

STUDIES OF FERTILIZATION¹

V. THE BEHAVIOR OF THE SPERMATOOA OF NEREIS AND ARBACIA WITH SPECIAL REFERENCE TO EGG-EXTRACTIVES

FRANK R. LILLIE

The Hull Zoölogical Laboratory, University of Chicago

FIVE FIGURES

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¹ The earlier studies of this series bore the general title "Studies of fertilization in Nereis."

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I. INTRODUCTION

The earlier studies of this series dealt exclusively with *Nereis* and concerned the cortical changes of the egg, partial fertilization, the morphology of the normal fertilization and the fertilizing power of portions of the spermatozoon. They yielded certain positive results which I need not review, but they had convinced me that other methods than the ones usually in vogue, including the methods of artificial parthenogenesis, are needed for a closer approach to some fundamental problems of fertilization. Some incidental observations drew my attention to the study of the behavior of the spermatozoa, and investigation of the subject soon showed that the reactions of these minute active reproductive elements might furnish evidence of considerable significance. This study was begun in the summer of 1911 and continued throughout the summer of 1912 at the Marine Biological Laboratory.

With the publication of Loeb's first study on artificial parthenogenesis the study of fertilization entered upon a new phase which has not yet run its entire course. The tendency during this phase of investigation has been to regard initiation of development as the fundamental problem of fertilization; and the aim has been to discover the way in which the spermatozoon induces development of the egg. Hence the term 'chemical fertilization'

has come to be used loosely as practically synonymous with artificial parthenogenesis, as though a salt solution could take the place, and play the rôle, of the spermatozoon. This it can do obviously only with reference to the initiation of development, which, so far from being the only function of fertilization, is more properly to be regarded as a secondary function, or better a separate phenomenon which is sometimes associated with fertilization, sometimes not. On the one hand we may have initiation of development without fertilization, as in parthenogenesis and all asexual modes of reproduction, and on the other hand the phenomenon of fertilization without initiation of development is extremely common, as in the so-called winter eggs of Cladocera, Aphids and Rotifera, where fertilization is followed by a long resting period; the Protozoa and unicellular algae also offer many instances in which fertilization is the immediate prelude to a long resting stage.

The study of initiation of development by chemical means has yielded results of prime importance, and the consequent absorption in these problems has been an inhibiting factor in the analysis of other problems of fertilization. Thus, as spermatozoa are not necessary for "chemical fertilization," the study of their behavior has been largely neglected. The problem of specificity has as a consequence been left almost entirely out of account, for there is no specificity in salts, or even in the blood sera of animals of other phyla; nevertheless specificity in reaction of sexual products is a much more nearly universal phenomenon of fertilization than initiation of development, and it is quite possible that the solution of this problem may furnish a valuable clue in the study of the latter problem. In any event, the time seems ripe for the development of new methods of attack on the fundamental problems of fertilization. The present contribution is a step in this direction. I have taken up the study of the behavior of the spermatozoa, because it represents, after all, considered in a broad sense, one-half of the problems of fertilization, and it seems probable that these small motile cells may prove better indicators of some of the reactions involved in fertilization than the slowly reacting egg.

There are three categories of behavior exhibited by spermatozoa that seem to me of importance for the problem of fertilization, because all are exhibited in response to egg secretions. These are (1) activation, (2) aggregation, (3) agglutination. The phenomena of activation are involved in those conditions that affect the activity of spermatozoa. The phenomena of aggregation are positive taxic responses, for the most part chemotactic. The phenomena of agglutination are exhibited in the presence of substances that cause the spermatozoa to adhere in masses. In a preliminary paper I have described some aspects of these phenomena (*Science*, N. S., vol. 36, October 18, 1912). We may consider the subject matter under these three heads.

The spermatozoa of marine animals in which fertilization takes place in the sea-water offer advantages for study probably greater than those of any other forms, because the conditions of normal activity are given in the sea-water itself, no secretions of accessory glands either of the male or of the female being requisite. Moreover, the spermatozoa may be obtained in large quantities. They offer, thus, material directly accessible to experimental work with the simplest possible facilities. Among the forms available for work, *Nereis* and *Arbacia* were soon found to be best adapted because the breeding season extends through most of the summer and they furnish material in large quantities. The present paper is therefore confined almost exclusively to these forms.

Suspensions of the spermatozoa in sea-water formed the material for all of the experiments. The reactions vary somewhat according to the density of the suspensions, and it may be important in future experiments to find some quantitative method of expressing the variations in density. But for the purposes of this paper it will be sufficient to indicate the extremes as opalescent, milky and creamy, with intermediate qualifications. An approximation to uniformity was attained in many of the experiments by adding a certain number of drops of the dry sperm² to measured quantities of sea-water.

² By 'dry sperm' is meant the sperm as it comes from the testes without the admixture of fluid.

II. ACTIVATION PHENOMENA

A. NEREIS

1. *The aggregation reaction*

We may begin the discussion of the behavior by describing a phenomenon which was used throughout the experiments with Nereis as a test of the activity of the spermatozoa. A drop of dry sperm from a mature Nereis is mixed in about 6 cc. of sea-water in a Syracuse watch crystal, making a uniformly milky suspension; in a few seconds clouds begin to appear and in fifteen to forty-five seconds these usually draw together in dead-white solid-looking masses uniformly spaced throughout the fluid. The intervening fluid becomes quite clear and the masses quickly settle on the bottom. The rate of formation of these masses, their number and size, depend on temperature, 'freshness' of the sperm and other conditions discussed beyond. Any sperm suspension that exhibits the aggregation phenomenon will be called 'aggregative' in the experiments that follow.

The appearance is of course due to the aggregation of the sperm in closely packed masses. Under a low power of the microscope each mass appears like a swarm of bees, owing to the intense activity of the peripheral spermatozoa. But those in the interior of the dense mass must be quiescent or the masses would break apart. After the aggregations have settled to the bottom of the crystal they tend to flatten out and may run together in time to a greater or less extent.

If, immediately after settling of the aggregations, the sperm is mixed up with a pipette, a perfectly uniform milky suspension is again produced, which may aggregate a second time, but more slowly than the first time, and in fewer aggregates; and the intervening fluid remains quite opalescent, showing that all the spermatozoa have not joined the aggregates. A third mixing up is not usually followed by aggregation until after the spermatozoa have settled to the bottom, and then only a very partial aggregation results.

A considerable number of variations of this theme can be produced by using sperm suspensions of varying density contained

in vessels of varying form, et cetera; under certain conditions the aggregations may arise in conformity with the water currents set up by the last emptying of the pipette, et cetera. But a description of these variations would be useless without the analysis of the causes of the phenomenon, which is taken up later.

All the experiments on *Nereis* to be described beyond were made with aggregative sperm, so that there was always a test, which had the advantage of being macroscopic and quick, of the activity of the sperm used in the experiments, and this has much to do with uniformity of results.

To give a more concrete idea I reproduce three photographs, natural size, of the phenomenon of aggregation. The first (fig. 1) was taken ninety seconds after mixing a drop of dry sperm in about 8 cc. of sea-water. The aggregations are quite uniformly distributed except in the upper right quarter where their arrangement marks out original currents produced by mixing with the pipette. Figure 2 was likewise taken ninety seconds after mixing; the effect of water currents on the arrangement of the aggregations is shown here quite well on the left. Figure 3 was taken three minutes after mixing, and the separate aggregations are beginning to fuse together on the bottom.

I propose to discuss in this section simply the conditions which modify the activity of the spermatozoa. In the case of *Nereis* such conditions may be inferred from two kinds of observations, namely: (1) The appearance of activity presented to the eye under the microscope and (2) the rate and degree of the aggregation reaction which is macroscopic. *Nereis* is the only form with which I am familiar that exhibits the latter reaction in any marked way. Its sperm is therefore better adapted than that of any other species for study of conditions of activity. The observations of different samples of sperm under the microscope are very difficult to compare as to degree of activity, as one is never sure of the successive subjective impressions, but in the case of *Nereis* these can be checked by the aggregation reaction.

The principle conditions that affect activity are 'freshness,' temperature, and the chemical constitution of the medium. These conditions will be considered not exhaustively at all, but

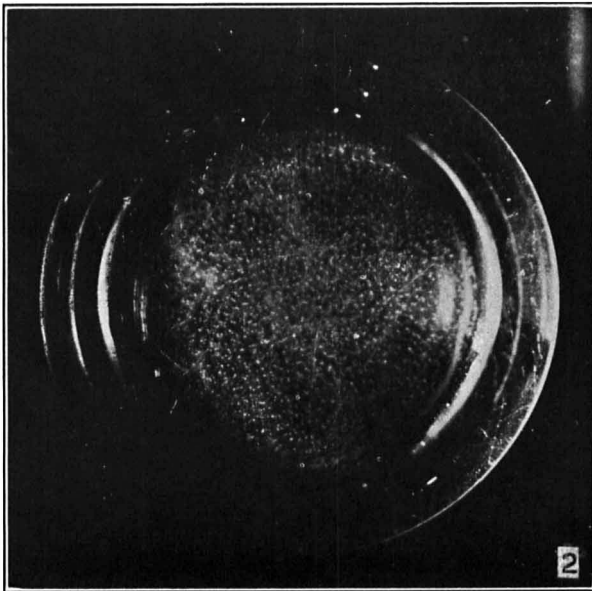
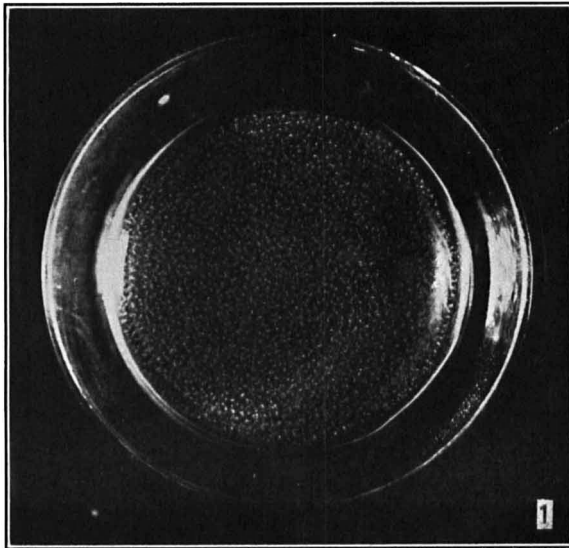


Fig. 1 Photograph of aggregation of a *Nereis* sperm suspension, taken 90 seconds after mixing the suspension; natural size; description in text.

Fig. 2 Another suspension photographed at 90 seconds; description in text.

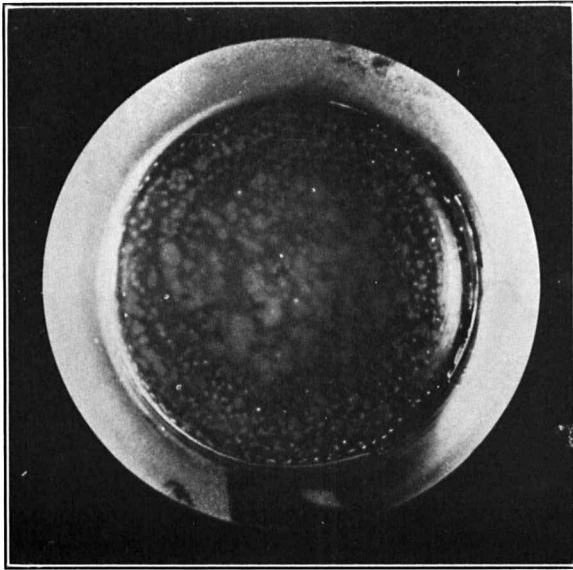


Fig. 3 Another suspension photographed 3 minutes after mixing; description in text.

only to the extent that they appear to be significant for the phenomena of aggregation and agglutination, which are the main problems for our consideration.

2. Individual movements of the sperm

To explain the various reactions of the sperm it is necessary to consider first some of the more obvious features of locomotion of individual spermatozoa. In their free movements through the water they describe, as is well known, spiral paths. In *Nereis* the successive turns of the spiral are rather close set. As soon as a spermatozoon comes in contact with a surface, the movements of translation cease, and circus movements begin. The sperm moves round and round in a circle of varying diameter in contact with the surface. In the case of a preparation beneath a cover slip on a slide, those in contact with the slide rotate anticlockwise, those in contact with the cover-slip clockwise. The direction of rotation is always the same. It is associated no doubt with structural asymmetry which I described briefly in Study III (Lillie

'12). The tail of the spermatozoon is attached not to the center of the middle piece, but on one side.

The movement is of course due to successive beats of the tail and it is a very interesting fact that under certain conditions of aggregation the successive beats of the spermatozoa forming an aggregation may become synchronous, and under such circumstances the number of beats approximates 120 a minute at temperature of 21°C., if the sperm be fresh. This phenomenon, which I have not yet attempted to analyze in detail, follows after the aggregations of sperm from a fresh suspension have settled on the bottom of the container and begin to spread out of their own weight. Its appearance may be accelerated by gentle agitation of the dish, which tends to spread out the aggregations. Synchronous movements appear when the sperm spread out in a kind of membrane from an aggregated condition. In such a case the synchronous beats spread over the membrane thus formed, like waves of contraction over a ciliated epithelium. In fact, a kind of synthetic ciliated epithelium is then established. The interest of the phenomenon in the present connection is that it furnishes a clear demonstration of the successive beats of the tail of the spermatozoa, which are not readily distinguishable, and certainly cannot be counted, in the case of a single spermatozoon.

The movements of the spermatozoa are, then, due to successive beats of the tail, which is so placed as to cause rotation in a definite direction. The movement when freely suspended in water is in a spiral path, but when in contact with a surface the translatory component of the locomotion is almost entirely eliminated.

The following account of the behavior does not deal directly with individual movements, but always concerns mass-reactions, from which the behavior of the individual spermatozoa may be inferred.

3. '*Freshness*'

The spermatozoa are absolutely immotile while they are in the body of the male, but become intensely active when suspended in sea-water. This expresses itself in the formation of aggregations; but, as already noted, aggregations form more slowly after

a second mixing up, and only to a slight extent or not at all after a third mixing. This condition of relative inactivity, or staleness, is reached in a few minutes, but varies more or less according to the density of the suspension, a very dense suspension exhibiting it more quickly than one less dense. The activity of the sperm may be restored, partly at least, by the addition of fresh sea-water, which shows that the staleness is not due to exhaustion, but to the accumulation of by-products of activity in the sea-water. Of these the chief is probably CO_2 , as will be shown by experiments described beyond. The formation of CO_2 by the activity of the spermatozoa themselves is indeed one of the chief causes that limits their activity when sufficiently concentrated to form milky suspensions. To obtain the best results with the experiments described it is necessary to work with fresh sperm; otherwise, the accumulation of CO_2 may obscure the reactions.

4. Temperature

In 1911 a series of observations were made on the effect of temperature on the aggregation reaction of fresh sperm. In general the results as tabulated are:

- 13°C. No aggregations form
- 15°C. Slight signs of aggregation in 4 minutes
- 18°-19°C. Aggregation in from 2 to 4 minutes; much fewer in number than at higher temperatures
- 20.5°C. Aggregations, numerous, in 1 minute
- 23.5°C. Aggregations, yet more numerous, in 30 seconds
- 26.5°C. No aggregations form at this temperature, but they form as the temperature falls to 23°C.

In general temperatures from 20° to 23.5°C. are optimum for the aggregation phenomenon. At 15°C. the movements of the spermatozoa are too slow, and at 26.5°C. the movements are extremely active, but apparently uncoordinated, so that the aggregation reaction is not given. These figures possess no absolute value, but they indicate approximately the limits of temperature within which the reaction may be expected. The normal temperature of the sea-water varies from about 18° to 22°C. at Woods Hole during the breeding season.

5. Chemical composition of the medium

The effect of the chemical composition of the medium upon the activity of the spermatozoa is a very complicated subject, and no attempt has been made to analyze it farther than was necessary for comprehension of the forms of behavior studied. Even the simplest experiments furnish convincing proof of the dependence of activity of the spermatozoa upon a constant chemical composition of the medium; and this extreme susceptibility is certainly a prime factor in the behavior of the spermatozoa. To determine something of its limits becomes therefore necessary.

One of the first questions that presents itself is obviously the relation of the activity to the various salts of the sea-water. This is, however, in itself a problem of so much complexity that I have hesitated to undertake it; especially as it is unnecessary for our present purpose, seeing that the behavior to be studied takes place in sea-water as its medium. The few observations made demonstrate that spermatozoa of *Nereis* are paralyzed in pure M/2 solutions of NaCl, KCl, CaCl₂ and MgCl₂. As these are the principal salts of sea-water, it is obvious that the activation of the spermatozoa in the sea-water is a question of balance of salts. I therefore tried Van't Hoff's solution, namely: 100 cc. M/2 NaCl + 2.2 cc. M/2 KCl + 2 cc. M/2 CaCl₂ + 12 cc. M/2 MgCl₂, but the spermatozoa did not activate in this solution either. Some other experiments were made, which did not materially help the problem, which was not followed farther. The later experiments all assume the sea-water as the given medium.

Some early observations in the course of this work had shown that the female excretes certain substances in the sea-water that have a strong inhibiting effect upon the activity of the spermatozoa. This is so marked that sperm suspensions made up in sea-water sufficiently charged with secretions of the females never exhibit the aggregation phenomenon, and their fertilizing power is markedly reduced. This fact repeatedly observed suggested tests on the susceptibility of the spermatozoa to CO₂ dissolved in the sea-water, and this formed the beginning of a series of tests that involved acids and alkalis and some other substances.

a. Susceptibility to acids, including CO₂. The susceptibility of spermatozoa of Nereis to acids was tested by opening a male Nereis in a dry watch crystal, and mixing a drop of the thick sperm, which flows out, in 6 to 8 cc. of the solution to be tested. The effect on the movements of the spermatozoa was then observed as rapidly as possible, first with the naked eye to control the aggregation reaction which is given only by very active spermatozoa, and second with the microscope to note the degree of activity if their movements were sufficiently slowed down to prevent the aggregation reaction. For each experiment a control suspension of spermatozoa in normal sea-water was run, and only those experiments are taken into account in which the control aggregated in ninety seconds or less. The acid solutions were made by adding a sufficient quantity of N/10 dilutions in distilled water to a measured quantity of sea-water to reach the dilutions tested. These were always so weak as not to involve the question of osmotic changes in the results. The results may be tabulated as follows:

	H ₂ SO ₄	HCl	HNO ₃	CH ₃ .COOH
N/1000.....	0	0	0	0
N/2000.....	1	1—	1	0
N/3000.....	2	2+	1	0
N/5000.....	3	4	3—	1
N/10000.....	4	4	4	4

In this table (0) stands for complete paralysis

- (1) represents minimum amount of movement; usually only a few spermatozoa moving
- (2) Fairly active, but no aggregations form
- (3) Active; aggregations form, but are few in number and require over two minutes to appear
- (4) represents maximum activity, aggregations forming at least as rapidly as in the control, i.e., in less than ninety seconds

While the four grades of activity noted are readily to be distinguished in Nereis, their relation to the grades of acidity in question is not to be taken as fixed and invariable. As a matter of fact the various observations show considerable variation with reference to the intermediate dilutions, N/2000 to N/5000, in the case of the mineral acids. But in the case of the extremes N/1000

always produces complete paralysis very quickly, and N/10000 always permits maximum activity. The range of activity with reference to these acids is thus marked out fairly well. It will be noted that acetic acid has a greater inhibiting effect than the mineral acids.

It is an interesting question in this connection whether there is a certain optimum amount of acidity which increases rather than decreases the activity of the spermatozoa. In the experiments now under consideration this could not be determined certainly. In some cases spermatozoa aggregated more rapidly in weakly acid solutions (N/5000 and under) than in the control; in others at the same rate or at a slightly less rate. In the experiments on chemotaxis, however, which involve an acid gradient, there is possible evidence of stimulation of weak solutions.

CO₂. The sensitiveness of the spermatozoa to CO₂ is considered separately because of its probable biological significance and also because it was impracticable to state the strength of the solution in molecular terms. The solutions were prepared empirically as follows: A certain quantity of sea-water was supersaturated with CO₂ in 'Sparklet' siphons. The charged sea-water was drawn as desired, and after the effervescence ceased it was diluted with measured quantities of sea-water, and the dilutions were expressed as percentages of the charged sea-water. These solutions were always prepared fresh for each experiment, and kept in stoppered bottles or otherwise covered as far as possible. The uniformity of the reactions obtained is adequate proof that the solutions used in the different experiments were equivalent.

A very large number of experiments was made with CO₂ during the course of the summer, so that the relations of the spermatozoa to CO₂ were more adequately ascertained than for any other substance. Here the question is only of the relation of CO₂ tension to the activity of the spermatozoa, and the results may be stated as follows:

One per cent of the charged CO₂ sea-water paralyzed the spermatozoa immediately; or rather a suspension of a drop of dry sperm in 6 to 8 cc. of this strength of CO₂ does not exhibit any activity. This is, however, very near the minimum paralyzing

dilution, and some samples of sperm will exhibit slight movements in it.

In 0.75 per cent CO_2 sea-water no aggregations take place, but the spermatozoa move feebly.

In 0.5 per cent CO_2 sea-water aggregations usually form slowly, but the activity is usually less than the control.

In 0.33 per cent CO_2 sea-water there is apparently no inhibition of activity as compared with the control.

Whether lower dilutions stimulate more than normal sea-water is difficult to say by the method used here. But the chemotaxis experiments possibly indicate stimulation at a certain optimum (see p. 535).

The sensitiveness of *Nereis* spermatozoa to CO_2 is thus surprisingly great, and it operates within very narrow limits. This is the more surprising when comparison is made with spermatozoa of other species. Thus I ascertained that the sperm of *Loligo* will move, though feebly, in 50 per cent CO_2 -charged sea-water, and that it is very active in 20 per cent, though less so than in normal sea-water. In the case of *Chaetopterus* it requires about $33\frac{1}{3}$ per cent to 40 per cent of the CO_2 sea-water to completely paralyze all the spermatozoa, though 10 per cent inhibits considerably. *Arbacia* sperm on the other hand is much more sensitive to CO_2 , being completely paralyzed in 3 per cent. But *Nereis* is very much more sensitive than any of these, and this involves some very interesting forms of behavior described later on.

b. Sensitiveness of spermatozoa of Nereis to alkalis. Alkalis above a certain concentration agglutinate the spermatozoa of *Nereis*, and cause them to stick together in masses. This is never seen in acids, however strong. I can best state the sensitiveness of the spermatozoa to KOH by giving the protocol of a single experiment (June 23, 1912) which followed some preliminary determinations. N/10 KOH in distilled water was used as the standard solution. Added to sea-water this solution produces a precipitate which redissolves up to about N/2500 KOH.

In the experiment a drop of dry sperm was stirred in about 8 cc. of each of the following dilutions in sea-water, and observations made as noted.

1. N/2500 KOH. Produces very rapid agglutination of the spermatozoa; free sperm between show some movement.
2. N/5000 KOH. No agglutination; no aggregation; sperms fairly motile.
3. N/7500 KOH. No agglutination; no aggregation; sperms more active.
4. N/12500 KOH. No agglutination; no aggregation; sperms very active.
5. N/25000 KOH. No agglutination; aggregations form slowly; but sperms are extremely active.
6. Normal sea-water. Control. Aggregations form in half minute. Very active sperm (maximum).

This experiment was carried out with the sperm of one male at one time, the solutions being prepared in advance. The limits of the agglutination effect are given. But it is improbable that the inhibiting effect extended to the lower limit, although aggregations were formed so slowly in N/25000. The reason for this, as will be shown later, is that the aggregation effect is due to positive chemotaxis to a weak acid, probably CO_2 , produced by the spermatozoa themselves. This is neutralized by the KOH so that in spite of the great activity noted in N/25000 KOH aggregation cannot take place until after neutralization of the alkali.

If the behavior of the spermatozoa be observed under the microscope at the moment they are put into N/2500 KOH, there is seen momentary great activity of the spermatozoa followed quickly by agglutination as described.

The relations to NaOH were essentially the same. There was slight agglutination in N/5000 NaOH, and the slightest appearance of aggregations in N/25000 NaOH.

c. To alcohol and ether the sensitiveness is as follows:

Alcohol:

- (1) 5 per cent, sperm are paralyzed
- (2) 2 per cent, some activity; no aggregations
- (3) 1 per cent, more active, some aggregations may form in five minutes
- (4) 0.5 per cent, few aggregations in about three minutes
- (5) 0.2 per cent aggregations in forty-five seconds
- (6) 0.1 per cent, aggregations in thirty-two seconds
- (7) Control, sea-water; aggregations in thirty seconds

(4), (5), (6) and (7) came from an experiment with the same sample of sperm.

The sensitiveness to ether is essentially the same, though the sperm did not aggregate even at 0.33 per cent. The chemotaxis experiments with ether indicate a possible stimulation of the sperms at an optimum concentration (see beyond).

As stated before, no attempt was made to carry the analysis of the relation of activity of the spermatozoa to known chemical substances very far. Experiments on chemotactic and other behavior phenomena of the spermatozoa were in progress at the same time, and the determinations already given seemed fairly adequate for the purposes of analysis.

6. Sensitiveness of spermatozoa to hypo- and hyper-tonicity of the medium

As regards the sensitiveness of spermatozoa to hypo- and hypertonicity of the medium, the following determinations may suffice:

August 18, 1911. Sperm of one male; one drop mixed in each of the following solutions, with results noted:

- | | |
|---|--|
| (1) 5 cc. sea-water + 2.5 cc. distilled water. The sperm are fairly active, but no aggregations form. | |
| (2) 5 cc. sea-water + 1 cc. distilled water | } Aggregations form in one minute;
a little better in 4 |
| (3) 5 cc. sea-water + 0.5 cc. distilled water | |
| (4) 5 cc. sea-water | |
| (5) 5 cc. sea-water + 1 cc. 5/2 N NaCl; sperm paralyzed | |

The spermatozoa will thus stand considerable decrease in osmotic pressure without much modification of activity. But increase in osmotic pressure induced in the experiment by addition of NaCl and in others by KCl, CaCl₂ or MgCl₂, rapidly paralyzes. The addition of this amount of KCl paralyzed every sample of sperm used and its effect is undoubtedly toxic; but some samples of sperm exhibited considerable, though decreased, activity, when the other salts were used.

B. ARBACIA

The tests concerning the relation of the activity of spermatozoa of Arbacia to the chemical composition of the sea-water were not so extensive as in the case of Nereis. Moreover we do not have any definite aggregation reaction here to serve as a measure of activity. However, a sufficient number of tests were made to show that Arbacia is much less sensitive to variations in inorganic constituents than Nereis.

CO₂. To afford comparisons we may use three forms here, Chaetopterus, Arbacia and Nereis:

	100% CO ₂	40% CO ₂	20% CO ₂	10% CO ₂	5% CO ₂
Chaetopterus....	paralyzed	few move slightly	many move	active	active
Arbacia.....	paralyzed	paralyzed	paralysed	paralyzed	paralyzed
Nereis.....	paralyzed	paralyzed	paralyzed	paralyzed	paralyzed
	2.5% CO ₂	1% CO ₂	0.5% CO ₂	NORMAL SEA-WATER	
Chaetopterus....	active	active	active	active	
Arbacia.....	traces of movement	fairly active	active	maximum activity	
Nereis.....	paralyzed	paralyzed	fairly active	maximum activity (aggregation)	

Thus, while compared to Chaetopterus, Arbacia, is extremely sensitive to the presence of CO₂, compared to Nereis it is relatively insensitive.

To other acids, H₂SO₃, HCl, HNO₃, and CH₃ COOH, Arbacia is also less sensitive than Nereis, exhibiting a fair degree of activity in N/1000 solutions in sea-water (compare table for Nereis, p. 526).

The sensitiveness to alkalis does not differ materially from that of Nereis. Agglutination of the spermatozoa is caused by N/2500 KOH, giving a very pretty precipitation picture in a vial. Such agglutinations are irreversible. Spermatozoa between the agglutinated masses may be in motion.

The relatively slight sensitiveness of Arbacia sperm to CO₂ is correlated with absence of any such striking aggregation effects

as are exhibited by suspensions of *Nereis* spermatozoa. But indications of the same kind of reaction may be seen under certain circumstances. Thus, a fresh suspension mounted beneath a raised cover will soon exhibit cloud effects due to differences in the density of aggregation, and this corresponds to the first stage of aggregation in *Nereis*. In the course of an hour or so all the spermatozoa retract from the edges into a central dense aggregation, and this is due, I believe, to the rising CO_2 tension towards the center. Reasons for this opinion are given under the head of the aggregation phenomena.

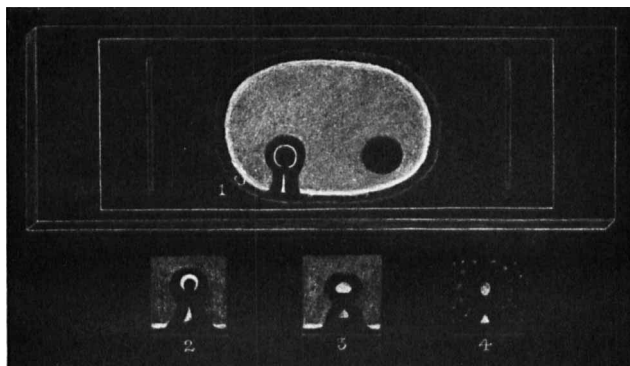


Fig. 4 Reaction of spermatozoa of *Nereis* to a drop of 1 per cent CO_2 sea-water; from an experiment of June 18, 1912. The original sperm suspension was made at 3.09; it aggregated clearly at 3.10 (fig. 1). It was then mixed up with a pipette and some drops mounted on a slide beneath a raised cover-slip as in 1. The drop of 1 per cent CO_2 sea-water was introduced at 3.12 $\frac{3}{4}$ (on left) and a drop of pure sea-water as control (drop to right). Figure 4, 1, shows the reaction at 3.13; 2, at 3.14; 3, at 3.14 $\frac{1}{2}$, and 4, at 3.16. In 4 the general suspension has aggregated. The final position of the reactive sperm is in the center of the introduced drop. No reaction takes place with reference to the drop of sea-water, which gradually becomes obliterated by inwandering of sperm. The figure shows also that the spermatozoa retract from the margin of the suspension.

III. AGGREGATION PHENOMENA

The spermatozoa of *Nereis* and *Arbacia* show very definite positive chemotaxis toward acids and egg-extracts of the same species, which may be demonstrated with striking clearness by the method first introduced by Jennings in studying the behavior of *Paramecium*. The method as applied to behavior of spermato-

zoa consists in mounting some drops of a sperm suspension beneath a long cover slip supported by glass rods, and injecting a drop of the fluid to be tested into the suspension. It then forms a clear drop within the milky suspension, and reaction at once begins at its borders. This method gives incomparably more delicate results than Pfeffer's method of using capillary glass tubes. The drop is confined above by the cover and below by the slide and diffusion takes place only at its margins; in this way a gradient is established. In the case of the capillary glass tubes diffusion is so slight from the open ends that no delicate reaction can be expected. So after a number of trials the capillary tube method was abandoned and the injected drop method was used exclusively.

A. NEREIS

1. Aggregation with reference to CO₂

As introduction, the reaction of a fresh suspension of the spermatozoa of Nereis to a 1 per cent dilution of sea-water saturated with CO₂ will first be described from a specific experiment:

June 26, 1912. A ripe male Nereis was placed in a dry watch crystal and snipped with scissors; two drops of the dry sperm were mixed in 10 cc. sea-water at 9.11½ A.M. and made a milky suspension which aggregated freely in thirty seconds. Some drops of this were then mounted beneath a cover slip supported by glass rods about 1 mm. in diameter. A drop of the 1 per cent CO₂ sea-water was injected at 9.13. In withdrawing the pipette a trail of the CO₂ sea-water is left extending to the margin. In a few seconds the following configuration developed (fig. 4-1). It consists essentially of a dense aggregation of very active spermatozoa in the form of a ring within the margin of the original drop, and a line extending from the drop to the edge where the pipette was introduced and withdrawn. In this case the ring is open below and a linear aggregation extends from the opening towards the margin of the suspension. The ring and the linear aggregation are separated from the general sperm suspension by a clear area devoid of spermatozoa 1.5 to 2 mm. in width. This area

belonged mainly to the original territory of the sperm suspension, and the ring owes its origin to migration of spermatozoa towards the drop. They do not, however, penetrate at first to the center of the drop, but their movements are arrested, hence the formation of the ring. The 'tail' of the ring is due to migration of spermatozoa to the trail of CO_2 sea-water left behind in withdrawing the pipette, leaving a clear zone marking the range of the effective stimulus. Control: No reaction is given to a drop of pure sea-water similarly introduced.

The migration of spermatozoa to the first formed ring continues for a short time; the ring thus grows broader and tends to close in the center (fig. 4-2 and 3). Shortly after the ring and tail aggregations have formed with reference to the introduced drop of CO_2 sea-water, the usual aggregations of the sperm, 1 to 2 mm. in diameter, form in the remainder of the suspension outside the drop evenly spaced throughout, if the sperm suspension is perfectly fresh (fig. 4-4). But if it is a little stale the general suspension remains homogeneous.

The detail of form of the ring and tail aggregations vary according to whether the introduced drop simply displaces a certain amount of the suspension, or is more or less mixed in the introduction; and this depends obviously on the size of the opening of the capillary pipette and the rate at which the drop is introduced. But the general form of the reaction is always the same.

The spermatozoa in the CO_2 aggregations are never in the least agglutinated and their behavior is in all essential respects the same as in the aggregations formed in any fresh suspension. I therefore early formed the hypothesis that the aggregation phenomenon is a chemotactic reaction to CO_2 produced by the spermatozoa themselves, and this hypothesis has been abundantly confirmed, as the series of experiments to be described will show.

The formation of the described configuration in a suspension of active spermatozoa with reference to an introduced drop is due to positive chemotaxis to the drop. If the clear margin be observed during the formation of the ring, the spermatozoa may be seen swimming across it to the ring head first. Under the low power of the microscope they appear to drift across it with a

dancing motion like motes in a sunbeam owing to their spiral path. If the external edge of the clear zone be carefully observed, the spermatozoa can be seen to detach themselves one by one from the general suspension and pass straight over to the ring. But only those freely suspended make the direct path; those in contact with the slide or cover continue their circus movements; the chemotactic stimulus seems unable to overcome the thigmotactic reaction.

The reaction is given most clearly and rapidly by a fresh sperm suspension, although one which has passed the aggregation stage still gives it; however, as the sperm suspension becomes stale the reaction becomes slower, and eventually ceases. Spermatozoa killed by gentle heat give no such reaction, thus excluding any purely physical diffusion effect as cause of the phenomenon.

In the case of this reaction in a somewhat stale non-aggregative suspension the movements of the spermatozoa on the outer margin of the ring are decidedly more vigorous than in the general suspension. This would appear to indicate that, at this place in the CO_2 gradient marked by the clear zone, the concentration of the CO_2 is stimulating rather than depressing; but when we consider that the CO_2 gradient must rise from the suspension across the clear zone to the ring, and that the relative inactivity of the sperm in the suspension is due, partially at least, to CO_2 , the conclusion is not so clear. In any event, if we attribute a stimulating action to a given CO_2 concentration on such evidence, we must regard the depression of activity in the general suspension as due partly to other excreta.

The conditions established by the experiment may be represented diagrammatically as follows (fig. 5). The injected drop is represented by the continuous line circle and continuation, the general suspension by the shaded area. By diffusion from the injected drop a CO_2 gradient is established outwards, and this must extend into the drop a certain distance because the gradient is established by loss of CO_2 from the drop. The concentric broken lines represent the gradient, or at least that part of the gradient which is affective in the reaction. The thick open circle and the similar linear extension represent the aggregations of the sperma-

tozoa. At the same time there is of course diffusion of substances peculiar to the general suspension towards the introduced drop; but that conditions thus arising are ineffective is shown by the fact that no reaction is given to the introduced drop of pure sea-water. We may, therefore, leave this centripetal diffusion out of account. It should be remembered that 1 per cent CO_2 sea-water is the minimum paralyzing strength for *Nereis* sperm.

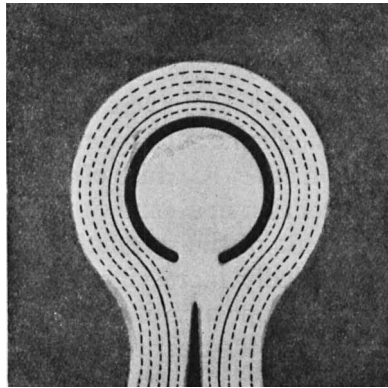


Fig. 5 Diagram of the reaction of a sperm-suspension of *Nereis* to an introduced drop of 1 per cent CO_2 sea-water; explanation in the text.

The diagram therefore shows, that migration of the spermatozoa proceeds up the gradient to, or near to, the point of paralysis of the spermatozoa; for in the case of the drop of 1 per cent CO_2 sea-water the ring forms well within the original margin of the drop. With higher and lower dilutions of CO_2 the width of the clear margin is practically the same.

The effects of greater and less CO_2 concentration than the 1 per cent used in the initial experiment are interesting. In general the use of a greater concentration involves a larger aggregation, and of a less concentration a smaller aggregation. Thus if a drop of sea-water saturated with CO_2 be introduced into a suspension of fresh sperm beneath a raised cover slip a border of dead or paralyzed sperm forms at its margin, and shortly a clear zone forms external to it; the spermatozoa migrate in large

numbers across the clear margin to the ring and are speedily paralyzed by the diffusing CO_2 ; this process continues very rapidly and as a consequence the central aggregation expands, and may in time absorb all the spermatozoa of the suspension.

The same phenomenon in less pronounced form is exhibited by the reaction to a 1/10 dilution of the saturated CO_2 sea-water. Here we may give a definite experiment with measurements of growth of the aggregation: July 1, 1912. Into a fresh sperm suspension beneath a raised cover a drop each, (a) of 1 per cent, and (b) of 10 per cent CO_2 sea-water was injected some distance apart. Drop (a) measured 5 mm. in diameter and drop (b) 3 mm. immediately after injection at 2.28 P.M. The aggregations caused by these drops measured at 2.32: (a) 3 mm. (b) 5 mm. That is to say, the aggregations formed *inside* the drop in case of the weaker solution, and outside in the case of the stronger. At 2.35 (a) still measured 3 mm. and (b) now 6 mm. In a repetition of this experiment drops (a) and (b) each measured 3 mm. at 2.37. The aggregations caused by them measured at 2.40, (a) 2 mm., (b) 5 mm.; at 2.47, (a) 2 mm., (b) 10 mm.

It is clear from these observations that the spermatozoa are positively chemotactic in a CO_2 gradient where the tension is above a certain point, and that the aggregation caused by the more concentrated drop grows because the diffusion of CO_2 from the center forms a widening ring of the necessary concentration. To furnish a gradient the concentration must exceed the CO_2 tension in the general suspension, which is a function of the age of the sperm suspension and the activity of the spermatozoa in it, and on the other hand a limit is set to the differential which furnishes the reaction by the fact that a concentration of about 1 per cent of the saturated CO_2 sea-water paralyzes the spermatozoa. The gradient that furnishes the reaction must, therefore, operate within very narrow limits.

Greater dilutions of the CO_2 sea-water than 1/100 will act positively in the case of fresh sperm suspensions. The ring forms within the margin of the drop in the case of 1/200 dilution, but it remains narrow, and tends to break into bead-like aggregations, proving that the spermatozoa by their own activity have produced

a greater CO_2 concentration within the ring, which furnishes centers of aggregation positive to the 1/200 concentration of the drop. Drops below this concentration or drops of pure sea-water furnish no reaction.

Summarizing; it would appear that the spermatozoa of *Nereis* follow a CO_2 gradient to the point of paralysis (about 1/100 saturation). The clear zone outside an aggregation represents the effective CO_2 gradient in every case. The various forms of reaction to drops of different concentrations follow from this simple principle.

2. Interpretation of the aggregation reaction

We are now prepared for the interpretation of the aggregation phenomena exhibited by fresh sperm suspensions described on page 519. The spermatozoa as they come from the body cavity are absolutely quiescent; as soon as they are suspended in sea-water they become intensively active, and consequently produce CO_2 very rapidly. Any area of greater concentration of spermatozoa, by producing more CO_2 than other areas, becomes a center of attraction, and aggregations of the spermatozoa once begun are bound to proceed to the limit, because the closer the aggregation the greater the CO_2 production and consequently the greater the chemotactic stimulation. If aggregations once formed are broken up and the spermatozoa evenly suspended once more, the CO_2 tension in the suspension is greater than at first and is evenly distributed. Hence, in the first place the activity of the spermatozoa is reduced, and, in the second place, the differential of the gradient between that of the general suspension and the point of paralysis is greatly lessened. Therefore aggregation takes place more slowly and less completely than before; and, after a second and a third stirring up, the CO_2 tension in the entire suspension has become too great to permit of sufficient activity to react to the slight possible differential gradient.

It is obvious that such a reaction can take place only in the case of spermatozoa that exhibit extreme sensitiveness to CO_2 . The spermatozoa of *Nereis* possess by far the greatest sensitiveness to CO_2 of any studied, as we have already seen. No other

spermatozoa exhibit the aggregation reaction in any marked form so far as I know; *Arbacia* which comes next to *Nereis* in point of sensitiveness to CO_2 among the forms studied, shows, under certain conditions, a cloud-like formation similar to the initial stage of aggregation in *Nereis*. These will be referred to beyond.

It may perhaps be objected that the aggregation reaction in *Nereis* is not necessarily caused by CO_2 excretion, but possibly by some other substance produced by the spermatozoa. And it would be difficult to meet this objection in any absolutely conclusive way. But the following considerations render the conclusion extremely probable. In the first place it can be proved that the spermatozoa exhibit positive chemotaxis towards some substance that they themselves produce. Thus July 31, 1912, the following experiment was made:

With the dry sperm of one individual two suspensions were made at the same time (8.57 A.M.) namely: *a* one drop of sperm in 9 cc. sea-water, *b* two drops of sperm in 6 cc. sea-water; the activity of the sperm in both suspensions being the same *b* should produce any attractive substance in much greater amount than *a*. This was tested by making preparations of *a* and *b* on separate slides beneath raised covers. A drop of *b* was then injected into slide *a*, and a drop of *a* into slide *b* at 8.59. On slide *a* there was a very quick beautiful positive reaction to the introduced drop, that is a clear border formed about the drop owing to positive chemotaxis of the spermatozoa *a* to the drop of denser suspension *b*. On slide *b* not only was such positive reaction absent, but the drop of introduced sperm actually lost its spermatozoa and became clearer, owing to the positive chemotaxis now being away from the drop. The same results were obtained also by using two suspensions of equal density, one of which was older than the other. The fresher suspension reacted positively to the older suspension.

In the second place, it is of course certain that the spermatozoa becoming suddenly active in the sea-water must produce CO_2 ; and as we have seen that the spermatozoa of *Nereis* react even to a 1/200 dilution of a saturated solution of CO_2 in sea-water, if it can be proved that a standard suspension of spermatozoa produces an equivalent amount, the probability that CO_2 is the agent involved in the aggregation effect become very great. Experiments directed to this end showed that a 1/100 dilution of CO_2

lies near the limit of demonstrability both by color reaction tests and also by gas-burette estimation. A number of tests of sperm suspensions were made with acid color indicators. In the case of neutral red a dilute solution in sea-water has a decided orange tinge due to slight normal alkalinity of the sea-water. The same dilution made from a standard concentrated solution by the addition of a sperm suspension, shows a decided rose color without any trace of orange. The spermatozoa then aggregated in the vial used and the aggregations sank to the bottom, forming a bright red precipitate, and the supernatant fluid, now merely opalescent on account of the few sperms remaining in it, was faint rose. There is thus a decided acid reaction of the sperm suspension. Tests with azolitmin and tropaeolin 000 No. 1 also gave clear indications of acid. The sperm suspensions were tested within two minutes or less after their preparation; the liberation of the acid takes place therefore very suddenly. It is liberated only when the sperm become active, and the change of color is not given if the sperm remain inactive. It is therefore very probable that CO_2 is the acid revealed.

Finally a large number of tests for CO_2 were made of the air in closed flasks containing considerable quantities of active sperm suspensions of *Arbacia*. The details of these tests made with a gas burette need not be given. They extended over a week, using the sperm of *Arbacia* which could be obtained in larger quantities than *Nereis*. Although the determinations came very near the limits of experimental error, there could be no question as to the presence of CO_2 in quantities above that contained in the air or in normal sea-water.

In consideration of the facts (1) that sperm suspensions of *Nereis* produce a substance to which spermatozoa of *Nereis* react positively (2) that an acid is present in the suspensions (3) that the production of CO_2 by the suspensions can be demonstrated and (4) that spermatozoa of *Nereis* react positively to dilutions of CO_2 in sea-water which are barely detectable by color indicator, or gas burette, it can hardly be questioned that the aggregation reaction in *Nereis* is due to positive chemotaxis to CO_2 .

3. Reaction to other acids

The sperm of *Nereis* exhibits the same positive chemotaxis to other acids as to CO_2 . It is hardly necessary therefore to enter into details. Sulphuric, hydrochloric, nitric, and acetic acids were tested. They agree very closely with respect to the effective dilutions; N/1000 dilution of any of these acids is an effective chemotacticum agreeing quite closely in the degree of the effect produced with 1 per cent CO_2 ; N/2000 may cause slight ring formation in a drop introduced into a fresh sperm suspension, but, if the suspension has reached the non-aggregative stage, no reaction ensues, owing to the fact that the acid concentration in question furnishes no gradient.

Drops of stronger concentration cause a ring-shaped aggregation which continues to grow until diffusion eliminates the acid gradient. None of these acids cause the least sign of agglutination of the spermatozoa whatever their strength.

A drop of N/10 acid introduced within a sperm suspension beneath a raised cover kills all the spermatozoa in its immediate neighborhood, as the acid diffuses the zone of dead sperm increases but as the margin of the diffusing acid reaches a dilution that is no longer fatal it becomes marked by a clear border which is due to the migration of sperm to it, even though they are carried into a fatal concentration; and so the drop continues to grow so long as an acid gradient remains.

There is never the least sign of negative chemotaxis with respect to any concentration of any acid, nor indeed of any other agent tested. This being the case it is obvious that the aggregation of the spermatozoa can not be by any trial and error method of behavior, but must take place through orientation.

4. Behavior with reference to alkalis

The spermatozoa of *Nereis* do not exhibit any chemotactic reaction, positive or negative, to drops of KOH or NaOH injected into a suspension beneath a raised cover-slip. The drop remains empty at first, and spermatozoa that enter it by chance are agglutinated, so that in a short time the drop becomes filled

with agglutinated sperm masses. The alkalis were used in concentrations of N/2500 and N/5000.

Though no chemotatic reaction takes place, yet an interesting reaction may be procured by injecting a drop of N/5000 KOH into a sperm suspension made in 1/100 dilution of saturated CO_2 sea-water. The sperm in this are nearly or quite paralyzed, and will not of course react to an injected drop of 1/100 CO_2 . But if a drop of N/5000 KOH be injected into such a suspension beneath a raised cover glass, motility of the spermatozoa returns at a short distance from the margin of the introduced drop, due evidently to neutralization of the acid, and aggregations of the active sperm may form outlining the KOH drop a certain distance from the margin. This experiment furnishes at once an interesting demonstration of recovery from CO_2 narcosis, and of the nature of the contrast between the acid and alkali reaction of the spermatozoa.

5. Reactions to other substances

It lay entirely outside of the scope of this work to attempt an exhaustive analysis of the behavior of the spermatozoa with reference to chemical substances. The investigation was undertaken to analyze the relations of the behavior of spermatozoa to fertilization; and when the principle of chemotaxis was once demonstrated, and the relation of this chemotactic reaction to the very striking aggregation reaction, it seemed better to turn at once to the subject of the behavior of the spermatozoa towards eggs and egg-secretions of the same species. It may be remarked incidentally that tests with alcohol and ether gave negative results, that is, no evidence of positive or negative chemotaxis was found. In some cases there was slight indication of ring formation near the margin of an introduced drop of 5 per cent alcohol or ether in sea-water, which may indicate a stimulating effect of the spermatozoa at an optimum concentration, but which, in the absence of a clear margin external to the ring cannot indicate chemotaxis.

6. *Thermotaxis*

The spermatozoa of *Nereis* do not exhibit any positive response to drops of sea-water at higher temperatures. Into suspensions of spermatozoa in sea-water at 21°C. under raised cover-slips drops of sea-water at 44°C., 52°C., and 84°C. were injected successively. No ring formation occurred with reference to any of these drops. They did not fill up with sperm as rapidly as drops of sea-water at room temperature, but this is no doubt due to the paralysis that sets in, as previously noted above about 28°C. The only observable effect of the heated drops was that aggregations formed a little earlier in the general suspension near the margins of the introduced drop; and this is attributable to increased activity of the sperm owing to rise of temperature, hence increased CO₂ production in the zone of increased activity; and more rapid aggregation as a consequence.

7. *Thigmotaxis*

In contact with any solid object *Nereis* spermatozoa tend to carry out circus movements in an anti-clockwise direction, when, fresh, but may soon come to rest. In any field of the microscope in a suspension beneath a cover glass one sees many of the spermatozoa in contact with the slide at rest, and many others carrying out the circus movements, while those that are freely suspended swim in spiral paths. The thigmotatic reaction then appears first to be the exaggeration of the rotation component of the ordinary locomotor movements, and second rest.

This reaction may of course come in conflict with the chemotactic reaction, as for instance in the clear margin external to the ring of spermatozoa produced in response to a drop of 1/100 CO₂ sea-water. Within this area all the freely suspended spermatozoa swim directly towards the ring, but those in contact with slide or cover-slip may continue their circus movements without any apparent directive effect from the CO₂ gradient. The thigmotactic stimulus appears thus to be more effective than the CO₂ gradient.

This thigmotactic reaction may be the starting point apparently of some of the aggregations formed in suspensions. Thus aggregations tend to form in the angle between the glass rod and the slide, which appear to owe their origin to the thigmotactic reaction; but when a considerable number of spermatozoa have accumulated in the angle their CO_2 excretion acts as a positive chemotactic stimulus on the sperm of the suspension, and a dense swarm soon forms along the rod, filling the angle and extending beyond it. Such a continuous swarm then tends to break into evenly spaced masses still in contact with the rod, owing to variations in CO_2 production. Thus thigmotaxis in this case is the initial cause of aggregations, which owe their subsequent growth to chemotaxis.

It may be that the thigmotactic reaction is a frequent cause of aggregations, particularly in suspensions that have produced considerable CO_2 when aggregations form only slowly and always in contact with the substratum. But in fresh suspensions this cannot be the case, for the aggregations first formed are freely suspended. In many cases aggregations may be seen to form with reference to firm strands or fibers of mucus in a suspension, and in such cases it appears probable that thigmotaxis and chemotaxis are combined.

8. Variations of reactions

So far as observed the behavior of sperm suspensions in sea-water may be quite fully explained by the forms of reaction described, and this brings the present section to a natural conclusion. But we may finally note certain variations of the reactions. The sperm suspensions were usually made, as stated, by mixing a drop or two of dry sperm with about 8 to 10 cc. of sea-water in a Syracuse watch crystal. This was done with a pipette, drawing in the suspension and squirting it out again until the sperm was evenly mixed. If this is done from one side, as is usually the case, a current is made across the dish to the opposite side and back along both sides, creating miniature whirlpools. Such currents of course come to rest in a few seconds, but when the aggregations become visible, ten to forty or more seconds later, they define very accurately the original currents. At first I thought natur-

ally of some rheotactic reaction. But on more careful examination and consideration the following explanation appears much more probable. Microscopic aggregations must begin to form while the water currents are still moving; they are then elongated by the friction in the direction of the current, and as they grow to macroscopic size the aggregations tend to preserve this form. The definition of the currents is due to the form of the aggregations rather than to their arrangement, and as they contract to spherical form the current-figures become less pronounced and very largely disappear.

Very interesting configurations may be produced in a sperm suspension of *Nereis* by dropping in dilute acids. In a few seconds quite complex wreath-like or festooned aggregations of spermatozoa appear at the site of the entering drop marking out accurately the distribution of the acid in the suspension. These of course vary with the strength of the acid, and the distance from which it is dropped.

If a few drops of a suspension of active *Nereis* sperm be mounted beneath a raised cover slip, it will be observed that the outer margin of the suspension for a width of 1 to 2 mm. soon becomes free from spermatozoa, thus tending to concentrate the suspension the same distance from the margin (fig. 4). This concentrated ring of the suspension then tends to form aggregations more rapidly than the more central parts. In the case of a suspension that is not perfectly fresh, aggregations may form only in this ring. The withdrawal of spermatozoa from the margin of the drop might at first thought be attributed to a negative chemotaxis towards oxygen. However, it is almost certainly not this, but a positive reaction towards the higher CO_2 tension of the interior of the drop. If a drop of sea-water saturated with oxygen be injected into a suspension beneath a raised cover, the spermatozoa avoid it in the same way that they do the free margin of the suspension.

The spermatozoa of *Nereis* make an acid indicator more delicate than any of the chemical dye indicators. In the course of some experiments I discovered quite accidentally, thus avoiding an awkward mistake, that the first few drops of water through any

available filter paper give a distinct acid test, using the spermatozoa of *Nereis* as indicator: A drop injected into a fresh sperm suspension invariably gave the ring formation with outer clear border, which is the characteristic and unmistakable acid reaction. This suggests the possible use of such cells as indicators in certain classes of experiments; some preliminary observations which I have made concerning CO_2 production of dividing eggs by this method are distinctly promising, though the results are complicated by the usual presence of other substances.

9. *Chemotaxis to egg-secretions*

The spermatozoa of *Nereis* exhibit positive chemotaxis to egg-secretions, which may be demonstrated in the same way as the positive chemotaxis to acids, but this subject, which is of course the most important part with reference to the fertilization problem, may be postponed to the next section dealing with agglutination phenomena, because it is always associated with agglutination.

B. ARBACIA

The reactions of *Arbacia* spermatozoa are essentially the same in principle as those of *Nereis*, but on account of the lesser sensitiveness of the spermatozoa, as noted in the section on activation phenomena, the reactions are much slower and less clearly defined. This may be illustrated by the notes on a single experiment: July 20, 1912. A fresh suspension of *Arbacia* sperm was made by mixing two drops of the dry sperm with 9 cc. of sea-water. The suspension appears milky, and the spermatozoa are decidedly active under the microscope. A portion was immediately mounted under a raised cover-slip and two drops of 5 per cent CO_2 sea-water were injected some distance apart. At first there appeared to be no reaction, as contrasted with *Nereis* in which ring formation is almost instantaneous under such circumstances. In two minutes the sites of the drops became more cloudy than the rest of the slide, and a faintly defined clear margin began to appear surrounding the drop. The picture gradually gained in definiteness until it became very clear. The central aggrega-

tions at first showed radiating strands of sperm visible to the naked eye, but then closed to form a solid drop, and this grew at its margins, preserving the clear external zone, until the clear margins of the two drops, at first several millimeters apart, ran together. Outside of the influence of the drops cloud formations appeared, corresponding to an early stage of the aggregations of *Nereis*.

The clear margin of the drops is not by any means so well defined as in *Nereis*. Moreover, the spermatozoa are so small that it is difficult to observe their behavior in the clear margin. However, there can be no doubt that the phenomenon is essentially the same as in *Nereis*, and that the aggregation in the drop, the appearance of the clear margin, and the growth of the aggregation are due to positive chemotaxis to CO_2 .

This reaction is given clearly only by a fresh sperm suspension. One ten minutes old does not give it, owing presumably to formation of CO_2 in the suspension.

A considerable number of tests were made. In some the reaction was much more rapid than in the experiment described. In one of these tests I injected drops of 20 per cent, 4 per cent and 1 per cent of the CO_2 sea-water near together. In the case of the 20 per cent a ring with external clear margin was formed in a few seconds. The ring did not close. The 4 per cent formed a ring which closed in its center. There was no reaction to the 1 per cent. The same suspension gave no reaction twenty-five minutes after it was mixed. In another case I got a faint reaction to 1 per cent CO_2 sea-water.

The fact of positive chemotaxis of *Arbacia* sperm to CO_2 dissolved in the sea-water was repeatedly demonstrated. In the case of a drop mounted beneath a raised cover it expresses itself by a gradual aggregation of the sperm towards the center, leaving the margins clear.

As is to be expected from the slower and less delicate reaction to CO_2 , as compared with *Nereis*, spermatozoa of *Arbacia* react also to other acids, but more slowly and not to so great dilutions. Thus in tests of N/10, N/50, N/250 and N/1000 H_2SO_4 , strong positive reactions were obtained for the first three, whereas only a faint shadowy reaction is given to N/1000. In the case of *Nereis*

it will be recalled that N/2000 gives a distinct reaction. Positive reactions were not secured with N/1000 HCl or HNO₃, but were with N/250. The ring formed in response to N/250 H₂SO₄ grows somewhat owing to diffusion of the acid; in the case of N/50 and N/10 H₂SO₄ the growth is much greater, in general in proportion to concentrations.

As in *Nereis* alkalis agglutinate, but cause no aggregation. KOH was tested in two ways: (1) addition of a small quantity of N/2000 KOH in sea-water to a vial of fresh sperm suspension caused macroscopic agglutinations which are irreversible. (2) a drop of N/2000 KOH injected into a suspension beneath a raised cover fills in a short time with sperms that agglutinate; but there is no chemotaxis, and in a short while the drop is left to one side by aggregation of the sperm away from it, owing to rise of CO₂ tension elsewhere.

Reaction to egg-secretions

As contrasted with the slowness of reaction of *Arbacia* spermatozoa to acids, the reaction to egg-secretions is instantaneous and clear cut. There is a most pronounced positive chemotaxis, as tested with drops even of very weak egg-extract injected into a sperm suspension mounted beneath a raised cover-slip; but this is always associated with agglutination, and is, therefore, best considered under that head.

IV. AGGLUTINATION PHENOMENA AND REACTIONS OF SPERMATOZOA TO EGG-SECRETIONS

1. INTRODUCTION

Following the demonstration of definite chemotactic behavior of spermatozoa of *Nereis* and *Arbacia* the question naturally arises what relation, if any, has this form of behavior to the union of egg and spermatozoon in fertilization. As is well known, the mere observation of the interaction of the sexual elements in fertilization led long ago to the theory that the egg attracts the spermatozoon to itself by chemotaxis, and fusion results from active penetration of the ovum by the spermatozoon. But in recent years there has been a tendency to deny both of these

principles as factors in the union. Chemotaxis has fallen into disrepute; and the theory that the spermatozoon bores into the egg has been rejected by several observers.

If chemotaxis is concerned in the union of ovum and spermatozoon the medium in which fertilization operates must contain the substance concerned. In the case of the eggs of *Nereis* and *Arbacia*, therefore, the hypothetical substance which attracts the spermatozoa must exist in sea-water which has been in contact with fertilizable eggs; and it must be possible to obtain a sufficient concentration of the substance in question in sea-water to demonstrate its presence by reaction of the spermatozoa, because, *ex hyp.*, the substance exists in effective amounts in the sea-water surrounding the eggs. If it were impossible to demonstrate the presence of an agent to which spermatozoa of the same species are positively chemotactic by such means the theory of chemotaxis would have to be abandoned. However, the presence of such a substance is readily demonstrated both in *Nereis* and *Arbacia*.

In the second place, if the union of the ovum and spermatozoon after they have come in contact operates not mechanically, but through some bio-chemical reaction between spermatozoon and ovum, the sea-water in which eggs have been standing should contain a substance also capable of reaction with the sperm, which should be an efficient indicator for it.

I was guided by some such ideas as these in the series of experiments which follow, and which showed at the very first trials that sea-water which has stood with fertilizable eggs of *Nereis* or *Arbacia* contains a substance to which the spermatozoa of the same species are positively chemotactic, and also a substance which agglutinates the spermatozoa of its own species. It may be that one substance is concerned in both reactions, but it is more probable that two are present. It is perhaps worth emphasizing here, for this is the fact that struck me at the start, that the sea-water which has stood over eggs³ combines both the effects of

³ To avoid the frequent repetition of such a circumlocution we may call sea-water, which has contained eggs and is charged with their emanation, egg sea-water; and the concentration of the substance in the sea-water may be expressed by writing the relative bulks of eggs and sea-water as a fraction. Thus 'egg sea-water 1/3' would indicate that the bulk of eggs was one-third the volume of the sea-water. Time is also a factor in the concentration, of course.

an acid (aggregation) and also an alkali (agglutination) on the spermatozoa. The comparison may, of course, be superficial, but it serves at least to emphasize the double action of the egg-secretion.

As contrasted with the difference in rapidity and delicacy of reaction between the spermatozoa of *Nereis* and those of *Arbacia* to inorganic substances, we may note in advance that the reactions to the egg extractives are as rapid and clear in the one case as in the other, and are entirely similar in principle, though there are certain secondary differences that will be noted in the proper place.

2. INITIAL EXPERIMENT

We may begin by describing the reactions to be observed in the case of an *Arbacia* sperm-suspension freshly made and mounted beneath a raised cover-slip, into which a drop of *Arbacia* egg sea-water 1/10 to 1/20 about half-an-hour-old is injected. The naked eye observation shows almost instantaneous formation of a ring at the margin of the drop, with simultaneous formation of a clear external zone about 1.5 to 2 mm. wide; the ring then breaks up into small agglutinated masses and so becomes beaded. The trail of substance left in withdrawing the pipette extends to the margin of the cover-slip. It also is a center of attraction and the ring is therefore prolonged by a chain of agglutinated masses to the margin.

One can observe the details of the reaction best under the microscope, using a low power, by bringing the point of the pipette into the field of the microscope and blowing in the drop with the aid of a flexible rubber tube held in the mouth, while looking through the microscope. The reaction takes place so rapidly that it requires repeated observations to observe all the details. In the first second the spermatozoa are aroused to intense activity and form small agglutinated masses within the drop; these then appear actually to 'rush' together (to use the language of my note book) to form larger agglutinations for a period of three to five seconds, after which no more fusion of masses takes place. The agglutinated masses thus range from relatively large to relatively small. While this has been going on in the interior of the drop, a ring

has formed at the margin, and a clear zone arises external to it. The ring is at first continuous, but it ruptures in numerous places in two or three seconds and each segment contracts quickly to an agglutination mass.

The agglutinated masses in the interior of the drop are smaller than in the ring, owing to the relatively low concentration of the sperm suspension within the drop, and they break up very quickly while the sperm is still extremely active. The movements of the spermatozoa then gradually slacken; in a few minutes the larger and more firmly agglutinated masses of the ring also begin to break up and in ten or fifteen minutes all are resolved.

This preliminary observation demonstrates a three-fold action of the egg-extractive: (1) it activates the spermatozoa; (2) it aggregates them through positive chemotaxis; (3) it agglutinates them. The phase of increase of activity lasts only a short time, a minute or two at the most, after which movements of the sperm slacken and become less than the control, or cease entirely. Positive chemotaxis (aggregation) is shown by formation of a clear zone external to the marginal ring. This is always a sign of chemotaxis, as we have seen in the preceding section.

The agglutination phenomenon is fundamentally different from the aggregation; in the latter the spermatozoa are merely loosely associated, and slight agitation is sufficient to scatter them. In the agglutinated masses the spermatozoa are stuck together and are not separated by shaking. In the case of *Nereis* where the agglutination is firmer than in *Arbacia* the masses may be broken up into smaller masses by needles, or preserved *en masse* in killing fluids. The breaking up of the ring into separate masses is a characteristic agglutination effect; the rings formed in response to an acid do not break up unless the acid is very weak (see p. 537). Finally an agglutinative substance produces its effect when shaken up and evenly distributed in a vial of sperm suspension, but an aggregative substance cannot of course exert a chemotactic effect in the absence of a gradient.

The same experiment succeeds well with *Nereis*. The eggs of this form give off a substance (or substances) into the sea-water, which causes aggregation and agglutination of the spermatozoa

when a drop of sea-water so charged is injected into a fresh sperm suspension beneath a raised cover slip. The activation is not so pronounced in this case as in *Arbacia*. The aggregation phenomenon is the same. The agglutinations are substantially permanent in *Nereis*; the spermatozoa stick together much more firmly.

In what follows we may leave the activation and aggregation phenomena out of account for the most part and confine ourselves to the problems of agglutination. The substance which causes agglutination of the spermatozoa we shall call the sperm agglutinin. The agglutination may be shown very strikingly in a vial of fresh sperm suspension. In the case of *Arbacia* the addition of two or three drops of egg sea-water $1/4$, which has stood half-an-hour, to about 2 cc. of a fresh milky sperm suspension causes formation of agglutinations 1 to 2 mm. in diameter in a few seconds. The agglutination may be so strong that the fluid between the white agglutinated masses appears perfectly clear. The masses gradually fade from view in a few minutes, but microscopic agglutinations may remain half-an-hour or more.

The degree of agglutination is of course dependent on the density of the suspension. This is shown by the following experiment: Ovaries and eggs of *Arbacia* were cut up in about four times their own bulk of sea-water and allowed to settle. Two cubic centimeters of the supernatant fluid was put in each of three vials. To one was added 3 drops of a fresh milky sperm suspension, to the next 12 drops of the same, to the third 36 drops. No visible agglutinations formed in the first; in the second agglutinations became visible to the naked eye almost immediately, in the third agglutinations were larger, more numerous, and apparently more solid.

3. OVA ALONE PRODUCE THE AGGLUTINATING SUBSTANCE

The eggs of both forms thus produce an agglutinin in the sea-water. The next question is whether the agglutinin is specifically an egg-product. A considerable number of experiments prove that this is the case. The large body cavity of *Arbacia* is filled with abundant coelomic fluid and this may be supposed to

contain substances from various tissues. But it invariably proved perfectly neutral to spermatozoa of *Arbacia*, even when taken from females with large ovaries, showing that the substance concerned in agglutination does not escape from the ovaries of the intact animal, or, if it does, that it is promptly destroyed. Nor was it possible to extract a sperm agglutinating substance for *Arbacia* by extracting the intestine in sea- or fresh-water.⁴ These experiments were repeated a sufficient number of times to be conclusive.

As illustrations: (1) Intestine extractives. August 31, 1912. The intestines of several *Arbacia* were cut up in about twice their bulk of distilled water, and were allowed to stand in it about an hour. The strongly amber-colored fluid was filtered off and rendered isotonic with the sea-water by addition of concentrated sea-water (four parts of the latter to six of the intestine extract). This fluid causes no agglutination in sperm suspensions. A similar extract of the ovaries caused immediate large dense agglutination masses. (2) Coelomic fluid: As is well known, the coelomic fluid of *Arbacia* contains large numbers of densely pigmented corpuscles. Outside the body the fluid quickly forms a loose clot which includes many of the corpuscles. The others can be separated from the remaining serum by centrifuging. The corpuscle-free serum was sometimes used, sometimes simply the clot-free serum. Repeated tests were made both by injecting drops into fresh sperm suspensions beneath raised cover glasses, and also by mixing with fresh sperm suspension in vials. Whether the coelomic fluid came from males or females it proved invariably negative, except for the faintest sort of agglutination reaction in one or two cases only, which may have indicated some individual differences. It is interesting in view of these facts that outside of the body the coelomic fluid becomes heavily charged with the sperm agglutinin if eggs are placed in it. It must be supposed therefore that the ovarian membrane is impermeable to the agglutinating substance in the intact animal.

⁴ The experiments this year simply opened up the problems, and it was impossible to make any quantitative tests or adequate chemical examination. For the purpose of the biological problem of the behavior of the spermatozoa with reference to the eggs the question of immediate importance was the behavior with reference to egg-extractives or secretions in the sea-water tested. Stronger agglutinating solutions were made by increasing the quantity of eggs with reference to the sea-water, or by crushing the eggs in sea-water. Distilled water was shown to extract more agglutinin from a given bulk of eggs than sea-water; and the coelomic fluid of *Arbacia* also proved to be a better medium for extracting agglutinin from the eggs than sea-water. It would be possible of course to establish quantitative values for all of these relations, and this problem should receive attention. The problems of solubility of the agglutinin in various media, and other chemical questions, also present themselves.

In the case of *Nereis* it was not possible to reach such conclusive results, because the sexually mature female is practically a bag of eggs, and one cannot obtain other organs for testing. I cut up five females that had shed their eggs in 10 cc. of sea-water. In spite of efforts to get rid of all eggs, a considerable number were in the water. For control I used the eggs of two females in 100 cc. sea-water. On test in half-an-hour the fluid from above the eggs was found to be about ten times as agglutinative as the fluid from the bodies of the spent females. So that it is certain that other tissues do not produce much sperm agglutinin and it is probable that they do not produce any. The small amount present could be accounted for by the few eggs included, and perhaps by egg secretions absorbed by the tissues.

4. FIXATION OF THE AGGLUTININ BY SPERMATOOA

The next question was whether the agglutination reaction as described has the usual characters of a chemical reaction? The general result is (1) that an agglutinated sperm suspension in which reversal has occurred is not capable of re-agglutination by addition of more of the agglutinating substance and (2) that the agglutinating substance disappears from an agglutinated suspension if not present originally in excess.

As regards the first point, the earlier experiments were concerned entirely with the form and conditions of the reaction, and the agglutinating substance was always used, as later results showed, in excess. It was not possible to get a repetition of the agglutination reaction under these circumstances. But one can get a repetition of the reaction in a sperm suspension by addition of successive small amounts of the agglutinating substance, until the reaction is complete, as the following experiment shows:

September 4, 1912. *Arbacia*. 2 cc. of a creamy active sperm suspension was agglutinated with 5 drops of an egg-extract prepared as follows: The ovaries of three females were cut up in about three times their volume of distilled water and allowed to stand about thirty minutes. Then the water was filtered off and made isotonic with sea-water by the addition of concentrated sea-water (proportions of 58 to 42 parts). This made a very strong agglutinating extract. After reversal of the agglutination described above the agglutination was repeated by addition of a drop of

another egg-extract. This time the agglutination was complete for it could not be repeated a third time. Other sperm suspensions gave similar results.

The result might be interpreted as a purely biological reaction, that is to say in terms of stimulation, were it not for the fact that the agglutinating substance disappears from an agglutinated sperm suspension, as shown by the following experiment:

September 12, 1912. *Arbacia*. Nine parts of a thick active sperm suspension was agglutinated by one part egg-extract. The agglutination produced was so strong that the fluid between the white masses appeared clear to the eye. In three or four minutes reversal of the agglutinations had begun. The agglutinated sperm suspension was then centrifuged until practically all the sperm was precipitated. The supernatant fluid was tested and agglutinin was shown to be absent. As control, a dilution of one part of the same egg-extract with 9 parts of sea-water was tested with the same sperm and proved to be strongly agglutinative. Three tests were made with each with uniform results.

There can be no doubt, as the result of this and other observations also, that the spermatozoa fix in some way the agglutinating substance, and it will be simplest to assume as a working hypothesis that the fixation is due to chemical union. I have not yet had the opportunity to ascertain if the agglutinin could be regained from the sperm precipitated in the centrifuge.

5. NATURE OF THE EFFECT ON THE SPERM

We have noted four effects of the egg-extracts on sperm of the same species, namely: (1) Stimulation of intense activity, which is of brief duration. This is more marked in the case of the sperm of *Arbacia*, than in the case of the naturally extremely active sperm of *Nereis*; (2) An orienting effect expressed in positive chemotaxis; (3) An agglutinating action; (4) Following these effects more or less complete paralysis of the sperm.

To what kind of change in the individual spermatozoa is the agglutination reaction due? We may note in the first place that the agglutination is between the heads of the spermatozoa, and that the tails are apparently unaffected, at least at first; it is only in later stages of the action of the agglutinating substance that the locomotor function is injured. The adhesion of the heads

demonstrates some change in the membrane that renders them sticky. The cells are so minute that it is difficult to observe any microscopical change in the case of *Arbacia*; in *Nereis* the spermatozoa are larger, and it can be seen that in agglutinated masses the heads of many of the spermatozoa are swollen into spherical form and have lost the normal strong refringibility. The change is in this case a very characteristic one, indicating a great increase in permeability. The spermatozoa which have undergone this change are usually motionless, and, when not fused with one another, appear to be glued to the slide or cover slip, never freely suspended.

Agglutination in itself is in no sense a specific reaction, but one that may be expected to accompany certain superficial changes of the spermatozoa, however caused, under conditions that bring the spermatozoa into contact. It occurs, to a limited extent, spontaneously in sperm suspensions that have stood for some time. It is particularly noticeable in *Nereis* under the following conditions: A fresh sperm suspension is allowed to aggregate on a slide beneath a raised cover slip and the aggregations remain undisturbed. In the course of ten or fifteen minutes small agglutinated masses may form around the margins of the aggregations or beneath the aggregations in contact with the slide. There may be twenty to fifty or more such masses associated with a single aggregation, and they are quite similar in their general appearance, to those produced suddenly by the agglutinin of the egg, though much smaller, on the average.

It is not probable that the agglutination is in any real sense toxic or cytolytic. It is true that the agglutinin inhibits movement after a few minutes, and it certainly lessens the fertilizing power of the sperm. But if an agglutinated mass of spermatozoa of *Nereis* be crushed under the microscope many of those liberated are active. Moreover, if a small quantity of an agglutinated suspension of spermatozoa be added to a relatively large quantity of sea-water fertilizing power is partially regained. This might be either because some of the spermatozoa of the agglutinated suspension had escaped combination with the agglutinin and were alone concerned in the actual fertilization, or because of recovery

from the agglutination effect. It is very difficult to form a definite opinion as to the real nature of the agglutination effect.

6. THERMO-RESISTANCE OF THE AGGLUTININ

The agglutinating agent is slowly destroyed at 95°C.:

August 28. *Arbacia*: (1) Ovaries and eggs of *Arbacia* were cut up in three times their bulk of sea-water, and let stand about an hour. The supernatant fluid is strongly agglutinative on *Arbacia* sperm suspensions. (a) Part of it was now taken and boiled about thirty seconds, and cooled. On test it proved as agglutinative as before. (b) Some more was then boiled five minutes and cooled. Its agglutinative power was apparently undiminished. (c) Another egg-extract similar to the first was then boiled and put in a beaker of boiling water for thirty minutes; the temperature stood about 95° during this process. On test its agglutinative power was shown to be greatly diminished. (d) In a fourth test some egg-extract was kept at 95° for sixty-six minutes. It still exhibited some agglutinative power, which, however, was very slight as compared with the control. The heated egg-extract exhibited a considerable change of color from the yellowish red of the control to a much brighter red.

Simultaneously with the loss in agglutinating power, it appeared also to gain in aggregating power: Drops of the heated and the unheated egg-extract were injected into the same sperm extract beneath a raised cover. The drop of the heated expanded its sphere of influence shown by immigration of the sperm about twice as rapidly as the unheated; this was tested several times; so that it would appear that the aggregative and agglutinative agents are probably distinct, and that the agglutinin inhibits aggregation to a considerable extent.

Nereis: The eggs of three females were inseminated in 9 cc. sea-water; the supernatant fluid has a slight amberish-green color, and is strongly agglutinative on *Nereis* sperm. (a) After keeping at 95° C. for ten minutes its agglutinative power was much reduced. (b) After twenty-two minutes at 95°C. the agglutinin was entirely destroyed. The color was entirely destroyed also.

Thus the agglutinating substances, whatever they may be, are either volatile being gradually driven off by heating, or they are slowly coagulated or disintegrated chemically by a temperature of 95°C. The agglutinin of *Nereis* is either more volatile or more labile than that of *Arbacia*. It is impossible to say definitely to what class of chemical substances these agglutinating

substances belong. It is, however, extremely improbable that they possess a degree of chemical simplicity sufficient to allow of volatilizing; it is more probable that they undergo slow chemical disintegration at the temperature employed. The thermostability of these sperm isoagglutinins is relatively very high, and this perhaps makes it doubtful whether they can belong to the same class of substances as the haem-agglutinins of vertebrate blood sera. This matter must therefore remain undecided, and it should be understood that the term agglutinin is used in the present paper in a purely descriptive sense.⁵

7. FERTILIZING POWER OF AGGLUTINATED SPERM

The powerful effect of the egg-extract on spermatozoa of the same species may be shown by a complete loss of motility as we have already seen, and also by a corresponding loss or diminution of the fertilizing power. The following experiments illustrate this:

1. *Arbacia*. The egg-extract used was made by cutting up the ripe ovaries in about three times their bulk of distilled water; in half-an-hour the water was filtered off and was then made isotonic with sea-water by the addition of 42 parts of condensed sea-water to 58 of the egg-extract. Five small watch crystals in a series contained (1) 8 drops of the egg-extract (2) 4 drops egg-extract, + 4 drops sea-water (3) 2 drops egg-extract, + 6 drops sea-water, (4) 1 drop egg-extract, + 7 drops sea-water (5) 8 drops sea-water. To each of these 3 drops of opalescent sperm suspension was added; and after twelve minutes a drop of a suspension of fresh eggs was added to each. We thus had the same quantity of eggs in sperm suspensions of the same density, but in graded amounts of egg-extract. The sperm suspensions were so dense that in the control (no. 5) the jelly became packed with sperm, forming dense halos around the eggs. In (4) a very few (about 1 per cent) had slight halos of sperm in the outer layer of the jelly, but in (1), (2) and (3) the paralysis of the sperm was so complete that they did not enter the jelly of the eggs at all. Ninety-seven minutes later none of the eggs in (1), (2), (3) or (4) had segmented; whereas at least 5 per cent of the control were now in the two-celled stage. The lot of eggs was rather poor in this case, but fertilization was confined entirely to the control.

If the experiment be made in another way some recovery of the spermatozoa from their state of paralysis may be observed. Thus: An

⁵ It should be borne in mind that but little is known concerning lysins or agglutinins of invertebrates. It is perhaps not to be expected that they should exhibit the same degree of thermolability as those of vertebrates.

active sperm suspension was divided in two parts, and one part was agglutinated by the addition of about 40 per cent of its own volume of the egg-extract described above, to the other an equal amount of sea-water was added. The first was strongly agglutinated; after reversal both suspensions were stirred up, and beginning thirteen minutes after agglutination a series of fertilizations were carried out by adding one drop of the agglutinated sperm suspension to a measured quantity of eggs in about 9 cc. of sea-water at 10 minute intervals. Each fertilization had a control of the same quantity of eggs fertilized with one drop of the control sperm. The consistent result was that about 16 per cent of the eggs fertilized with the agglutinated sperm segmented and at least 33 per cent of the control. The non-agglutinated spermatozoa are about twice as effective as the agglutinated. But a considerable degree of recovery of some spermatozoa of the agglutinated suspension is shown.

2. *Nereis*. Experiments with *Nereis* did not give such a marked reduction of the fertilizing power of agglutinated sperm as in *Arbacia*. There was, however, a marked delay in the formation of jelly when agglutinated sperm was used as compared with normal sperm.

It is somewhat difficult to make a satisfactory interpretation of the effect of agglutination on fertilizing power. On the one hand we may suppose that a certain proportion of spermatozoa resist the agglutination effect, and are alone concerned in any fertilizing power of an agglutinated suspension; on the other hand it might be supposed that the agglutinating effect does not modify fertilizing power except as it decreases the motility of the spermatozoa or that the effect is reversible under the condition of the experiments. Either assumption would be consistent with the facts.

8. CONDITIONS OF FORMATION OF THE AGGLUTININ BY THE EGGS

The conditions of excretion of the agglutinating substance by the eggs into the sea-water is quite different in the two forms. In *Arbacia* the agglutinin is excreted continuously by unfertilized eggs in such amounts that I have not succeeded even by repeated washings in removing it all. Fertilization does not appear to increase or decrease the quantity. In *Nereis*, on the other hand, unfertilized eggs secrete but little of it, and one or two washings in sea-water will completely remove it so that the eggs secrete no more in detectable quantities; but at the moment of fertilization, on the other hand, it is poured forth in advance of the jelly in

large quantities and the eggs then appear to have disposed of their entire store, for washed fertilized eggs no longer produce it. The conditions are important in their bearing upon the question of permeability of the egg-membrane with reference to fertilization. The spermatozoa are very efficient indicators of substances leaving the egg. In the case of *Nereis* it can be shown that there is a sudden increase of permeability at the moment of fertilization, but in *Arbacia* such evidence is lacking.

The conditions in *Arbacia* may be shown by the following experiment: Eggs were washed free from all fragments of ovary and placed in about 20 times their own bulk of sea-water and divided in three lots, *a*, *b* and *c*. The sea-water over them agglutinates *Arbacia* sperm instantaneously. This test was made at 9.30 A.M. The supernatant fluid was then removed and the eggs washed in 20 times their bulk of sea-water. At 9.53 the supernatant water was tested and found agglutinative. 9.58, *a* and *b* were again washed. 10.04, supernatant fluid again agglutinative. 10.05, lot *a* was fertilized with 2 drops of sperm. 10.10, *a* and *b* tested again; both agglutinate. 10.16, *a* and *b* washed again. 10.23, supernatant fluid of both agglutinates. 10.30, *a* and *b* washed again. 10.36, both agglutinate sperm; *b* is rather more effective. 10.40, washed *a* and *b* again. 10.45, both agglutinate; *b* more effective. 10.51, washed *a* and *b* again. 11.00, both agglutinative; *a* more than *b*. 11.08 to 11.20, other tests of *a* and *b* show *a* somewhat more effective.

The experiment shows both fertilized and unfertilized eggs of *Arbacia* to be constantly secreting a substance into the sea-water which agglutinates the spermatozoa. The substance must be effective in very minute quantities for the amount of water used in each of the eight washings was at least ten times the bulk of the eggs, yet as soon as the eggs had settled to the bottom of the vials used the supernatant fluid contained the agglutinin in appreciable amounts. How long this process keeps up in *Arbacia* I cannot say; and too much reliance cannot be placed on the result for the fertilized eggs because a large proportion of the eggs failed to fertilize or at least to segment.

The conditions in *Nereis* are quite different; the experiments showed that shortly after the eggs are taken they charge at least ten times their bulk of sea-water with an easily detectable amount of sperm agglutinin. But if the eggs are washed once usually no more agglutinin can be demonstrated. If now the eggs are stirred

up in the vial, fertilized and allowed to settle, the supernatant fluid is very powerfully agglutinative. The experiment was repeated a sufficient number of times to make certain of this result.

A number of tests were also made with the object of determining if the eggs of *Nereis* continued to produce agglutinin after fertilization. These showed that the eggs cease very quickly their production of agglutinating substance. None could be detected during the maturation period, but apparently there is a second production about the time of the first cleavage. On this point I wish to be understood to speak with reserve. The swelling of the jelly secreted at fertilization makes the eggs very bulky, and the jelly itself takes up any egg secretion, so that there are considerable technical difficulties in making satisfactory tests.

The fact that stands out perfectly plainly in the case of *Nereis* is the sudden increase in secretion of agglutinin into the seawater just after insemination, followed by cessation of its production. The spermatozoa give absolutely positive tests. As will be shown later, the observations on the normal fertilization are in complete harmony with this.

The difference between *Nereis* and *Arbacia* in these respects is thus sharply marked. However, it should be remembered that the unfertilized eggs of *Arbacia* have formed both polar bodies, whereas those of *Nereis* are in the stage of the germinal vesicle. It may be that eggs of *Arbacia* in the germinal vesicle stage are relatively impermeable in the same sense as those of *Nereis*.

9. HETERO-AGGLUTINATION AND THE QUESTION OF SPECIFICITY: REACTIONS BETWEEN *NEREIS* AND *ARBACIA*

The demonstration of intraspecific sperm-agglutinating substances derived only from the ova having been made, the question arose whether these substances were essentially the same in both species, or different. If the same, the egg-extract of each should agglutinate the sperm of the other. A number of tests were therefore made which demonstrated conclusively that the substances are decidedly different with reference to their cross-agglu-

tinating effects, and this result has raised a number of questions, the most important of which relate to the question of specificity, but which could only be defined in the time at my disposal and not definitely answered.

The first suggestion that the sperm agglutinating substances of *Arbacia* and *Nereis* are different came from the following experiment: A raised coverslip preparation of *Nereis* sperm was made in the usual way and into it were injected a drop each of (1) *Arbacia* egg-extract in sea-water, (2) drop a of coelomic fluid from a female *Arbacia*, (3) a drop of coelomic fluid from a male *Arbacia*. All three caused very extensive firm agglutinations of the *Nereis* sperm.

Thus the egg-extract of *Arbacia* contains an agglutinating substance for the *Nereis* spermatozoa as well as for its own; but the coelomic fluid of *Arbacia* also causes agglutination of the *Nereis* spermatozoa, whereas it is perfectly neutral with respect to its own. This demonstrated, therefore, the existence of at least two sperm-agglutinating substances in *Arbacia*, namely: One in the egg-extract agglutinative for its own sperm and that of *Nereis*, and one in the coelomic fluid not agglutinative for its own sperm, but agglutinative for the foreign sperm of *Nereis*. The probability is, therefore, that the egg-extract contains both substances seeing that it is agglutinative for both kinds of spermatozoa. This was afterwards demonstrated.

The reciprocal experiment proved the difference of the *Nereis* and *Arbacia* agglutinating substances conclusively, for it was shown that *Nereis* egg-extracts strongly agglutinative for sperm of *Nereis* had no agglutinating effect on *Arbacia* sperm within the limits of attainable concentrations (several experiments). The *Arbacia* fluids are extremely toxic apparently to the *Nereis* sperm for the agglutinations were more solid than those caused by the iso-agglutinating substance. The absence of a reciprocal effect, that is, of *Nereis* extracts on *Arbacia* sperm, is therefore all the more striking.

The same difference may be shown by the reactions of sperm suspensions of both species to one another. The experiment was made in the following manner:

July 28, 1912. *a.* A suspension of active *Arbacia* sperm was made at 11.23 A.M.

b. A suspension of active *Nereis* sperm was made at 11.24 1/2.

The two suspensions were made of equal density as far as possible.

Part of each suspension was then mounted on a slide beneath a raised cover-slip.

A drop of the *Nereis* sperm was then injected into the *Arbacia* slide (slide 1) and a drop of *Arbacia* sperm into the *Nereis* slide (slide 2).

Slide 1 gave a very faint reaction; only slight evidence of a ring formation at the margin of the *Nereis* drop.

Slide 2, on the other hand, gave a very pronounced reaction due to inwandering of the *Nereis* sperm into the *Arbacia* drop, followed by agglutination of the inwandering sperm.

The difference in reaction is due to two circumstances: (1) The *Nereis* sperm exhibit a more pronounced chemotaxis than the *Arbacia* sperm, hence they tend to enter the drop of *Arbacia* sperm, whereas on the other slide the *Nereis* sperm tend to diffuse from the drop into the *Arbacia* suspension. (2) The *Nereis* sperm that wander into the drop of *Arbacia* sperm are agglutinated, but there is no reciprocal reaction of the *Nereis* sperm on any inwandering *Arbacia* spermatozoa.

It is demonstrated, therefore, first that *Arbacia* fluids in general are toxic for *Nereis* spermatozoa to the extent, at least, that they cause agglutination; but on the other hand, that no secretion of *Nereis* appears to cause agglutination of *Arbacia* spermatozoa; and second that the eggs of each species produce an agglutinin for the sperm of its own species.

Agglutination is not in itself a specific process; it may take place spontaneously to a certain extent under some conditions; it is caused by increase of alkalinity of the sea-water in the case of *Nereis* and *Arbacia*, or by the action of certain foreign sera as in the case of the action of *Arbacia* fluids on *Nereis* sperm. On the other hand, the class of specific immune agglutinins, characterized by limitation of their action to the specific form of blood or sperm used as antigen is well known. The question naturally arises, therefore, to which class the iso-agglutinating substances produced by ova of *Arbacia* and *Nereis* belong.⁶

⁶ In the latter case, fertilization itself would have to be regarded as an immunizing process, the sperm acting as antigen after entrance into the egg. It seems, in

The fact that the egg secretions of *Arbacia* cause agglutination of *Nereis* sperm as well as *Arbacia* sperm seems at first sight to indicate the iso-agglutinating substance of *Arbacia* is not specific. But the fact that other *Arbacia* fluids likewise agglutinate the sperm of *Nereis*, but not those of *Arbacia*, raises the question whether the egg-secretion does not contain in addition to the iso-agglutinating substance, also another which is agglutinative for *Nereis* sperm like the substance in the coelomic fluid, but not for its own sperm.

If there are two substances present in the egg secretion it ought to be possible to separate them by various means. They might exhibit different heat-lability, so that one might be destroyed at a temperature that would leave the other still active. Or if they have different affinities it might be possible to fix the *Nereis* agglutinating substance by *Nereis* sperm, leaving the iso-agglutinating substance intact. Neither of these experiments could be performed this year, owing to the disappearance of the necessary material.

However, it was possible to show in another way that the *Nereis*-agglutinating substance of *Arbacia* egg-extract is distinct from the iso-agglutinating substance: An egg-extract of *Arbacia* seventeen days old was found to have entirely lost its power of agglutinating *Nereis* spermatozoa, while it retained undiminished its power of agglutinating *Arbacia* spermatozoa. Originally it agglutinated both kinds of spermatozoa. Now the change that took place in the egg-extract on standing is not a mere weakening of action as might be supposed, because the iso-agglutinating action was noted as undiminished, whereas the hetero-agglutinating action was entirely lost. The only possible conclusion, therefore, is that the egg-extract contained two agglutinating substances at least, namely: An iso-agglutinin and a hetero-agglutinin, and that the latter is relatively labile, the former relatively stable. Unfortunately the experiment could not be repeated on account of the total disappearance of the material.

fact, an almost necessary conception on the general principles of immunity phenomena that the sperm should so act. The question would be, of course, whether there is a connection between any antibodies so formed and the sperm iso-agglutinins produced by the next generation of ova.

The experiment in detail was as follows:

September 15, 1912. One small male Nereis available. Its sperm is aggregative and very active. The Arbacia egg-extract was of August 29 and was made by cutting up ovaries and eggs of Arbacia in four times their volume of sea-water. After settling of the eggs the supernatant fluid was poured off, and had been kept in a stoppered vial since.

1. A drop of the Nereis sperm and a drop of the egg-extract were placed side by side on a slide and connected; the sperm diffused into the egg-extract and swam around in it; no agglutination.

2. A raised cover mount was made of the Nereis sperm and a drop of the egg-extract injected. The sperm entering the drop swam around, and were not agglutinated.

Controls: 1. The sperm of Nereis was agglutinated immediately by extract of Nereis eggs kept since September 8.

2. The egg-extract of Arbacia agglutinated Arbacia spermatozoa with no apparent diminution in the strength of the reaction.

Conclusion: The nonspecific agglutinating substance has been destroyed by the chemical changes in the extract in the course of seventeen days; but the iso-agglutinating sperm substance still remains.

This experiment does not demonstrate that the sperm iso-agglutinin of Arbacia egg-extract is specific, but merely that it is without effect on the Nereis sperm, just as the iso-agglutinin of Nereis eggs is without effect on Arbacia sperm. It is of course still possible that the iso-agglutinating substances might have an agglutinating effect on some other varieties of sperm, and a crucial test of specificity must await the securing of new material.

However, it seems to me that the probabilities in the case lie strongly on the side of specificity of these sperm iso-agglutinating substances. Quite apart from the value of the evidence already adduced, we must consider the general fact that ova and spermatozoa of the same species do behave in a specific way with reference to one another in the process of fertilization. This must have some chemical basis and on the chemical side the only reactions that exhibit a corresponding degree of specificity are those between antigens and anti-bodies in the field of immunity. We have two parallel instances, therefore, and the slight evidence which I have so far been able to bring forward in favor of the specificity of the sperm iso-agglutinins of ova gains immensely in weight by its association with the universal principle of specificity in fertilization, and the known class of specificities in agglutination reactions.

The agglutination of spermatozoa is, of course, in itself of no significance for the problem of fertilization; the spermatozoa unite in fertilization with the egg, not with one another. The agglutination reaction is, however, an indicator of an important change in the spermatozoon in the presence of egg secretions, and therefore evidence of a change that any spermatozoon must undergo when it comes in contact with the egg. The adhesive property that the sperm develops under these circumstances may be an important factor in binding the sperm to the egg until it can be incorporated. But, if the reaction be specific, it is much more than this; it is evidence of an intimate chemical combination of sperm and egg constituents which begins at the very moment of union.

Von Dungern's experiments ('02) are the only ones, so far as I know, in which the production of sperm agglutinins by ova was investigated, and he discovered only hetero-agglutinins, no iso-agglutinins. He did, indeed, describe the loss of motility of spermatozoa in egg-extracts of the same species, but he entirely missed the phenomenon of agglutination and its reversal. He reveals the reason for this failure by his remark that he always examined for the effect of the 'egg-poison' about half-an-hour after its addition to the sperm; but the phenomenon of agglutination and its reversal are completed in about five minutes.

Von Dungern also made experiments on the production of immune sera by injection of ova and spermatozoa separately into rabbits, and found that both caused the production of a sperm agglutinin in the rabbit's serum. From this he concludes that both kinds of reproductive elements possess chemically identical complexes of molecules in the protoplasm. While this may be admitted as at least a very probable conclusion, his farther conclusion that fertilization does not depend upon any specific antagonism between ovum and sperm, but is conditioned by the similarity of their protoplasms, is not well founded, for the egg is a very complicated chemical system, and it certainly contains molecules antagonistic to sperm, even if, as Von Dungern's experiments indicate, it also contains some that are not.

10. INTERPRETATION OF SOME PHENOMENA OF NORMAL FERTILIZATION IN NEREIS

To observe all the details of normal fertilization it is desirable to inseminate in a suspension of India ink which will define the transparent substances exuding from the egg on insemination. the following observations can then readily be made on mixing a drop of the eggs in the ink suspension with a drop of opalescent sperm suspension: Hundreds of spermatozoa become attached to each egg almost immediately; those in contact with the egg do not show much activity, but are usually definitely oriented radially; the spermatozoa external to these are in active movement. In about a minute a clear fluid begins to exude from the egg and surrounds all attached spermatozoa and involves the immediate neighbors. The first exudate is quite fluid for it flows around the spermatozoa and does not sweep them away, but the movements of all the spermatozoa within the exudate cease suddenly. The flow continues, and then most of the spermatozoa are swept away from contact with the egg, for the later exudate is gelatinous in consistency. However, a good many spermatozoa remain in contact with the egg for some time, but these are detached one by one as the flow of the jelly continues, until only one remains. Some of the supernumerary spermatozoa are not carried away until five or more minutes after insemination.

The immediate prevention of polyspermy in *Nereis* appears to be due to the paralyzing effect of the egg-exudate poured out in response to the stimulus of the first effective spermatozoon. Polyspermy could take place in *Nereis* only under two conditions, namely, (1) if two or more spermatozoa *simultaneously* give the stimulus to the egg that causes excretion of the agglutinin; for the condition of stimulus appears to be that the spermatozoon be securely anchored to the egg; and all spermatozoa not securely attached at the moment the egg begins to secrete are prevented from securing attachment by the resulting paralysis; (2) if the reaction of the egg be slow and therefore localized at first to the region of an effective spermatozoon, opportunity will be afforded for attachment of other spermatozoa.

In a short time the egg develops a physiological condition in which union of spermatozoa is no longer possible. The immediate protection against supernumerary spermatozoa is, however, afforded by the paralyzing action of the egg-secretion.

Union of ovum and sperm, prevention of polyspermy, and the attainment of a condition of insusceptibility to other spermatozoa are phenomena so closely related in time sequence that a casual connection must be postulated. These can be brought under one head, in the case of *Nereis* at least, if we assume that the substance that paralyzes all the sperm in the vicinity of the egg is necessary for the actual fusion of the spermatozoon and egg and is completely used up in the cortical changes that follow immediately on insemination: the condition of insusceptibility would be due to loss of a necessary substance, the immediate prevention of polyspermy to paralysis of all ineffective spermatozoa, and the penetration of the sperm to a chemical change of the effective sperm and the neighboring egg-cytoplasm involving physical alterations in surface tension, viscosity, et cetera.

In the case of *Arbacia*, I have been unable to demonstrate an increase of secretion from the egg into the sea-water at the moment of insemination nor yet cessation of such secretion soon after insemination; however, as indicated before (p. 560), this failure may be due to the presence of unfertilized eggs in the experiments, which require to be repeated.

11. SUMMARY: PART IV

1. The ova of *Nereis* and *Arbacia* give off into the sea-water a substance (or substances) which agglutinates the sperm of their own species. The sea-water, which has the agglutinating substance in it, has also a substance to which the spermatozoa of the same species are positively chemotactic.

2. The eggs alone produce the sperm agglutinating substance; It cannot be extracted from other tissues.

3. The agglutinin disappears from a mixture of sperm suspension and agglutinin if not present in excess; the disappearance is attributed to chemical combination.

4. The agglutinating substances are highly thermostable, but are slowly destroyed by temperatures above 95°C.

5. In the presence of excess of the agglutinating substance spermatozoa of *Arbacia* lose their fertilizing power.

6. Eggs of *Arbacia* give off the agglutinating substance in the sea-water in large quantities prior to insemination; but eggs of *Nereis* give off only small quantities until inseminated, or until the cortical change analogous to membrane formation in the sea-urchin egg is somehow produced.

7. The egg-extract of *Nereis* does not agglutinate *Arbacia* spermatozoa.

8. The substance in the egg-extract of *Arbacia* that agglutinates *Nereis* spermatozoa is distinct from the iso-agglutinating substance.

9. The coelomic fluid of *Arbacia* contains a substance which agglutinates the spermatozoa of *Nereis* but not of *Arbacia*. Presumably this substance is the same as the hetero-agglutinating substance of *Arbacia* egg-extract.

10. Two arguments in favor of the specificity of the iso-agglutinative reaction were brought forward, namely, (a) The fact that the iso-agglutinin of *Arbacia* is distinct from the hetero-agglutinin in the case of *Arbacia* and *Nereis*; (b) that fertilization is fundamentally a specific reaction, and that the phenomena of agglutination belong in a class of phenomena in which specificity exists, and between elements which react specifically in fertilization. While admittedly not demonstrative these arguments appear to me to be cogent.

V. DISCUSSION

Since Pfeffer's fundamental investigations concerning chemotaxis of spermatozoa of ferns and mosses with reference to the secretion of the archegonia a similar explanation of the behavior of animal spermatozoa with reference to the eggs of the same species has been anticipated, and indeed has been postulated by many writers without any other experimental basis. However such actual experiments as have been performed have not been favorable to such an interpretation. Thus Buller ('00) experi-

mented with the spermatozoa of sea-urchins by the method of Pfeffer and came to the conclusion that "the spermatozoa of the Echinoidea are not attracted to the egg by means of any special substance excreted by the latter. The vast number of spermatozoa and the large size of the eggs are sufficient to ensure the necessary contact taking place." Von Dungern ('02) also rejects the idea of an egg-secretion attracting the spermatozoa in the case of sea-urchins and starfish. Morgan, Payne and Brown ('10) also accept Buller's interpretation, and there has recently been a tendency among biologists to reject chemotaxis of the spermatozoon as a factor in the fertilization of the egg.

Previous observers have worked either with Pfeffer's capillary tube method, or with the eggs themselves. I made a sufficient number of experiments with capillary tubes to convince myself that this method of experimentation is many times less effective than the method I employed. As illustration: July 4, 1912: I filled three pieces of capillary tubing with a concentrated solution of CO_2 in sea-water, with 10 per cent and 1 per cent of this solution and broke off short pieces—neither end of which had been in the solution—to be tested. These pieces were then introduced into an active sperm suspension of *Nereis* beneath a raised cover-slip. In the course of a few minutes a decided positive reaction was obtained with the first and second tubes, the sperm appeared to stream into the open mouth of the capillary tubes and soon formed white plugs at the mouths of the tubes. The tube containing 1 per cent CO_2 sea-water and a control tube with sea-water alone showed no reaction. In a repetition of this experiment the 1 per cent CO_2 sea-water and control were negative, and the 10 per cent showed only slight reaction. The diameter of the lumen of the tube was 0.48 mm. A much finer tube of 10 per cent CO_2 sea-water showed no reaction at all. With tubes of the size first used 10 per cent CO_2 sea-water is near the minimum for a positive reaction. But a drop of 0.5 per cent CO_2 sea-water injected into a similar suspension causes chemotactic response. Tubes of the size used are therefore about twenty times less effective as indicators of the chemotactic reaction, stated in terms of percentage of CO_2 required, than the injected drop method. It

is obvious that the size of the tube is a fundamental condition of the experiment, both because the diffusion is a factor of size and also because of the interference of thigmotactic reactions of the spermatozoa at the mouth of the tube with the purely chemotactic response. Buller does not state what was the size of the tubes that he used in his experiments. But, if the delicacy of the reaction was reduced to one-twentieth by the tube, his failure to get a positive reaction in tubes containing sea-water taken from over eggs is not surprising.

Von Dungern drew his conclusions from observing the behavior of spermatozoa mixed with eggs. Many embryologists, like myself, have made hundreds of observations of this kind; but it is obvious that the conditions thus created render an analysis of the behavior of the spermatozoa impossible. In some experiments I introduced a drop of eggs in sea-water into a sperm suspension beneath a raised cover, and obtained the typical ring formation of spermatozoa with reference to the group of eggs considered as a whole. But within the group any evidence of chemotactic reaction is clearly impossible.

As to the rôle that chemotaxis as a principle may play in the fertilization of the ova in nature it is difficult to form a clear conception. It may be little and it may be considerable. In the first place it may be noted that, although the echinids have been favorite subjects for research, but little appears to be actually known concerning their breeding behavior. In the second place we do not know the distance to which the secretion from an isolated egg will diffuse. But even if we assume that it extends effectively only a short distance in terms of the egg-diameter the result would be essentially to immensely increase the chances of scattered spermatozoa to become entangled in the jelly of the egg. Measurements of the effective radius of diffusion of the egg-secretion could, I believe, readily be made by the method employed in my work and the results of this might enable us to form some clearer idea of the possible significance of chemotaxis taken by itself in the meeting of the germ-cells.

The present results merely show that it may be a factor of some significance. The quickness and readiness of the reaction of

spermatozoa of *Arbacia* and *Nereis* to the secretion of their own kind of eggs is certainly surprising.

Another effect of their secretions that should be taken into account in this connection is the stimulating effect on the spermatozoa. This is more marked in some animals than in others. Thus the spermatozoa of *Nereis* are so active in the sea-water alone that but little effect of the egg-secretions can be noted; in the case of *Arbacia*, although the sperm are quite active in pure sea-water yet the egg-secretions greatly increase their activity for a brief time. In the case of the star-fish, according to Von Dungern's account, the spermatozoa tend to be very inactive in pure sea-water, but are aroused to intense activity by the secretions of the ova.

In different animals, therefore, we may expect to find some difference in the effect of egg-secretions on the activity of the spermatozoa. But the fact that in such widely separated forms as *Arbacia* and *Nereis* secretions of the egg cause strong positive chemotaxis of the spermatozoa inclines one to the view that such a reaction may be very wide spread. In a form in which egg-secretions are both activating and directing in their action, the importance of such secretions in favoring the preliminary steps in fertilization can hardly be doubted.

The experiments, like Pfeffer's earlier ones, indicate that the factor of specificity is probably subordinate in the purely chemotactic response. CO_2 and acids are in no sense specific, but they are very effective chemotactic agents with *Nereis* spermatozoa. But the case of *Arbacia* serves to indicate that substances of the egg, whether specific or not, are more generally effective than simple chemical substances, for it requires such substances, apparently, in the case of *Arbacia* to produce a reaction of the spermatozoa comparable in quickness and precision to the reaction of *Nereis* spermatozoa to acid, CO_2 , or the secretions of its own eggs.

We have seen that in some respects the chemotactic behavior of spermatozoa of *Nereis* and *Arbacia* is different depending on their relative sensitiveness to CO_2 and other agents. The much greater power of resistance of spermatozoa of *Chaetopterus* and

Loligo to CO_2 (see p. 528) indicates still greater differences in characteristic behavior. And it may be that the differences of chemotactic behavior of the spermatozoa of various animal phyla will turn out on investigation to be extensive. I am very far, therefore, from wishing to generalize any of the principles that we have found to hold true for *Nereis* and *Arbacia*. Sound generalizations must be based on much more extensive work. I have made some observations on the sperm of *Platynereis megalops* which demonstrate great differences as compared with *Nereis* in spite of the close relationship, correlated, no doubt, with differences in breeding behavior. While the common form of organization of flagellated spermatozoa points to fundamental principles of behavior in common, yet it must not be forgotten that each kind of spermatozoa has the chemical composition of the species, and may therefore have entirely specific forms of behavior.

The agglutination of the spermatozoa by normally formed egg-exudates of the same species indicates the possibility of studying the chemistry of fertilization directly through use of the spermatozoa as indicators. The very few and incomplete results which I was able to obtain in the time at my disposal seem to me to indicate a fruitful line of work. It would be interesting, for instance, to investigate whether or not the ova of hermaphrodite animals produce a sperm auto-agglutinin, that is, an agglutinating substance for spermatozoa of the same individual. Morgan's work on *Ciona* has shown that the failure to self-fertilize is in this case due to failure of penetration of the spermatozoon. It is difficult, as he points out, to explain this on any mechanical grounds. But if a specific agglutination is a necessary step in union of ovum and spermatozoon, the failure to produce an auto-agglutinin would explain the failure of self-fertilization. We would have in this event a precise parallel to the usual failure to produce blood auto-agglutinating substances in experiments on immunity, though iso-agglutinins are readily produced.

Godlewsky ('11) has shown that there is an antagonism between the sperm of certain animals (*Chaetopterus* and *Echinids*) which destroys the fertilizing power of each when mixed together for a certain length of time. He compares this to the antagonistic

action of heterogenous haemolytic sera on one another; and concludes that his results strongly confirm Loeb's theory that the spermatozoon initiates development by means of a lysin.

Without discussing the interpretation, and considering only Godlewsky's most interesting results, another parallel is furnished to immunity phenomena. We may confidently expect, therefore, that study of the reactions of spermatozoa will break a new path into the field of fertilization.

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