LEMAIRE

SELF-FERTILIZATION INDUCED BY ARTIFICIAL MEANS.

BY

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It has long been known that the pollen of some plants will not fertilize the ovules of the same plant. The cause of this impotence has not yet been detected.

It is also known that pollen from another plant is often prepotent in those cases where normal self-fertilization may occur. It has further been shown, especially by Darwin, that the offspring from self-fertilized ovules are in general not so vigorous as those from cross-fertilized ones.

There are here two problems, which, even if they should prove to be fundamentally related, can be most profitably examined separately;— first, the problem of the inability of the male element to fertilize the female germ-cells of the same individual; and, second, the effect of self-fertilization (in those cases in which it occurs) on the offspring. Both problems appear to be within the range of experimental examination.

There are only a very few cases known amongst animals where conditions similar to those in plants have been found to prevail, although very few hermaphroditic animals appear to have been examined in this respect. Close inbreeding, which is commonly supposed to bring about deterioration in some cases, is perhaps not very dissimilar to self-fertilization. Whether in the case of inbreeding there is ultimately a loss of power to fertilize the egg, or whether the egg fails to develop after it has been fertilized, has not, so far as I know, been determined.

Castle discovered in the ascidian, *Ciona intestinalis,* a case apparently similar to those in plants. The eggs are generally incapable of self-fertilization, yet can be readily cross-fertilized; *i.e.,* the spermatozoa of an individual will not fertilize the eggs of that individual, but have the power to fertilize the eggs of any other individual.

My object in undertaking a study of this problem was, in the first place, to determine if possible the nature of the conditions that prevent or interfere with self-fertilization; and in the second place, I was not without hope of being able to find some way in which self-fertilization could be artificially induced. As will appear in the sequel, these two questions are not two sides of the same problem: for, while it has been possible to discover the means of bringing about self-fertilization, it still remains to be definitely determined what conditions in the egg normally prevent the entrance of the spermatozoa of the same individual.

Since Castle's observations had shown that the ascidians offer favorable material for a study of this sort, I first turned my attention to this group, using the three most available species found at Woods Hole, or in the vicinity; namely, *Ciona intestinalis, Molgula manhattensis,* and *Cynthia partita* (*Styela* sp.). The work was done while holding the Bryn Mawr Table at the Marine Biological Laboratory, from June to September, 1903. Owing to the scarcity of Ciona I have not been able to work out completely a number of important problems connected with one of the two main questions that I examined. In the near future I shall hope to complete this side of the investigation.

EXPERIMENTS WITH CIONA INTESTINALIS.

The ovary of Ciona is a sac-shaped body of fair size lying loosely attached in the coil of the intestine. It can easily be removed without cutting into the testis. Its lumen contains some of the ripe eggs, but the majority of these are in the oviduct. The oviduct can readily be opened and the eggs set free without cutting into the vas deferens, which follows a course parallel to the oviduct. If the animal is kept isolated for 24 hours the oviduct becomes greatly distended with eggs, and after another 24 hours even more eggs may have accumulated. The eggs are laid normally in the early morning, at dawn, and Castle has recorded that Ciona deposits its eggs and sperm with the regularity

of the rising sun. The rough handling incidental to removal and isolation appears to cause Ciona to retain its eggs for several days. The individuals to be used were isolated, as a rule, from 24 to 48 hours, and in most cases were rinsed in fresh water before opening. It was not found necessary to boil the water; for check experiments showed that eggs left to themselves were never fertilized by stray spermatozoa in the sea-water. Since Ciona deposits its eggs only in the very early morning, the chances are very slight that functionally active spermatozoa would be present in the sea-water in the late morning and in the afternoon when the experiments were carried out.

The eggs of Ciona are surrounded by a rather thick membrane. Standing out like broad spikes over the surface of the membrane, and forming a beautiful aureole around the egg, are the transparent follicle cells, each with a shining drop in its outer end.

A number of preliminary experiments confirmed Castle's conclusion that self-fertilization is rarely possible in Ciona intestinalis. The evidence, however, on which Castle based this conclusion is not altogether satisfactory, since he records many cases in which self-fertilization occurred. Instances are cited in which isolated individuals gave 90, 25, 16, 5, 4, 0 per cent. of self-fertilized eggs. Castle supposes that, in the first of these cases at least, the spermatozoa of one day fertilized the eggs of the next, but it has not been shown that the spermatozoa have this power if left so long in sea-water. The same individuals that had been used for these isolation experiments were killed (after being washed in 90 per cent. alcohol), and the eggs and sperm of each taken out and mixed together. The results gave 50, 4, 1, ⅓, 0 per cent. of self-fertilized eggs. The same experiment repeated with fresh individuals gave 50, 12½, 10, 5, 2, 0 per cent. of self-fertilized eggs. From these figures it is clear that in some cases a considerable amount of self-fertilization occurred, unless there was some source of error in the experiment. In fact, Castle believes that in those cases where a large number of eggs were fertilized there was some contamination. My own results with Ciona have never given so large a percentage of self-fertilized eggs, and I am inclined to attribute this result in part to the precaution that I took to isolate the individuals the day before they were to be used. I have rarely seen more than from 1 to 10 per cent. of self-fertilized eggs segment, and in the greater number of cases not a single egg segmented. On the other hand I found, as did Castle, that as a rule 100 per cent. of cross-fertilized eggs develop, to which statement I should add, provided the spermatozoa are in "good" condition.

What is the meaning of these remarkable facts? Why do not the sperm fertilize the eggs produced by the same individual, and yet fertilize those of any other individual? A number of possibilities readily suggest themselves, and since the following pages record an attempt to test these suggestions they may be briefly mentioned here:

1. That the spermatozoa are not made sufficiently active by secretions from the eggs of the same individual, but by those from the eggs of any other individual.

2. That the spermatozoa are not "attracted" to the eggs of the same individual.

3. That the egg contains, or secretes some substance that lessens the activity of the spermatozoa of the same individual.

4. That some mechanical difficulty prevents the spermatozoön from entering the egg of the same individual.

5. That even if the spermatozoön enters, it can not fertilize the egg of the same individual, in the sense of causing the egg to begin to develop.

In order to discover if the lack of power to self-fertilize the eggs is due to the absence of some substance around the eggs that excites the spermatozoa, the following experiment was carried out. The eggs of an individual (A) were taken from the oviduct. Similarly the eggs of another individual (B) were also taken out. Then the ovary of (A) and that of (B) were crushed separately, and a little sea-water was added. The eggs of (A) were then allowed to soak in the crushed ovary extract of (B) and those of (B) in the extract of (A). After a short time the sperm of (A) with a little water was added to the (A)-eggs, and the sperm of (B) to the (B)-eggs. If the sojourn of the eggs in the extract of the ovary of another individual has

the postulated effect, or if the presence of the extract of the ovary of another individual has the postulated effect on the sperm, fertilization ought to have occurred. The results showed, however, that fertilization did not take place.

This experiment was performed four times, giving eight sets in all. In six of these sets not a single egg segmented. In two others a very few eggs segmented (6 per cent. in one, 5 per cent. in the other), but this sometimes occurs in self-fertilized eggs not treated in any special way. Moreover there may have been contamination in the latter case.

Another experiment similar in some respects to the last was also carried out. The heart of one individual was opened and the blood collected. The eggs of another individual were put into this blood and allowed to stand. Later, sperm of the same individual was added in sea-water, but no fertilization occurred in one set and only one per cent. in the other. Check eggs were also kept in this experiment to make certain that no sperm had accidentally gotten into the blood. That none were present was shown by the fact that no fertilization took place. It is evident from this experiment that self-fertilization can not be brought about by soaking the eggs in the extract from the ovary or in the blood of another individual, although the somewhat high percentage of self-fertilized eggs that segmented in two cases after treatment with the ovarian extract may have resulted from the influence of the extract on the spermatozoa.

If the spermatozoa are excited to greater activity by the presence of the eggs of another individual it seemed not improbable that this might be directly observed. Therefore, I placed some of the sperm with the eggs of another individual and more of the same sperm with the eggs of the same individual, and compared the two preparations under the microscope. The spermatozoa of Ciona are not very active as a rule, nor do they accumulate in crowds around the eggs, as they do in many other animals, or at least not to any marked extent. It seemed to me in both cases that sometimes the spermatozoa were more active immediately in the vicinity of the eggs, and in the spaces between the follicle cells, but as they also show the same activity around pieces of the tissue of the body of the same or of another individual I have not laid much stress on this observation, or accredited the results to the presence of an exciting substance. At times I have thought that the spermatozoa were more active around the eggs of another individual than around the eggs of the same individual, but as there is no very accurate means of determining their relative motility, unless very marked, I should not wish, as yet, to give a final answer to this question. It is certain that there is no such great difference in the behaviour of the spermatozoa in the presence of the eggs of the same and of another individual as to suggest that the difference in the result is connected with this factor. And even if this were the case, the influence probably extends for only a short distance from the surface of the egg, as the following experiment shows.

The eggs were taken from the oviduct, great care being taken not to injure the sperm-duct. The eggs from another individual were collected in the same way. An equal number of eggs from each were put together and fertilized with the sperm from one of the individuals. In another dish another lot of the same eggs were mixed half and half, and these fertilized with the sperm from the other individual. In each of these two sets half at least of the eggs should be fertilized by the other sperm, but half should not be fertilized unless the eggs of one individual exert some influence that causes the sperm to fertilize the eggs of the same individual also. It was found that only about half of the eggs were fertilized. This result shows that the fertilization is probably not due to some substance set free by the eggs that acts on the sperm or at least that if such a substance is set free its action is confined to the immediate vicinity of the egg. The experiment does not show, however, whether the egg, or its membranes, may not contain some substance that prevents the spermatozoa from entering the eggs of the same individual. Even if such a substance is set free from the eggs it may not have had time in my experiment to accumulate sufficiently in the surrounding water to have prevented the spermatozoa from fertilizing the other eggs, which may be quickly entered. This view can be tested by letting eggs stand in a small amount of water for a

long time, then taking out some sperm from the same individual, first making it active by placing it in sea-water, and then putting it into the water in which the eggs have stood. On the hypothesis these sperm should soon be brought to rest, and if then the eggs of another individual are added, they should not be fertilized, or at least not in the same proportion as when the sperm is taken directly from the oviducts, put into sea-water, and then added to the eggs.

THE INFLUENCE OF ETHER ON CROSS-FERTILIZATION. EXPERIMENTS WITH CIONA.

My first experiments with ether were made in order to determine whether when eggs are etherized it might not be possible to self-fertilize them. The results turned out somewhat differently from what I had anticipated, for although I found that it was possible to self-fertilize the eggs in ether-solutions, the result seemed to be due to the action of the ether on the sperm rather than on the eggs.

The experiment was first made with Cynthia, which in most cases has very sluggish spermatozoa. I observed that the first effect of the ether was to make the sluggish sperm very active, and even greatly quickened the activity of already active sperm. Furthermore I found that spermatozoa that scarcely moved at all in sea-water became active in the ether-solutions. Finally I found that in ether-solutions of certain strengths the eggs of Cynthia and of Ciona could be self-fertilized. The eggs behave in this respect so capriciously that I was obliged to carry out a large number of experiments in order to determine the conditions that lead to the self-fertilization of eggs in ether-solutions. The outcome was only partially satisfactory, but the experiments opened up a field for research, in which it may be possible to obtain further results of interest.

The experiments with ether were carried out as follows: At first I used a nearly saturated solution of ether and diluted it a half, or a fourth, etc. In the later experiments I used solutions of known strength. It was found by trial that the solutions were effective between 0.25 and 5 per cent. Some of the results may now be given in detail.

Experiment I. The eggs were removed from an individual that had been isolated 20 hours. The sperm was also taken out, and, together with the eggs, was put into ether-solutions, 5, 2, 1, 0.7, 0.5 per cent. in sea-water. After 5 minutes, and again after 10 minutes, the eggs were removed to pure sea-water. The eggs were injured by the ether in the strongest solution, but nevertheless one segmented. In all of the other solutions about 80 per cent. of the eggs divided; the most in the weaker solutions.

Experiment II. In this experiment the eggs and the sperm were put together into ether solutions of 5, 2, 1, 0.7, 0.5 per cent. Some of the eggs were transferred to water after 5 and 10 minutes, but others were left in the solutions. In the strongest solution the eggs were killed. In the others the following results were obtained:

Ether	Eggs segmented 5 minutes in ether.	Eggs segmented. 10 minutes in ether.
2. per cent.	20 per cent.	25 per cent.
1. "	5 "	75 "
0.7 "	2 "	2 "
0.5 "	10 "	5 "

It is clear that the stronger solutions gave the best results, and that ten minutes immersion was better than five minutes. None of the eggs that were left in the ether-solutions segmented. This does not mean that they were not fertilized, but that the ether so injured the eggs after a long immersion, that they failed to develop. Several check experiments were also made in this case. In one the eggs were not self-fertilized but were put into a 5 per cent. ether-solution, and transferred after ten minutes to sea-water. They did not segment, nor did a few that were left behind in the ether solution. In another check series the eggs were not fertilized, and were left in sea-water. None segmented, which shows clearly that the ether in the preceding experiment was in some way responsible for the self-fertilization of the eggs. It should also be recorded that tadpoles developed from all the fertilized eggs that had been in the ether-solutions.

Experiment III. The eggs and sperm of the same individual

were put into ether-solutions of 3, 2, 1, 0.7, 0.5 per cent., and were removed to sea-water after 10, 20 and 30 minutes.

Ether	10 min.	20 min.	30 min.
3. per cent.	20 per cent.	20 per cent.	0 per cent.
2. "	33 "	17 "	90 "
1. "	50 "	25 "	10 "
0.7 "	50 "	20 "	0 "
0.5 "	20 "	20 "	12 "

The table shows that eggs segmented in all of the solutions, best however in the stronger solutions, although in one case the eggs became so injured by the ether that they did not develop further than the segmentation stages. In a check series, in which the self-fertilized eggs were put into sea-water, about ten per cent. of the eggs segmented. There may have been some source of contamination, or else, and this seems more likely since the individual had been isolated 20 hours, self-fertilization took place on a larger scale than usual.

Experiment IV. Eggs and sperm were mixed in ether-solutions of 4, 2, 1, 0.5 per cent.

Ether	2 min.	4 min.
4.	0	0
2.	40	1
1.	35	30
0.5	5	1

These results show that the 4 per cent. solution was too strong, while the 0.5 per cent. solution appears to have been too weak. The injurious action of the 4 per cent. solution appears to have been mainly on the sperm rather than on the eggs, for these eggs after they had been in sea-water 4 hours were capable of being cross-fertilized, and 25 per cent. of them developed.

Experiment V. Eggs and sperm were put into a 2 and into a 0.5 per cent. ether-solution and removed after 5 minutes.

Ether	5 min.
2.	90
0.5	90

It is interesting to note in this case, in which so large a percentage of the eggs were self-fertilized in ether, that of several hundred eggs of the same individual, to which sperm was added, but which were kept in sea-water, not one segmented. It was also found, and will be referred to again later, that when the sperm alone was put into a 2 per cent. soluton of ether for five minutes, and was then added to eggs of the same individual in sea-water, 70 per cent. of the eggs segmented.

Experiment VI. In this experiment the eggs and the sperm were put into ether-solutions of 2 and of 0.5 per cent. and removed after ten minutes. In one lot the eggs were self-fertilized, in the other they were cross-fertilized.

Ether	Self-fert. 10 min.	Cross-fert. 10 min.
2.	0	100
0.5	0	100

In this case although no self-fertilization took place, all the crossed eggs which had also been in the ether-solution developed, showing that the solutions have no baneful effect on cross-fertilization. The lack of self-fertilization shows that the sperm were not sufficiently acted upon by the ether-solutions employed to effect self-fertilization.

Experiment VII. This experiment shows how slight a difference in the conditions may cause great differences in the result. The individuals had been isolated 48 hours. One lot of self-fertilized eggs was kept in water and allowed to stand there 20 minutes. The eggs with the surrounding sperm were then put into ether-solutions of 2, 1 and 0.5 per cent. for 20 minutes, and then returned to sea-water. None of these segmented. Another lot of eggs from this individual were mixed with sperm of the same individual and put into a 2 per cent. ether-solution for ten minutes and then carried back to sea-water. Here 95 per cent. of the eggs segmented. On the other hand some of these same eggs taken from the ether after 5 minutes did not divide. The following experiments were also carried out with other self-fertilized eggs of the same individual.

Ether	15 min.	30 min.
2.	45	0 (only 3 eggs)
1.	30	40

Experiment VIII. The eggs and the sperm of one individual were mixed in ether-solutions of 4, 3, 2, 1, 0.7, 0.5 per cent., and removed after 10, 20, 30, 60 minutes to sea-water. It was noticed that the spermatozoa were very sluggish in sea-water, and although somewhat more active in the ether solutions, yet their activity was not marked. Of the eggs, which appeared to be in excellent condition, only three segmented, two in the 1 per cent. (20 minutes) and one in the 0.5 per cent. (20 minutes.)

The eggs that had not segmented after the ether treatment were fertilized, after they had stood 6 hours, with sperm from another individual, and three quarters of an hour later nearly all had divided normally into two cells. It was observed that the spermatozoa of this second individual, used for cross-fertilization, were also very inactive in their own fluid. They seemed to be more active in the extract from the ovary of the first individual. This ovary had also stood 6 hours in sea-water.

At the same time another experiment was made in which the eggs of another individual, that had been isolated for 24 hours, were put into ether solutions of 3, 2, 1 per cent. for 10, 20, and 60 minutes, and then returned to sea-water. None of these eggs divided, except one in the 3 per cent. solution (60 minutes). It was observed in this case that the sperm was inactive even in the ether-solutions, but nevertheless this same sperm, not in ether, cross-fertilized the eggs of the other individual in the preceding series, and also, as stated, appeared to be somewhat active in the extract of the ovary of the other individual.

Experiment IX. The eggs and sperm together were put into ether-solutions of 5, 4, 0.7 per cent. The sperm were active even in sea-water. The individual had been isolated for two days.

Ether.	10 min.	20 min.	30 min.
5.	0	12	0
4.	10	5	(7 out of 8 eggs)
0.7	12	10	(9 out of 10 eggs)

There was another series in this set in which the ether was stronger (about one-half saturated). None of the eggs from this solution segmented, but they became filled with clear spots. In another check series, self-fertilized but kept in sea-water, none of the eggs developed.

Experiment X. Sperm alone was put into ether solutions of 6, 4, 1, 0.5 per cent. It was removed (along with some of the surrounding fluid) and added to the eggs after 2 and 10 minutes.

Ether	2 min.	10 min.
6.	0	0
4.	0	0
1.	20	10
0.5	4	50

It appears from this experiment that it suffices to put only the sperm into the ether-solutions to bring about self-fertilization, but it should not be overlooked that a certain amount of the ether is carried over with the sperm when the latter is added to the eggs. The amount, it is true, will be small, since the eggs stand in water which further dilutes the ether, but so long as this source of error is present, and it is very difficult to remove it entirely, the result does not show conclusively that the ether acts on the sperm alone, although I think this is the more probable interpretation.

A check series of experiments was also made in which both eggs and sperm were put into solutions of the same strength as those given above, for 15 minutes and then removed to water.

Ether.	15 min.
6.	0
4.	0
1.	100 (but only ten eggs present.)
0.5	90

It is evident from both of the foregoing tables that only the weak solutions were effective, and from the first table it appears that this must have been the result of injury to the sperm. It can easily be seen that the eggs also are killed in a few minutes by a 6 per cent. solution of ether.

Experiment XI. Eggs from the oviduct were put into ether solutions of 1 and 0.5 per cent. for one-half and three-quarters of an hour; then washed in a small amount of fresh water and fertilized with the sperm from the vas deferens of the same individual. The experiment was carried out primarily in order to see if the eggs were affected by the solutions, so that they could be subsequently self-fertilized, but it is obvious that this test is not a good one, since the eggs will carry with them, despite the partial washing in water, some of the ether which may then act on the sperm. The results were as follows:

Ether	½ hour	¾ hour
1.0	0	0
0.5	4	5

Without a check series, which unfortunately was not made, it is difficult to decide whether the small number of eggs that were self-fertilized was due to the action of the ether on the eggs or on the sperm. The experiment must be repeated on a more elaborate scale.

Experiment XII. In this experiment with two individuals, weaker solutions of ether were used. In one lot the sperm alone was put into ether, and then added to the eggs. In the other lot both eggs and sperm were put together into the ether. I omitted recording the time in the ether, but it was probably about five minutes.

Ether	Sperm and Eggs in Ether	Sperm only in Ether
1.0	0	0
0.5	0	2
0.25	0	0

Ether	Sperm and Eggs in Ether	Sperm only in Ether
1.0	20	0
0.5	50	0
0.25	85	4

This experiment shows that the sperm of the first individual was incapable of self-fertilization, even with the ether present. In the other individual, the sperm was good, and there was a great deal of it; hence, no doubt, the excellent results in the first column. What is especially significant is that the best results were obtained when the eggs and the sperm were put at the same time into the solution together. This may mean the ether has some effect on the eggs as well as on the sperm, or that the most effective period of activity for the sperm is immediately after it comes into contact with the ether. My experiments do not suffice to settle this point, but that the spermatozoa are still capable of cross-fertilizing, after they have been in the ether for some time, is shown by the following result. After four hours the eggs of the first individual were mixed with the eggs and the sperm of the second individual. Later it was found that all the unsegmented eggs had been fertilized. The ether had no doubt largely evaporated.

The preceding twelve experiments with ether-solutions gave definite results, although in a few cases the number of eggs self-fertilized was small. It should be stated that there were ten other individuals in which self-fertilization in ether did not take place. This does not detract, I think, from the value of the successful experiments, because, as has been shown, the sperm is sometimes incapable of fertilizing even the eggs of another individual. The following experiments were carried out in order to examine this question further. It will be observed that parallel experiments with ether were also performed.

In each series five individuals were used. The eggs of each were fertilized with the sperm of every other individual. The following scheme shows the order in which the eggs were crossed. An individual having been opened, the eggs were removed from its oviduct and distributed in five dishes, A-A. Another individual was then opened (using, of course, different scissors, pipettes, etc.) and its eggs distributed to the next line of dishes, B-B. The same method was followed for the other three individuals. The sperm, a, of the first individual was then taken out and put into a small amount of water. It was then distributed to one set of eggs from each of the other individuals, B, C, D, E; then the sperm of B was taken out and applied to another set of eggs. The process was repeated until all the eggs were supplied with sperm. The

sperm in each case is indicated in the table by the small letter used as an exponent. The first set of A-eggs was as a rule fertilized with the e-sperm and the last set of E-eggs with the a-sperm.

Experiment XIII.—

E^a 100	E^b 100	E^c 100	E^d 100	E^a 85
D^a 100	D^b 100	D^c 100	D^e 100	D^e 100
C^a 98	C^b 100	C^d 75	C^d 75	C^e 100
B^a 99	B^c 100	B^c 100	B^d 100	B^e 100
A^e (omitted to add sperm)	A^b (omitted to add sperm)	A^c 100	A^d 85	A^e 99

In this experiment practically all of the eggs were fertilized by the sperm of another individual. When fewer than the total number segmented (fertilized), immature eggs may have been present. As a check series A, B, C, D and E were self-fertilized. None segmented, except in E, where two eggs out of the twenty present, *i. e.,* 10 per cent. divided.

The two ether series (self-fertilized) of these same eggs gave the following results:

Ether	A	B	C	D	E
0.5	10	1	0	80	30
1.0	50	2	0	0	0

Experiment XIV. An experiment similar to the last was carried out, with five other individuals, and gave the following results:

E^a 0	E^b 0	E^c 30	E^d 100	E^a 4
D^a 0	D^b 0	D^c 0 (one egg)	D^e (no ripe eggs)	D^e 100
C^a 0	C^b 0	C^d (no eggs)	C^d 100	C^e 100
B^a 0	B^c 0	B^c 0	B^d 50	B^e 100
A^e 0	A^b 0	A^c 0	A^d 100	A^e 100

Despite a few slight discrepancies in this table, the main result is clear. In only two of the five individuals was the sperm capable of cross-fertilization, namely, the e-sperm and the d-sperm.

There was also a self-fertilized series of these eggs, and in this not any of the eggs segmented. The ether series gave the following results:

Ether	A	B	C	D	E
0.5	0	0	0	0	10 (only ten eggs.)
1.0	0	0	?	2	50

It becomes evident from this result that the frequent failure of the sperm and eggs (mixed together) to self-fertilize in ether is due to the poor quality of the sperm. The poor sperm does not cross-fertilize, and presumably for the same cause it can not always be made to self-fertilize even in the ether. That sperm that is too poor to cross-fertilize may sometimes self-fertilize with the help of ether I hold to be possible. I regret that I did not attempt to determine whether poor sperm, that will not cross-fertilize, can be made to do so by means of ether, but other experiments lead me to think that it would often do so.

Experiment XV. In the following experiment all of the sperm appears to have been good except that of A, whose eggs, however, were in excellent condition.

E^a 2	E^b 85	E^c 90	E^d 0 (unfertilized)	E^a 0
D^a 1	D^b 100	D^c 20	D^e 90	D^e 100
C^a 70	C^b 100	C^d 100	C^d 100	C^e 90
B^a 5	B^c 100	B^c 100	B^d 100	B^e 100
A^e 0	A^b 70	A^c 100	A^d 100	A^e 100

It is clear that the a-sperm was poor, although it did well in C^a in which 70 per cent. of the eggs divided.

There was also a self-fertilized series in which none of the eggs segmented, except 5 per cent. in B. (In C, 90 per cent. of the eggs divided, but this may have been due to accidental contamination.) In the ether series the following results were obtained.

Ether	A	B	C	D	E
0.5	0	25	50	0	2
1.0	0	0	10	0	0

In this case although the spermatozoa of B, C, D, E were capable of crossing, they self-fertilized in ether very poorly, except in C, where good results followed.

Experiment XVI. In this experiment again only the first in-

dividual produced poor sperm, yet it did fairly well in one case, and in ether gave some results.

E^a 5	E^b 100	E^c 80	E^d 100	E^a 70
D^a 0	D^b 100	D^c 10 (few eggs)	D^e 100	D^e 30
C^a 0	C^b 100	C^d 100	C^d 100	C 100
B^a 25 (only 4 eggs)	B^c (no eggs)	B^c (no eggs)	B^c (no eggs)	B^e (no eggs)
A^e 75	A^b 100	A^c 100	A^d 100	A^e 100

In the self-fertilized series no eggs segmented. The ether series gave the following results:

Ether	A	B	C	D	E
0.5	2	4	2	30	0
1.0	50 (only 8 eggs)	12 (only 8 eggs)	0	10	30

The experiments recorded in Exp. XIII to XVI show that the sperm is at fault when cross-fertilization does not take place. In fact, eggs in the oviduct seem always to be capable of cross-fertilization. It is also evident that it is more difficult to get results with ether when the sperm does not cross-fertilize well, than when it does act well in this way. From this it seems to me very probable that when the ether fails to bring about self-fertilization the fault lies with the sperm. We may perhaps even go further and conclude that the action of the ether in bringing about the self-fertilization is on the sperm alone, but I am not in position to prove positively that the action of the ether on the eggs may not also enter into the result.

In concluding my account of these experiments on Ciona, I should like to point out that I had constantly in mind the possibility that the ether might produce parthenogenetic segmentation, and that the sperm had in reality nothing to do with the result. It was abundantly shown, however, that this was not the case, and in the few experiments in which I put this view to the test, by keeping eggs without sperm in ether-solutions of various strengths, I got no results when the eggs were returned to water. It should be noted in this connection that Lyon[1] has recently

[1] American Journal of Physiology, IX, July, 1903.

recorded that he was unable to cause artificial parthenogenesis in Ciona intestinalis at Naples by any of the ordinary means that excite this development in other eggs.

I shall discuss later the view as to whether eggs may be entered by the sperm of the same individual, but fail to develop unless incited to do so by some external agent.

It has been pointed out in the preceding pages that the eggs of Ciona may be fertilized after they have been in sea-water several hours. I made a test of this again in the following experiment:

Experiment XVII. Some eggs were cross-fertilized at once, others after 30, 80, 125 minutes, with fresh sperm from the same individual. All the eggs developed. A striking fact was observed in this case. The eggs fertilized late began to segment after a shorter interval than did those fertilized at once, so that at the 32-cell stage those fertilized last were only one division behind the first set, and no doubt soon caught up. It appears that a ripening process goes on in the egg as it stands in the sea-water, so that it begins to segment more quickly after it is fertilized than does an egg fertilized as soon as removed from the oviducts. It even appeared that after the first cleavage the rhythm of division was quicker in the eggs whose fertilization had been delayed, but this point needs a special examination which I have not yet made. The discovery is all the more significant because the first polar spindle is already formed in Ciona while the egg is in the oviduct, and the spindle remains resting in the equatorial plate stage until the egg is fertilized; hence the difference in time of segmentation can not be accounted for by the time required for the breaking down of the egg-nucleus and for the formation of the polar spindle after the egg has been removed from the animal. Some change must take place in the sea-water, which, while it does not cause the polar spindle to pursue its development, yet causes the developments that take place after the spermatozoōn enters to go on more rapidly.

EXPERIMENTS WITH CYNTHIA

The ovaries of Cynthia extend far forward, and have a very short oviduct. Each ovary—there appear to be two in each individual—is double, the halves being united at the distal end. Owing to the close proximity of the ovary to the surrounding tubes of the testis, it is possible only by very careful manipulation to get the eggs out of the cavity of the ovary without cutting into the testicular tubes. When it was necessary to separate the eggs from the sperm of the same individual, I have carried out this operation, but in general the ovaries and the testes were cut up together.

For the purpose of studying the effects of self-fertilization Cynthia is in many respects inferior to Ciona because self-fertilization takes place to a very large extent. On the other hand, if check experiments are used for each individual, this factor can be estimated, and the very fact that Cynthia does self-fertilize its own eggs to such an extent gives an opportunity to examine other aspects of the problem. A much more serious difficulty is met with in that artificial cross-fertilization is often unsuccessful in this species. Even when the eggs and sperm from a large number of individuals are mixed together, fertilization may not take place; but in curious contrast to this result are the following observations on the egg-laying processes of this animal kept in aquaria. On several occasions a number of individuals were put together in the same dish. About 5 o'clock in the afternoon one after another began to send out jets of eggs and of sperm producing the effect of a lively cannonading. Under these circumstances it was found that every single egg was fertilized. Perhaps only ripe individuals sent out their eggs and sperm, or perhaps the eggs were mature in all individuals, and the sperm from one or two individuals may have sufficed to fertilize all of the eggs. In general it is, I think, the sperm of Cynthia that is not good. Certainly the spermatozoa are often very sluggish when taken from the testis and put into water. May it not be possible that when the eggs are laid, Cynthia secretes some other fluid that makes the sperm active? This point needs further investigation.

The best means that I found to determine the extent to which self-fertilization of the eggs of Cynthia may take place was to isolate some of the individuals early in the day, and observe in those that emitted eggs and sperm in the late afternoon the percentage of eggs that segmented. The following four records were obtained in this way: For August 11—33, 10, 100, 95, 95, 75, 10 per cent. For August 16—30, 30, 10, 1, 75, 90, 85 per cent. For August 19—33, 0, 10, 4, 4, 0. For August 20—12, 4. A much larger number of individuals gave off neither eggs nor sperm, and some produced sperm and no eggs, and *vice versa*. The results in the above list show all conditions from perfect self-fertility to absolute self-sterility, although some of the latter cases may have been due to no sperm being given off.

A few preliminary trials were made with two (A and B), and with three (A, B, and C) individuals. The scheme of crossing is given in the following diagrams:

For Two Individuals.

A^a	B^b
A^b	B^a

For Three Individuals.

A^a	B^b	C^c
B^a	A^b	B^c
C^a	C^b	A^c

A few examples of the results with two individuals are as follows:

A^a 0	B^b 0	A^a 0	B^b 0
A^b few	B^a 15	A^b 10	B^a 10
A^a 0	B^b 0 (later rarely one)	A^a 0	B^b 0 (one egg)
A^b few	B^a 20	A^b few	B^a few
A^a 0	B^b 0 (one egg)		
A^b 50	B^a 0		

Comparing the self-fertilized eggs with the crossed-eggs, it is clear that while self-fertilization did not take place in nine cases, and in only one egg in the other case, yet cross-fertilization more frequently occurred, but never so completely as when many individuals normally deposited their eggs and sperm together. In addition to these cases there were three others in which none of the eggs, neither self- nor cross-fertilized, segmented. One of the results with three individuals is given in the next table:

A^a 0	B^b 2	C^c 0
B^a few	A^b very few	B^c 25
C^a 75	C^b rare	A^c 4

In this experiment the a- and c-sperm did not self-fertilize, but the former did well with C- and the latter with B-eggs. The b-sperm self-fertilized to a slight extent, but did no better with the A- and with the C-eggs.

In the next series the results are more striking:

A^a 0	B^b 0	C^c 0
B^a 95	A^b 50 (test not ripe)	B^c 1
C^a 50	C^b very few	A^c 50

Here none of the sperm self-fertilized the eggs. The a-sperm did quite well with the B- and C-eggs (95 and 50 per cent). The b-sperm did well with the A-eggs, but not with the C-eggs. The c-sperm did well with the A-eggs, but not with the B-eggs. It may appear from the preceding table that there is something more involved than simply the question of good sperm, for the same sperm appears to act differently with different eggs.

Another experiment with three individuals gave no eggs self-fertilized, but good cross-fertilizations with the c-sperm; less good with the b-sperm. These experiments should be carried out on a larger scale, and at different times of the year, but they suffice to show that self-fertilization is very infrequent when the process is an artificial one. It takes place to a considerable extent in some cases when eggs are normally laid. Moreover the artificially crossed eggs do not segment nearly so well in Cynthia as in Ciona.

The next experiment shows the action of ether on self- and cross-fertilized eggs. Some of the eggs and sperm of one individual, A, were removed and put into sea-water. Other eggs, A^a, were self-fertilized in an ether-solution, and a third lot, A^b, were crossed with sperm from B (A-sperm was also present). The same process was carried out with B which was crossed with sperm from A.

A 0	B 0
A^a few	B^b few
A^b very few	B^a very few

The results show that the self-fertilized eggs in ether did as well as those that were crossed, but none of the eggs in water alone, with their own sperm, segmented. Another similar experiment with two other individuals gave the following results:

A 0	B 0
A^a 0	B^b 0
A^b 50	B^a (only one egg 0)

In another set the ether appears to have been too strong, yet 50 per cent. of A^b divided.

In another experiment, 10 per cent. of the self-fertilized eggs in ether segmented, and 50 per cent. of the crossed.

In another, 5 per cent. of the self-fertilized eggs in ether segmented, and 75 of the crossed.

The next set is more instructive:

A 0	B 0
A^a 100	B^b 0
A^b 2	B^a 4

It is clear that the ether had a marked effect in A^a, making all of the eggs self-fertilize. This is all the more interesting because none of the eggs without ether self-fertilized. Both eggs and sperm of the B- set appear to have been in poor condition, so that the sperm did not cross-fertilize, or the eggs become cross-fertilized, to any extent.

In searching for other substances that might act on the spermatozoa as does the ether, I tried, amongst other things, a solution of ammonia in sea-water, and this I found made the spermatozoa even more active than the ether. Dilute solutions of alcohol from 1 to 10 per cent. also excite the spermatozoa to greater activity. Certain salt-solutions, ammonium chloride (1, ½, ¼ per cent.), magnesium chloride (2 per cent.), and sodium chloride (1 per cent.) appeared also to act on the sperm, but much less effectively than does ether, alcohol, or ammonia. In the alcohol series of 1, 3, 5, 6, 10 per cent., it was found that 1 per cent. made the sperm very little more active; 3 per cent. more so; 5 per cent. most active; 6 per cent, less; 8 per cent, no effect; 10 per cent., no effect. The last two solutions undoubtedly injured the sperm. In another series, 7 per cent. gave the best results.

A few experiments were carried out in order to see if the sperm made active by the alcohol, would self-fertilize the eggs when it would not do so without the stimulus. Here, as in the preceding series, the same lettering will be used in the tables. A^a self-fertilized in sea-water, A^a self-fertilized in alcohol-solution, A^b crossed in alcohol-solution.

A^a 0	B^b 0
A^a [Alcohol] few	B^b [Alcohol] several
A^b 20	B^a 50

In this experiment while no eggs were self-fertilized in sea-water, a few or several (the percentages were not recorded) were self-fertilized in alcohol, but even more developed in the crossed lots.

In another experiment only one individual was used. The eggs, self-fertilized in sea-water, did not segment, but 10 per cent. did so in a 3 per cent. soluton of alcohol, and 50 per cent. in a 5 per cent. solution of alcohol.

Solutions of ammonia gave similar results. Sperm and eggs were mixed together in very dilute solutions of ammonia. Many eggs divided and of these most appeared, from their method of division into several cells at once, to be polyspermic. Some of the sperm from the last lot was added to eggs in sea-water. Fewer eggs were fertilized, but several that were fertilized were polyspermic. Eggs (not separated from their own sperm) were crossed in ether. All of these were polyspermic. Another set gave almost identical results.

It is clear from these experiments that those solutions that make the spermatozoa more active often induce fertilization of the eggs, when such a fertilization does not take place without the use of the solutions. The activity of the sperm and the fertilization of the egg appear to be directly connected. This point will be more fully discussed later.

EXPERIMENTS WITH MOLGULA.

On each side of the body of Molgula there is an ovary surrounded by a testis. It is very easy to open the central cavity of the ovary, and remove the eggs without cutting the testis.

A few preliminary experiments showed that the sperm of Molgula fertilizes the eggs of the same individual. The following illustrations will show the great powers of self-fertilization of this species:

A^a 85	B^b 90	A^a 100	B^b 2
A^b 90	B^a 100	A^b 100	B^a 90 [irregular
A^a 100	B^b 100	A^a 0	B^b 100
A^b 100	B^a 100	A^b few	B^a 0
A^a 90	B^b 0 [1]		
A^b 100	B^a 100		

These cases make it clear that the sperm is capable of fertilizing the eggs of the same individual. Whether the sperm of another individual is prepotent I did not attempt to determine. There were only a few cases in which neither self- nor cross-fertilization was effective, and whenever good crossing was accomplished self-fertilization was also realized, showing that when the sperm is good, it will readily fertilize the eggs of the same individual. Since similar results were obtained when three individuals were used it will not be necessary to give the latter cases. The experiments were not extensive enough to show whether good sperm affects the eggs of certain individuals better than it does others, but Molgula is not well suited to test this point.

It occurred to me as possible that in Cynthia and in Molgula the power to self-fertilize the eggs might be due to the eggs coming from the ovary on one side of the body, and the sperm from the other side. Conversely, if this were true, the lack of self-fertilization in Ciona might be connected with the presence of only one ovo-testis. I examined this possibility for Molgula. The eggs from the small ovo-testis were fertilized with sperm from the same side, and other eggs with the sperm from the other side. In both cases all the eggs were fertilized. Conversely, the eggs from the large ovary were fertilized with sperm from the

[1] In B the sperm was probably bad. The A^b must therefore have been self-fertilized. The same conditions hold also for the second couple.

same side, and others with sperm from the opposite side. Here also all the eggs segmented. It is perfectly evident, therefore, that the question of self-fertilization in Molgula is not connected with the double condition of the ovo-testis.

EXPERIMENTS WITH OTHER FORMS.

In order to find out how generally ether, alcohol, and ammonia excite to greater activity the movements of cilia, of flagella, and of the spermatozoa of other animals, I made a few experiments on certain protozoa and on the spermatozoa of the frog and of the rat.

Ether, 5 per cent. stops the movements of paramoecium, and kills stentor; 3 per cent. slows up the movements of the former, and causes stentor to throw off its outer layer; the movements of free swimming vorticellae seemed to be increased; 2 and 1 per cent. hasten the movements of paramoecium and of stentor.

Alcohol of 6 and of 8 per cent. slow down the movements of paramoecium and stylonychia, and cause stentor to disintegrate; 10 per cent. kills; 4 per cent. appears to be near the limit, and seems to increase their activity; 2 per cent. clearly increases their activity.

Ammonia 1/200 per cent. kills paramoecium, stentor, and stylonychia; and even 1/2000 also kills; 1/5000 per cent. seems to make these protozoa somewhat more active, but I have not sufficiently tested this solution.

Some of the same solutions were used with euglena, which moves by means of an anteriorly directed flagellum. Ether 5 per cent. makes them somewhat more active; 3 per cent. less so, and 2 per cent. gives no very noticeable effect. Alcohol 10 per cent. kills; 8, 6, and 4 per cent. make them swim more actively; 2 and 1 per cent. give no definite result. Ammonia 1/200 kills; 1/2000 per cent. does not appear to make euglena more active, but other strengths should be tried.

A male spotted frog (*Rana halecina*) was killed in November; its testes opened, and the immobile sperm squeezed out into normal salt-solution. It was found that it took some minutes to get a noticeable effect. Ether 5 and 2 per cent. caused the spermatozoa to show some movement in the course of 15 minutes. Alcohol gave better results. A 10 and a 6 per cent. solution awakened the spermatozoa to activity; a 4 per cent. gave the best results of all. In no case, however, was the activity very great. No movements were detected in ammonia-solutions, but only two strengths were used.

These scattering and incomplete observations show that these substances are in all probability general stimulants for protoplasmic activity of certain kinds.

I have also made a few experiments with the spermatozoa of mice. The spermatozoa were taken directly from the testis of a mouse that had just been killed. The solutions were added to a drop of the sperm squeezed out from the testis into a drop of physiological salt-solution, consequently the dilution is greater than actually given by the percentage. In certain strengths of ether (5 per cent.) and of alcohol (8 per cent.) it appeared that the movement was increased; with ammonia I did not get satisfactory results. The observations are made more uncertain here because, when the testes are opened, spermatozoa in all stages of development are found, and are consequently acted upon differently by the solutions. It would be more satisfactory to use a larger animal and take the spermatozoa from the vasa deferentia, where they are all fully formed. It is certain, however, that alcohol and ether do not produce as great effects on these spermatozoa as they do on the spermatozoa of the ascidians and of some other marine animals that I have examined.

In one of the preparations of the mouse testis the water began to run out at one side and it became apparent at once that the spermatozoa all turned and headed up-stream. It has been recorded by Kraft that spermatozoa swim in the opposite direction to that in which the cilia of the oviducts act. My observation suggests that movement in this direction is not due to the spermatozoa swimming against the direction of the greatest action of the cilia, but against the stream that is produced by the cilia. The movement may be a simple physical phenomenon—the lighter tails of the spermatozoa being swept backwards by the current so that the heads are turned up-stream, and the contraction of the tail then causes the spermatozoön to travel in this direction.

In later experiments the sperm was taken from the vasa deferentia, and put first into distilled water where the spermatozoa remained quiescent. If a drop of salt-solution (water 100, NaCl 0.75) was added to a drop of water containing the spermatozoa, they became active in the course of a minute or less, and their activity continued to increase for several minutes longer, when they remained active for some time. If a drop of a 5 per cent. ether solution is added to a drop of water containing quiescent spermatozoa, no result is seen at first, but after ten minutes I have observed a slight vibration of the spermatozoa. If now after the ether has been added, a drop of the salt-solution is also added, the spermatozoa become active, but it is difficult to determine whether they become more active than when the salt-solution alone is present. Certainly there is no marked difference. If a drop of 8 per cent. alcohol is added to a drop of water containing the spermatozoa no activity is observable, but if then a drop of salt-solution is also added the spermatozoa begin to swim, showing that the alcohol had not injured them, although it had failed to arouse them to activity. Several strengths of KOH (3 per cent. and weaker) were tried, but without effect; yet if salt-solution was added later some slight activity was seen.

In another series of experiments the spermatozoa quiescent in water were first made active by adding the salt solution. If ether was then added no decided effect on the sperm could be seen when their activity was compared with that of check preparations of salt-solution only. It appeared sometimes as though the ether did make the activity more pronounced, and the movement of the spermatozoa appeared somewhat different in the two cases. In the ether the motion was more jerky, and in the salt solution more sinuous and normal.

The following solutions were also tried: The sperm was first put into a drop of water, and then a drop of the solution was added. $NaHCO_3$ 0.625 per cent. caused the sperm to vibrate rather actively; Na_2CO_3, 5.0 per cent. caused a little activity after five minutes; KCl, 0.5 per cent. caused greater activity than did the sodium carbonate, while CaCl caused somewhat less vibration.

These, and some other experiments that need not be described here, show that salt-solutions of various kinds have a marked effect in arousing to activity the inactive spermatozoa of the vasa deferentia. They also make active, spermatozoa that are quiescent in distilled water. On the other hand ether, alcohol, and ammonia, which proved so efficient for the spermatozoa of the sea-urchin and starfish, appear to have little effect on the spermatozoa of the mouse.

The more fundamental physiological question as to the nature of the action of these different substances I shall not attempt to discuss without a further basis of observation and experiment to go upon. Enough has been seen, however, to suggest that the substances act as a "stimulus," which is perhaps not dissimilar in kind from that which causes some eggs to begin to develop, or a nerve impulse to start, or a muscle to contract. Here also we may urge, as I have urged elsewhere[1] in opposition to Loeb's conclusion in regard to the action of certain agents in causing artificial parthenogenesis, that the nature of the stimulus is of such a kind that the result depends much more on the structure or the composition of the living thing than upon the kind of stimulus employed. So unstable is the living organization that the slightest change brought about in it by chemical or by physical means suffices to set into action a perfectly definite and pre-arranged series of events.

HISTORICAL REVIEW.

The action of ether, ammonia and alcohol on the speramtozoa of Ciona, arousing them to greater activity and thus, under certain conditions, bringing about the fertilization of the egg, raises the question as to whether in the higher animals a similar action may not result from the application of these and of other substances, and also whether the secretions of some of the glands connected with the reproductive system may not have a similar effect on the spermatozoa.

When I tried to find some substances that might bring about self-fertilization in Ciona I was not aware that there had already

[1] Science. N. S. XI. 1900. Pp. 178-180.

been made several experiments on the action of solutions on the spermatozoa of other animals. I find that there are quite a number of observations of this sort, although none of the observers have had in view the same question with which I was especially concerned.

Kölliker in 1856 carried out an extensive series of experiments on the effect of different solutions on the spermatozoa of the bull, dog, rabbit, horse, and also made a few observations on the spermatozoa from a human cadaver. He found that water alone quickly brings spermatozoa to rest, but does not kill them. They can be aroused to activity by adding, for instance, a 10 per cent. solution of disodium phosphate.[1] Many other substances were found favorable to the activity of the spermatozoa, such as blood-serum, sugar in certain strengths, sodium chloride, caustic potash, etc.

The caustic alkalies (potassium, sodium, and ammonium hydroxide) were found to be especially powerful excitants. Kölliker also tried a number of other solutions, such as three different kinds of sugar, glycerine, gum, etc., which in certain strengths cause increased activity; also urea, gall, morphine, strychnine (nitricum), which have an indifferent effect. He also tried alcohol, creosote, chloroform, ether, alkaloids, and tannin, which have an injurious effect. Kölliker also examined the action of the secretions of the glands of the male reproductive organs—the uterus masculinus, prostate and Cowper's glands. He found that these secretions excite the spermatozoa to greater activity.[2]

The much more recent experiments of Steinach bear even more directly on the present problem. He found that after removal of the glandulae vesiculares ("receptaculum seminalis" of some writers) of the male rat, that, although the sexual instinct remained, the number of young that were born was much decreased. When

[1] Kölliker gives the formula $2NaOHOPO_5$, which is no doubt disodium phosphate, now written Na_2HPO_4.

[2] Moleschott and Richetti are quoted by Kölliker as recognizing the favorable action of sodium salts on the spermatozoa. Quatrefages found that the spermatozoa of the weasel showed a "surexcitation" in 64 parts sea water to one part sea salt. Newport found that potassium carbonate, and also 1/480 of potassium salt made the activity of the spermatozoa of the frog greater.

this gland, as well as the prostate, was removed no young at all were born, although frequent union with the females took place. The results may be due to the semen being insufficiently diluted when it is not mixed with the secretions of the glands, or else to the absence of proper excitation of the spermatozoa when the gland-secretion is removed. That the spermatozoa may be normally acted upon by the secretion of the glands was shown by Steinach in the following way: Sperm from the vas deferens was mixed with a physiological salt-solution. A drop was placed under a cover slip and the edges sealed to prevent evaporation. The preparation was kept at a temperature of 35° to 37° C. A similar preparation was made with the secretion of the prostate. In the former the spermatozoa began to lose their activity in one and a half hours, and after three hours had come completely to rest. In the other preparation, that containing the extract from the prostate gland, the spermatozoa were active after 11 hours, and ceased to move altogether only after 22 hours. This experiment shows that the secretion of the gland prolongs greatly the period of activity of the spermatozoa. Whether it excites them to greater activity is not stated, but Kölliker's results leave no doubt on this score. The decrease in the fertilizing power when the glands were removed may well be connected, as suggested above, with the lessened activity of the spermatozoa.

Buller has recently studied the question as to whether the spermatozoa of the sea urchin are attracted to the egg,— in other words, whether, as some authors have assumed off-hand to be the case, there is a chemotactic action of the egg on the spermatozoa. He points out that although Strasburger claimed that the egg of Fucus excretes a substance that attracts the spermatozoön from a distance of two diameters of the egg, Bordet and Buller himself have failed to confirm this statement. Massart thinks that in the case of the frog the meeting of the spermatozoön and the egg is purely accidental. Buller finds for the sea-urchins, Arbacia, Echinus, and others, that when the spermatozoa are set free near the egg they show no tendency to swim towards it. The dense collection of spermatozoa that forms around the egg is due to those that happened to run into the jelly sticking there. These

spermatozoa then proceed to bore into the jelly; most of them in a radial direction, although a few can be seen to go in obliquely, or tangentially. The same phenomenon occurs in unripe eggs, and in eggs that have been killed in weak osmic acid and the acid washed out. It is improbable, therefore, that chemotaxis has anything to do with the result.

In order to see if any substance is given off by the eggs that attracts the spermatozoön, the eggs were taken from the ovary, carefully washed, and allowed to stand for 2 to 12 hours in a small amount of sea-water. Capillary tubes were then filled with this water and placed in a drop containing spermatozoa. The spermatozoa did not show any tendency to collect around the openings of the tubes. Several other substances were tried in the tubes in the same way,— salts, sugar, ferments, acids, alcohol, etc.—but no chemotaxis was discovered.

The spermatozoa of the sea-urchins swim in spirals. Coming into contact with a surface, the spiral is changed to a circular movement due to contact. Buller considers whether the radial path taken by most of the spermatozoa after they have entered the jelly is due to stereotropism. He reaches the conclusion that while theoretically this assumption will explain the phenomenon, yet conclusive evidence in favour of this view is lacking. He suggests that it may be possible to find a purely physical solution of the problem.

Von Dungern has examined the question of cross-fertilization from the point of view of the different substances contained in the egg, and has reached some conclusions of the greatest interest. He finds that the egg of the starfish, *Asterias glacialis,* contains a substance that acts as a poison on the sperm of the sea-urchin (Echinus or Sphaerechinus). The minimal lethal dose for the sperm mixed with 2 ccm of sea-water varies considerably with the individual; for Echinus between 1/800 to 1/6400 part is fatal in half an hour. Von Dungern tried to obtain an antitoxin from the blood of the rabbit that would neutralize the effect of the poison of the eggs, hoping that it might be possible in this way to bring about the cross-fertilization of the egg of the starfish by the spermatozoön of the sea-urchin. He found, however, that the serum of the normal rabbit already contains a substance that has a powerful antitoxic action on the poison of the starfish, so that it was not necessary to obtain an antitoxin by injecting the poison into the rabbit. The antitoxin of the rabbit's serum was added to water containing the eggs of Asterias, and then sperm from a sea-urchin was supplied. Von Dungern often obtained two- and four-cell stages in this way, but the results were uncertain, and he could not decide whether fertilization had or had not taken place. It seems not improbable, I think, that the outcome may have been due to artificial parthenogenesis which occurs very readily in the eggs of certain starfish; in fact, it is very difficult to prevent its occurrence, unless the eggs are very carefully handled.

The same poison that is present in the eggs of the starfish is also secreted by the skin. It is also rendered harmless by the rabbit's serum. In the sea-urchin there is a poisonous substance in the gemmiform pedicellariae, which is very injurious to the sperm of the starfish. If 100 of the pedicellariae of Sphaerechinus are rubbed up in one ccm of sea-water, the solution will destroy in a quarter of an hour the sperm contained in ten to twenty litres of sea-water. The minimal lethal dose for 2 ccm is 1/5120 to 1/16240 ccm. The spermatozoa of Sphaerechinus itself are killed by this fluid, but a much stronger dose is necessary. On the other hand an extract of the egg of Echinus, Sphaerechinus, Strongylocentrotus, or Arbacia does not kill the spermatozoa of the starfish even in the strongest solutions. What then prevents the spermatozoa of the starfish from entering the eggs of these sea-urchins? There is another factor, Von Dungern thinks, that interferes with this combination. The egg membrane of these urchins has an agglutinizing effect on the spermatozoa of the starfish. This agglutinizing effect appears to be the same phenomenon as that seen "whenever cells of any kind are introduced into the body of another animal." So far as this process is involved in the union of germ-cells, Von Dungern thinks that under certain conditions it might assist the fusion, while under others it might interfere with it. Thus two naked and equivalent cells might be helped to unite, while an egg surrounded by an agglutinizing jelly would fail to be fertilized. The substance in the sea-urchin's egg that agglutinizes the starfish sperm can be rendered ineffective by the rabbit's serum. Not all starfish spermatozoa are agglutinized by the jelly or by the egg-substance of all the different sea-urchins. In Sphaerechinus it fails to occur. Therefore in this case the failure to cross-fertilize must be due to some other factor, and, in fact, Von Dungern claims to have found still another substance in the sea-urchin's egg that excites to greater activity the immature and quiescent spermatozoa of the starfish. These immature sperm, made active by this substance, are then capable of fertilizing the eggs of the starfish. He found that weak doses of chloral hydrate and of cocaine also make these quiescent spermatozoa active, and that rabbit's serum has a marked effect. Von Dungern believes further that these exciting substances may actually prevent, in certain cases, the cross-fertilization, because they may change the kind of reaction shown by the sperm. He observed that the spermatozoa of those species that do not normally show rotational movements when they come in contact with surfaces, usually do so when the exciting substances just mentioned are present. It does not appear to me, however, that this is an altogether satisfactory explanation of the failure of cross-fertilization in these cases.

Von Dungern also examined the question as to whether the egg secretes a substance that favours fertilization by its own sperm. He believes that he has also discovered such a substance. The eggs of Echinus (or of Sphaerechinus) are rubbed up and mixed with pieces of jelly that have been carefully washed. When sperm is added to the water in which such pieces lie they stand vertically to the surfaces of the pieces. If on the other hand the pieces of jelly are not mixed with the substance from the egg, the spermatozoa simply rotate on the surface of the jelly, and do not stand vertically. Starfish spermatozoa with Arbacia jelly behave as with simple jelly alone, *i. e.*, they do not stand vertically. The vertical position of the spermatozoa is due, Von Dungern thinks, to the presence of some substance in the extract that lowers the excitability of the spermatozoön to contact, and hence it takes a vertical position. He also points out that this same substance causes the spermatozoa to lose their power of movement in a short time. Thus, while Von Dungern finds no evidence of a substance in the egg that attracts the sperm, he believes that there may be present in some eggs a substance that favours the fertilization of the egg, by causing the spermatozoön to assume that position in the jelly that is most likely to bring them to the surface of the egg.

Loew has attempted to show by an experiment, which is not, I think, well suited to prove his point, that the spermatozoa of the rat are attracted to, *i. e.*, that they are positively chemotactic to, the slime layer of the uterus and also to the alkaline mucosa of the digestive tract, but not to the acid slime of the vagina. His method of experimenting was as follows: A piece of the mucosa of the uterus was put on one side of a slide and a piece of the vagina on the other. A drop containing the sperm was placed in the middle of a cover-slip, and this put over the pieces on the slide. It was found that the sperm collected more on the side near the piece of the uterus, and from this Loew infers that they have been attracted to this side. In the light of the other experiments described above it will be clear, I think, that the greater accumulation of the sperm on one side by no means establishes the conclusion that they have been attracted to this side. Loew tried to show that filter paper saturated with alkaline substances acts chemotactically on the spermatozoa, in the sense that they move towards such substances, but, as in the preceding case, it does not necessarily follow because spermatozoa collect around or in certain substances, therefore they must have moved towards these substances. The recent work of Jennings on the protozoans shows that their accumulations in certain areas is not due to the action of substances that cause the individuals to swim towards those substances, but on the contrary to their action being such that those individuals that enter areas containing these substances are unable to leave them. The *result* is the same as when the spermatozoa touch the jelly of the egg and stick to it, although the *means* by which the accumulations are formed in the two cases are entirely different. It would be interesting to see if spermatozoa may not behave towards certain solutions as do the protozoans.

THEORETICAL.

It has been often assumed by embryologists that there exists some sort of attraction between the eggs and the spermatozoa of the same species. This idea would readily suggest itself to anyone who saw spermatozoa collecting in crowds around the eggs, but it by no means follows that this phenomenon is really due to an attracting substance emanating from the egg. The result may be due to the membrane of the egg, to which those spermatozoa stick that come accidentally into contact with it. In fact I have observed similar collections of spermatozoa in the ascidian around pieces of the body tissue, where the result had every appearance of being due to some sticky substance, exuding from the piece, rather than to an attraction exerted by the piece on the spermatozoa.

Pfeffer's oft-quoted experiment with the antherozoöids of ferns, liverworts, etc., appears to support the idea that the antherozoöids are attracted to the malic acid that is present in the neck of the archegonia, but in the light of the recent experiments of Jennings and others, as to the way in which unicellular forms accumulate in a drop of acid, we can readily see that the results may have a very different interpretation from that usually given to them. Confining our discussion to the results obtained with the ascidians, I offer the following tentative analysis of the problem:

The failure of the spermatozoön of Ciona to enter the egg of the same individual may be conceived as due to some physical obstacle. It is conceivable that pores may exist in the egg-membrane, or even in the surface of the egg itself. This is the argument that Pflüger[1] used in the case of cross-fertilization of the frog's egg. If in the ascidian there existed a correlation of such a sort, that the size of a spermatozoön of a given individual is always greater than the pores of the eggs of the same individual, then self-fertilization could not take place. That this is not the real explanation is shown by the fact that good spermatozoa are apparently capable of fertilizing the eggs of all other individuals. This would certainly not be the case if the exclusion of the sperma-

[1] Archiv. f. die gesammte Physiologie, XXIX., 1882.

tozoön from the egg of the same individual was due to the size of the pores, because there would be eggs of some other individuals having pores as small or smaller. Another possibility that suggests itself is that the surface tension of the egg is of such a sort that it excludes the spermatozoa of the same individual, but this idea does not appear to give a satisfactory solution, for, aside from the fact that it is difficult to imagine how such a relation could exist, there would also occur cases in which the surface tension of the eggs of other individuals would exclude certain sperm, and this does not appear to be the case. It is true that the addition of the ether to the water may cause a difference in the surface tension of the egg, and it might be made to appear that this was the way in which the self-fertilization is effected in the ether-solutions, but I can not believe that this is the explanation of the results, because other experiments show that a considerable amount of ether is necessary to cause self-fertilization.

It seemed to me that violent shaking might so affect the surface of the egg that self-fertilization might take place. A number of eggs from the oviduct were violently shaken for a few minutes in a small vial, and then sperm from the same individual was added. No segmentation took place, and the presumption is therefore that the eggs were not fertilized.

Turning to the chemical side we find a number of possibilities that demand consideration. The inactivity of the immature spermatozoa, and the lack of power of such sperm to fertilize the egg, their becoming active in certain solutions, and their power then to fertilize eggs that they did not fertilize before, as best shown in Cynthia, suggests that normally the eggs may secrete certain substances that make more active the spermatozoa, which then become capable of fertilizing the eggs. This view appears all the more attractive in the present case on account of the observed lethargy of the spermatozoa of these ascidians, and the apparent connection in such cases between this condition and the impotence of such sperm in fertilization. Yet after careful consideration I am not prepared to advocate this view as the only solution, although I realize that it might be made to give the appearance of a ready explanation of my results. Not that this induced activity may not

be one of the factors to be taken into consideration, but it is not, I think, the whole explanation. My reasons for regarding this view as insufficient are the following: It was found that sperm that appeared to be very little active was sometimes capable of cross-fertilizing the eggs of another individual. Possibly this may be due to somewhat greater activity induced by something secreted by the eggs of the other individual, yet on the whole I can not claim that direct observation gave any convincing evidence in favour of this assumption. More significant are the results of the experiment of mixing eggs from two individuals, and subsequently fertilizing them with the sperm from one of the individuals. Half only of the eggs segmented, presumably those cross-fertilized. If some substance that makes the sperm active were really thrown out by the eggs, then we should expect that all the eggs would have been fertilized, unless indeed the secretion loses its power a short distance from the surface of the egg that secretes it; but this does not seem to be a probable interpretation.

A different point of view is that the egg secretes some substance that attracts the spermatozoa. On this view we must suppose that the substance secreted by the egg of Ciona has no attraction for the spermatozoa of the same individual.

The little evidence that I have to offer, based on experiments with ascidians, is not favorable to this idea, that the cross-fertilization is due to some attractive substance secreted by the egg. In the species that I have examined there is no such marked accumulation of spermatozoa around the eggs as is seen in many other animals, and nothing in the behaviour of the cross- and self-fertilized egg to suggest that the difference in the results is due to an attraction in the one case, and to the absence of an attraction in the other. In other forms where there is a better opportunity for examining this question the most recent observations go to show, as has been pointed out in detail above, that there is no sufficient evidence for the view that the egg attracts the spermatozoön.

Conversely, it may be supposed that the egg secretes some substance that *repels* the spermatozoa of the same individual. I observed nothing that would support such a conclusion, and this interpretation of the process would be foreign to what we find in general in connection with fertilization even in cases where the sperm of one species does not fertilize the eggs of another.

We come now to a more subtile argument, and one that we are scarcely in position to discuss profitably in our present state of ignorance concerning the union of egg and spermatozoön. It may be assumed that there is some sort of "chemical affinity" between the egg and the spermatozoön that causes the two to unite when they come together. On this assumption we should have to suppose in Ciona that this affinity does not exist, or at least is less strong, between the egg and the spermatozoa of the same individual than between those of different individuals. Such a statement carries us no further, however, than the facts, and in the case of Cynthia we should have to assume that the affinity is so nicely balanced that sometimes the spermatozoön can unite, and sometimes it can not. In the case of Molgula the affinity must be assumed to suffice to bring about self-fertilization. Until we can give some more tangible form to this idea it does not appear to have any greater value, than the mere statement of the facts, and indeed may have less value, since it may give a wrong impression as to the real factors at work.

Finally there might be advanced what may be called the electro-chemical hypothesis. The union of the egg and the spermatozoön may be supposed to be an electrical phenomenon, connected with a difference in the chemical composition of the two elements. The sperm head is almost pure nuclear chromatin, while the surface of the egg is protoplasmic. Possibly the spermatozoön and the egg have different electrical charges and unite with each other if brought near enough for the charges to become effective. But on this supposition it is not clear why the eggs and the sperm of the same individual would not unite. Here also we get no light on the absence of self-fertilization in Ciona.

I have kept constantly in mind while at work on this problem the possibility that the spermatozoön may really enter the egg, but fail to develop there, or fail to start the development of the egg, because, coming from the same individual, it was not sufficiently different in composition to supply the necessary stimulus. The ether might be supposed to make the sperm sufficiently different from the egg to start the cleavage, or the ether might itself supply the stimulus which is capable of starting the development of the egg *after* the spermatozoön has entered.

The test of this view should be found in direct observation of the eggs themselves. I prepared therefore a series of eggs of Ciona, some unfertilized for check series, others self-fertilized, but not put into ether, and others like the last, but put into ether.

The difficulties of determining whether the spermatozoa can enter the eggs of the same individual, but fail to start the development, are greater than may appear at first sight. The sperm head is so minute that if after it entered no changes were affected in the protoplasm about it, its presence might be readily overlooked, and since the spermatozoön of Ciona enters the egg in a granular zone that colors more deeply in certain stains than does the rest of the egg, the difficulty is thereby increased. Of course I have been on my guard against cases where the surrounding sperm have floated over the section, as sometimes happens, or have been carried over it by a defect in the knife, and I have also been careful to exclude all cases where specks of foreign matter may have been on the slide, or in the fixative. There are also two further precautions to be taken. When the egg withdraws from the membrane and the test-cells are extruded, as it were, from the outer zone of the egg, the protoplasm is sometimes drawn out in mamiliform processes that stain deeply and resemble the entrance cone formed by the spermatozoön penetrating certain eggs. Even when the protoplasm does not protrude, deeply staining spots are generally present and are especially obvious after iron haematoxylin. Careful staining with Delafield's haematoxylin shows clearly that these spots have nothing to do with the entrance of spermatozoa. Furthermore these spots are found in unfertilized eggs. After iron haematoxylin minute deeply staining bodies, flattened against the outer surface of the egg, can generally be found, and these strongly suggest spermatozoa. That they are not such is shown by their presence in unfertilized eggs, and by their absence after the Delafield stain. I mention these points because they might easily lead one after only a casual examination to conclude that spermatozoa enter the eggs. My best results have been obtained by drawing out the iron haematoxylin until the protoplasm has lost all of its color, or better still by using the Delafield stain, and also thoroughly extracting the color from the protoplasm.

Although I have examined a large number of preparations I have not seen a single definite case without ether in which a spermatozoön has entered the egg of the same individual. Difficult as it admittedly is to be absolutely certain on this point, yet if the spermatozoa had entered and had begun to enlarge I feel certain that I should have detected their presence. That undeveloped sperm-heads may be present I must admit as a possibility, but I have not detected them, and believe that I should have been able to do so were they present. It is also a point of some importance that I have not found any spermatozoa within the egg membrane, although quantities of them may lie outside.

There is a further point in this connection, the importance of which I did not appreciate until I had closed the experimental part of my work. In the eggs of many animals a change takes place in the egg, after the penetration of one spermatozoön, of such a sort that the entrance of more spermatozoa is prevented. I have found in Ciona that, after the sperm has stood with the eggs of the same individual and has failed to fertilize them, these eggs could still be readily fertilized by spermatozoa from another individual. If a spermatozoön of the same individual really enters the egg it does not in consequence bring about such a change in the egg that other spermatozoa can not enter, and therefore many spermatozoa of the same individual from which the eggs were taken should be expected to gain entrance, but I am quite certain that this, at least, does not occur. From this consideration also it may be inferred that the spermatozoa do not normally penetrate the eggs of the same individual.

In the light of these observations it seems probable that whenever a spermatozoön enters the egg, the egg begins to develop regardless of whether the spermatozoön comes from the same or from another individual. The ether must therefore induce a change

of some sort that directly effects the entrance of the spermatozoön into the egg, and at present I see no other interpretation that is left than that this entrance is due to the greater activity of the spermatozoön that causes it to overcome some resistance, either on the surface of the egg itself, or in the membrane surrounding it. The nature of this resistance I did not detect, and this must be the next step in the analysis. One method by which this view may be tested is obvious, and has already been referred to. The spermatozoa made active by sea-water must be placed in an extract of the eggs (or body-tissues) of the *same* individual, and then, after a time, the eggs of another individual added. On the hypothesis these eggs *should be less likely* to become fertilized than eggs placed directly in contact with the fresh sperm.

It has been found that certain substances secreted by the glands of the reproductive organs of the male mammal arouse the spermatozoa to greater activity. It has also been found that many other substances have a similar effect on spermatozoa. It would be equally interesting to discover if the secretions of other parts of the genital ducts of the male or of the receptacula of the female, when such are present, may not bring the spermatozoa to rest, or keep them quiescent until some other exciting agent arouses them. It seems almost certain that this must be the case in those animals in which the spermatozoa of the male are stored up in receptacula of the female, as for instance in the honey bee, or in such a hermaphroditic animal as the earthworm. The length of life of the spermatozoa in some of these forms would seem to make some assumption of this sort necessary. Experiments can easily be made that would decide this question. Kölliker has shown, in fact, that water quiets the spermatozoa of mammals without killing them.

In the ascidians it is probable that the spermatozoa in the vas deferens are quiescent. It is significant that in these hermaphroditic forms the oviduct in which the eggs are stored takes a course parallel to the male duct. Possibly the proximity of the two ducts may be connected with the lack of power of self-fertilization of the eggs, because the egg may be saturated with the same substances that keep the sperm quiescent. It may be, however,