasmuch as it is introduced at one point the lysin must diffuse with a speed and evenness that renders it effective simultaneously, and very quickly, at all points on the surface of the egg. This conception seems to me to be inconceivable, for the theory of lysin action presupposes a union molecule for molecule over the affected surface. We should also have a large spermatozoon for large eggs and a small spermatozoon for small eggs on the basis of a lysin theory, which is not the case.

These considerations are arguments not only against the lysin theory but also against any theory that presupposes that the spermatozoon is the bearer of a substance acting directly in causing formation of the cortical changes of fertilization.⁵

I may be allowed finally to point out that, even if some form of sperm extract should be shown to be effective in the production of the cortical change, such a result would be as consistent with the theory of indirect action or activation of an ovogenous substance as with the theory of direct action and would be no argument against the preceding considerations.

⁵ Although Professor Loeb has committed himself very definitely to the lysin theory, he has not failed to note the theoretical possibility of the activation hypothesis (see Loeb 1913, p. 235; earlier reference given here).

4. THE THEORY OF PARTHENOGENESIS

A theory of parthenogenesis must also be a theory of fertilization, at least for the phenomena common to both. Similarly a theory of fertilization must be consistent with the facts of parthenogenesis. This has been generally recognized and it is one of Loeb's great services to have brought about the recognition of this principle and to bring the problem of fertilization definitely into the field of physiology. That the fertilizin theory is consistent in this respect is sufficiently obvious; indeed it makes the action of the sperm itself into a kind of parthenogenesis, for the sperm activates the fertilizing substance already present in the egg. The egg is self-fertilizing.

However, it lies beyond the province of this paper to consider the various methods of artificial parthenogenesis in detail and to examine the question of their consistency with the theory. It is obvious, I think, that the fertilizin theory from its very nature is more elastic with reference to this problem than any other, and is in general, therefore, more consistent with the established fact of the great variety of proved parthenogenetic agents.

Obviously it suggests certain lines of experimentation in parthenogenesis. Glaser has already followed one of these lines with some success, viz.: The effect of exposure of eggs to extracts or secretions of eggs of their own species; and he has ascertained that such extracts are rather efficient parthenogenetic agents in the case of *Arbacia*, especially when followed by treatment with hypertonic sea-water. He did not, however, observe the formation of membranes in eggs so treated, which leaves the mechanism of action rather obscure; farther analysis of the result is desirable.

But the whole procedure in experiments in parthenogenesis must take on a different aspect as soon as it is realized that the entire process, and not merely part of it, consists in acceleration of possible combinations in the egg, or increase in avidity of certain chemical groups.

It is not possible to separate the problem of fertilization from the general problems of cellular physiology; I may therefore be

allowed to point out the impossibility of regarding the interchange between the egg and its environment as controlled exclusively by diffusion through a semi-permeable membrane. However significant the rôle of permeability of the membrane may be, my experiments show that the egg cell is by no means bound in its exchange to diffusible substances; but that the membrane must be regarded as an active factor, and not only a passive one. Twenty years ago diffusion was assumed to play the chief rôle in the absorption from the intestine or in excretion by the kidney epithelium. Today we know that the excretion of urea means work by the kidney cell; it is an active, not a passive, process. Similarly the secretion of fertilizin by the egg cell, and possibly other cortical phenomena, must be regarded as active processes.

The ordinary chemical analysis of the cell begins with destruction of its more highly organized living constituents; it is obvious that such methods are inadequate for the investigation of the immediate reactions in living protoplasm. The results of these experiments may then gain a still broader interest if they may be taken to indicate a method of studying such reactions by the use of living cells as indicators.

LITERATURE CITED

- ALLYN, HARRIET M. 1912 The initiation of development in *Chaetopterus*. Biol. Bull., vol. 24, pp. 22-72.
- DELAGE, YVES 1901 Études expérimentales sur la maturation cytoplasmique et sur la parthénogénèse artificielle chez les Echinodermes. Archiv. de Zool. Expér. et Gén., Ser. 3, T. 9, pp. 285-326.
- GLASER, OTTO 1913 On inducing parthenogenesis in the sea-urchin (*Arbacia punctulata*) together with considerations on the initiatory effect of fertilization. Science, N. S., vol. 38, no. 978, p. 446.
- 1914 a A qualitative analysis of the egg-secretions and extracts of *Arbacia* and *Asterias*. Biol. Bull., in press.
- 1914 b Auto-parthenogenesis in *Arbacia* and *Asterias*. Biol. Bull., in press.
- GODLEWSKI, E. 1910 Ueber den Einfluss des Spermas der Annelide *Chaetopterus* auf die Echinodermeier and über die antagonistische Wirkung des Spermas fremder Tierklassen auf die Befruchtungsfähigkeit der Geschlechtselemente. Bull. de l'Acad. de Sc. de Cracovie, pp. 796-803.
- 1911 Studien über die Entwicklungserregung II. Antagonismus der Einwirkung des Spermas von verschiedenen Tierklassen. Archiv f. Entw'mech. der Organismen, Bd. 33, pp. 233-254.
- HERLANT, MAURICE 1912 a Recherches sur l'inhibition reciproque de deux spermes provenant d'espèces éloignées. Bull. de la Soc. Roy. des Sc. Méd. et Nat. de Bruxelles, no. 10.
- 1912 b Recherches sur l'antagonisme de deux spermes provenant d'espèces éloignées. Anat. Anz., Bd. 42, pp. 563-575.
- LILLIE, FRANK R. 1911-1912 Studies of fertilization in *Nereis*. I. Cortical changes in the egg. II. Partial fertilization. Jour. Morph., vol. 22, pp. 361-391. III and IV, Jour. Exp. Zool., vol. 12.
- 1913 Studies of fertilization. V. The behavior of the spermatozoa of *Nereis* and *Arbacia* with special reference to egg-extracts. Jour. Exp. Zool., vol. 14, pp. 515-574.
- 1913 The mechanism of fertilization. Science, N. S., vol. 38, no. 980, pp. 524-528.
- LOEB, JACQUES 1909 Die chemische Entwicklungserregung des tierschen Eies. Berlin, Julius Springer, pp. 259.
- 1913 Artificial parthenogenesis and fertilization. University of Chicago Press, pp. 312.
- DE MEYER, J. 1911 Observations et expériences relatives à l'action exercée par des extraits d'oeufs et d'autres substances sur les spermatozoïdes. Arch. de Biologie, T. 26, pp. 65-101.

- ROBERTSON, T. B. 1912 Studies in the fertilization of the eggs of a sea-urchin (*Strongylocentrotus purpuratus*) by blood-sera, sperm, sperm-extract and other fertilizing agents. *Archiv f. Entw'mech. d. Organismen*, Bd. 35, pp. 64-130 (see pp. 80-82).
- WILSON, E. B. 1903 Experiments on cleavage and localization in the Nemertine egg. *Archiv f. Entw'mech. d. Organismen*, Bd. 16, pp. 411-460 (see pp. 417-419).