ANTIMITOTIC SUBSTANCES FROM OVARIES [1]

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In a paper published two years ago (Heilbrunn, Wilson and Harding, 1951), it was shown that a powerful antimitotic substance could be extracted from the ovaries of the common starfish (*Asterias forbesii*). This substance tends to exert a liquefying influence both on the cortex and on the interior protoplasm of marine eggs, and because it prevents the mitotic gelation in somewhat the same way that heparin does, we were led to believe that it might possibly be a heparin or heparin-like substance. However, we had very little direct evidence to support this belief.

Accordingly, we have been eager to find out what we could as to the nature of the antimitotic substance. The work is part of a broad general program in which we seek to establish that all living material contains substances which favor protoplasmic clotting and those which tend to prevent such clotting. It is now clear that the colloidal behavior of protoplasm is quite similar to the behavior of vertebrate blood; and if this is true, it would be logical to suppose that the anticlotting substances of living cells include heparins and heparin-like substances. For a detailed discussion of protoplasmic clotting and how it influences not only cell division but other vital processes as well, see Heilbrunn (1951, 1952a, 1952b).

The fact that we can obtain a potent antimitotic substance from the starfish ovary is perhaps not surprising, for the eggs in the ovary do not divide until they leave it. In our earlier paper (Heilbrunn, Wilson and Harding, 1951), it was suggested that "perhaps the ovaries of many organisms are rich in heparin-like substances." As will be seen later, this idea is apparently a fruitful one.

Materials and Methods

In general, extracts were prepared in much the same manner as in our previous work, except for the fact that instead of merely cutting up the ovaries, we homogenized them before extraction with acidified sea water. Specific details concerning the preparation of various individual extracts are given in relation to individual experiments. When the extracts were dialyzed, cellulose dialysis tubing was used. This was purchased from the Arthur H. Thomas Co. of Philadelphia, and they state that according to the manufacturer, the Visking Corporation of Detroit, the average pore diameter of the cellulose material is 24 Ångstroms. The purity of the cellulose is said to be very high, but it contains some glycerine and approximately 0.1 per cent sulfur. During the process of dialysis, the tubes were agitated on a shaking apparatus.

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In testing for antimitotic action, in most cases we used the eggs of a marine worm, *Chaetopterus pergamentaceus*. As in earlier works, the eggs were kept in a constant temperature bath maintained at a temperature of 21° C.

Protoplasmic viscosity tests were made with an Emerson-type centrifuge. For information about the use of the centrifuge in viscosity determinations, see Heilbrunn (1950), Wilson and Heilbrunn (1952).

Results

The substance we extract from starfish ovaries is presumably responsible for the inhibition of mitosis in the ovary. As is well known, as soon as the starfish eggs leave the ovary and enter sea water, the large nucleus of the immature egg, that is to say, the germinal vesicle, breaks down and the maturation divisions begin. In order to prevent the breakdown of the germinal vesicle and the subsequent maturation divisions, it is only necessary to leave the eggs in contact with the ovary. Thus in one experiment, the ovaries of a starfish were cut up in 25 ml. of sea water. Left in this sea water in the presence of the minced ovaries, only 2 per cent of the eggs showed germinal vesicle breakdown. However, with progressive dilution of the sea water which had been in contact with the ovary, there was a progressive increase in the percentage of germinal vesicle breakdown, so that when the original fluid was diluted 64 times, the percentage of germinal vesicle breakdown rose to 69 per cent. The inhibitor effect of the ovarian substance is largely reversible. As a matter of fact, this effect of ovarian substance both on maturation and cleavage divisions has long been known to students of marine eggs, and they commonly wash eggs two or three times before experimenting with them; that is to say, they pour off the sea water over the eggs and replace it with fresh sea water, and then repeat this operation several times.

Table I

Effect of starfish ovary extract on division of Chaetopterus eggs. Eggs exposed two minutes after fertilization

Dilution	Per cent cleavage
1/100	0
1/200	0
1/400	93
1/800	97
Control	97

In the work reported previously (Heilbrunn, Wilson and Harding, 1951), the extracts from starfish ovaries that we studied, when diluted to more than 1 part in 10, did not have much effect on Chaetopterus eggs. But in the extracts that were prepared from homogenized ovaries, a dilution of 1 to 200 was still effective. This is shown in Table I, which illustrates the effect on cleavage of one of our extracts. To prepare this extract, 100 ml. of acid sea water at pH 5.8 were added to 50 g. of starfish ovaries, and the ovaries were then homogenized in a Waring blendor. (The acid sea water was prepared as in our previous work.) The homogenate was centrifuged in a Sorvall centrifuge at about 15,000 g. and the resultant supernate was neutralized with 0.1 N NaOH so that its final pH was 7.0.

Nature of the antimitotic substance in starfish ovary extract. What is the nature of the antimitotic substance in the extract from starfish ovaries? If we knew that, then we might go ahead to discover various other antimitotic substances in the hope that one or another of them would be useful in the treatment of cancer. From the beginning, our suspicion has been that the potent substance in our extracts was a heparin-like compound. Let us summarize the old and new evidence in support of this opinion. Some of this evidence will later be presented in more detail by individual members of our group.

1. The extract from starfish ovaries is strongly metachromatic, just as heparin is. That is to say, the extract gives a reddish color with dilute solutions of toluidine blue. Tests for metachromasy are best made in calcium-free sea water or distilled water, for the calcium ions tend to prevent the metachromatic reaction.

2. The metachromatic reaction with toluidine blue disappears in the presence of protein, just as the metachromatic reaction of heparin disappears in the presence of protein (Kelly, 1951). If the crude extract from starfish ovaries is salted out with varying concentrations of ammonium sulfate, the activity appears in the globulin fraction and not in the albumin fraction. If now the globulin fraction is re-suspended in 0.3 molar sodium chloride solution and digested with trypsin, the resultant solution is metachromatic. This solution, after boiling to destroy trypsin, exerts a strong anticoagulant action on sheep plasma and it also prevents cell division in Chaetopterus eggs. Also if the re-suspended globulin fraction is dialyzed for 48 hours against 0.3 molar sodium chloride, a metachromatic reaction is obtained in the dialysate, and the dialysate is likewise effective in preventing cell division in Chaetopterus eggs. These facts will be discussed more fully by one of us (Dunn). The fact that the potent substance is able to pass through the dialysis sac indicates that it is not a substance of high molecular weight. Perhaps a correlation is to be found with the fact that, as Chaet (1952) has shown, ordinary heparin in solution can break down and yield substances capable of passing through a dialysis membrane and nevertheless capable of powerful physiological activity. Chaet's experiments, so far presented only as a preliminary note, will be published *in extenso* before long.

3. The active substance is heat-stable. Solutions containing it can be heated to 99.5° C. for 30 minutes and still retain their activity. On the other hand, when the substance is combined with globulin as a result of the salting out procedure described above, its activity is lost after exposure to a temperature of 80° C. for 20 minutes. (Following such inactivation, the active substance can no longer be separated off by dialysis.)

4. The activity of the starfish ovary extracts is destroyed by dilute solutions of periodate. Potassium periodate was added to potent extracts. Then the excess periodate was removed by dialysis. The control, containing extract without periodate, was dialyzed in similar fashion. The precipitates that formed were all removed by centrifugation and the potency of the extracts was tested on Chaetopterus eggs. The periodate was completely successful in destroying the antimitotic activity of the extracts. This is shown in Table II. These experiments are consistent with the idea that a polysaccharide is responsible for the activity of the extracts, but they do not constitute absolute proof of such an idea, for substances other than polysaccharides may also be destroyed by periodate.

TABLE II

Effect of potassium periodate on potency of starfish ovary extract

% periodate	% cleavage			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
0 (control-extract alone)	0	0	0	0
0.25	96	84		100
0.5	93	92	94	
0.75	98	95	96	
1	96	96		
Control (no extract)	99	96	98	100

5. The dialysis behavior of the extracts is similar to that of heparin. As already noted, Chaet (1952) has found that when preparations of commercial heparin are dialyzed, a potent substance keeps coming through the dialysis membrane. He believes that heparin is continually breaking down to produce more of this substance. Similarly, when starfish ovary extract is dialyzed, a substance passes through the membrane and this substance is strong in antimitotic properties. However, the material that remains in the dialysis sac also prevents cell division. Table III shows the results of an experiment on Chaetopterus eggs. Both the substance or substances remaining in the sac after prolonged dialysis and the substance that diffused through the sac into sea water completely inhibited cell division. The active substance is not completely removed by a single dialysis

TABLE III

The effect of dialysis on the potency of starfish ovary extract in preventing cell division. (The extract was first dialyzed against double its volume of sea water for 7 hours, then against running sea water for 9 hours)

	% cleavage	
	Exp. 1	Exp. 2
Contents of sac after dialysis	0	0
Dialysate	0	0
Control (dialyzed sea water)	99	99

TABLE IV

The effect of repeated dialysis on the potency of starfish ovary extract in preventing cell division. (The extract was dialyzed against an equal volume of sea water for 12 hours and the antimitotic effect of the dialysate tested. Then the contents of the sac were dialyzed against running sea water for 12 hours, following which the contents were dialyzed against an equal volume of sea water for 12 hours and the antimitotic effect of the second dialysate tested)

	% cleavage
Contents of sac (after 2 dialyses)	0
First dialysate	0
Second dialysate	0
Sea water dialysate	95
Second sea water dialysate	90
Control (sea water)	96

operation. This is shown in Table IV, which gives the data on an experiment in which a second dialysate was still strongly antimitotic and completely prevented the division of Chaetopterus eggs. In this experiment, control tests were made with dialyzed sea water, for it is sometimes found that dialysis tubes give off substances that have some slight antimitotic action. (This might well be expected from the fact that, as previously noted, the cellulose dialysis tubes consist of polysaccharide containing a little sulfur.) In another experiment, an active antimitotic substance continued to pass through the dialysis membrane after seven successive dialyses. It is quite possible that when a living cell is exposed to a heparin-like substance or to a combination of such a substance with protein, breakdown products of the heparin-like substance diffuse into the cell, whereas the components of larger molecular size remain outside.

6. When an active extract of starfish ovary is placed in a dialysis sac, carbohydrate diffuses through the sac into the surrounding fluid. This is shown clearly by chromatographic tests. The carbohydrate is a polysaccharide. Chromatographic tests of the dialysate also indicate the absence of nucleic acids and amino acids. Details of these tests will be published later (by Dunn).

7. The ultraviolet absorption spectrum of starfish ovary extract is similar to that of heparin. This is shown in Figure 1. In this figure, the open circles show the absorption spectrum of a 0.17 per cent solution of sodium heparinate, kindly supplied by the Upjohn Co. of Kalamazoo, Michigan. The closed (completely black) circles show the absorption spectrum of a 2 per cent solution of the globulin fraction of starfish ovary extract in 0.5 *M* NaCl. In the preparation of this fraction, 100 g. of starfish ovary were washed in 0.5 *M* NaCl for one hour to remove excess mucus; the washed ovaries were then suspended in 200 ml. 0.3 *M* NaCl and homogenized in a Waring blendor. Following centrifugation, the globulin fraction of the supernatant solution was salted out with half-saturated ammonium sulfate solution, and the precipitate was made salt-free by dialysis against distilled water. The triangles show the spectrum of a highly dilute solution of starfish ovary extract. This was prepared from an extract made by extracting 20 gm. of homogenized starfish ovary in 40 ml. of acid sea water (pH 5.8) and then neutralizing the resultant solution. This extract was then diluted with ordinary sea water until it was only 0.26 per cent of its original strength. The dilution was made in order to obtain a curve at about the same position on the graph at the heparin curve. The readings on the (Beckman) spectrophotometer were made by Dr. Lester Goldstein.

The general similarity of the three curves in Figure 1 is obvious, and although this similarity does not provide proof that the starfish ovary extract does actually contain a heparin-like substance, it is certainly consistent with such a view.

Indeed, no one of the seven arguments that we have presented is in itself very cogent, but taken as a whole they do indicate rather strongly that the starfish ovary extract contains a heparin-like substance and that this substance is responsible for its anticlotting and antimitotic activity.

Antimitotic substances in fish ovaries. In our thinking about the starfish ovary extract, we were bothered by two facts. In the first place, we knew of no evidence in the biochemical literature of heparin or heparin-like compounds splitting to form compounds of lower molecular weight capable of passing through dialysis membranes. Secondly, and this from a practical clinical standpoint is more important,

we were soon led to believe that the starfish ovary extract which had so drastic a liquefying action and so strong an antimitotic effect on invertebrate marine eggs, was rather powerless on vertebrate cells and tissues. The starfish ovary extract acts on starfish eggs, Chaetopterus eggs, eggs of the sea urchin Arbacia and eggs of the clam Spisula; but we found no very great antimitotic activity on frog eggs, and the antimitotic action of the extract on embryo mouse cells in tissue culture was much less than that of ordinary commercial heparin. [The studies on tissue culture cells were made by Carol Bocher and were presented by her as a Master's thesis (Bocher, 1952).] Moreover, although ordinary heparin, or a breakdown

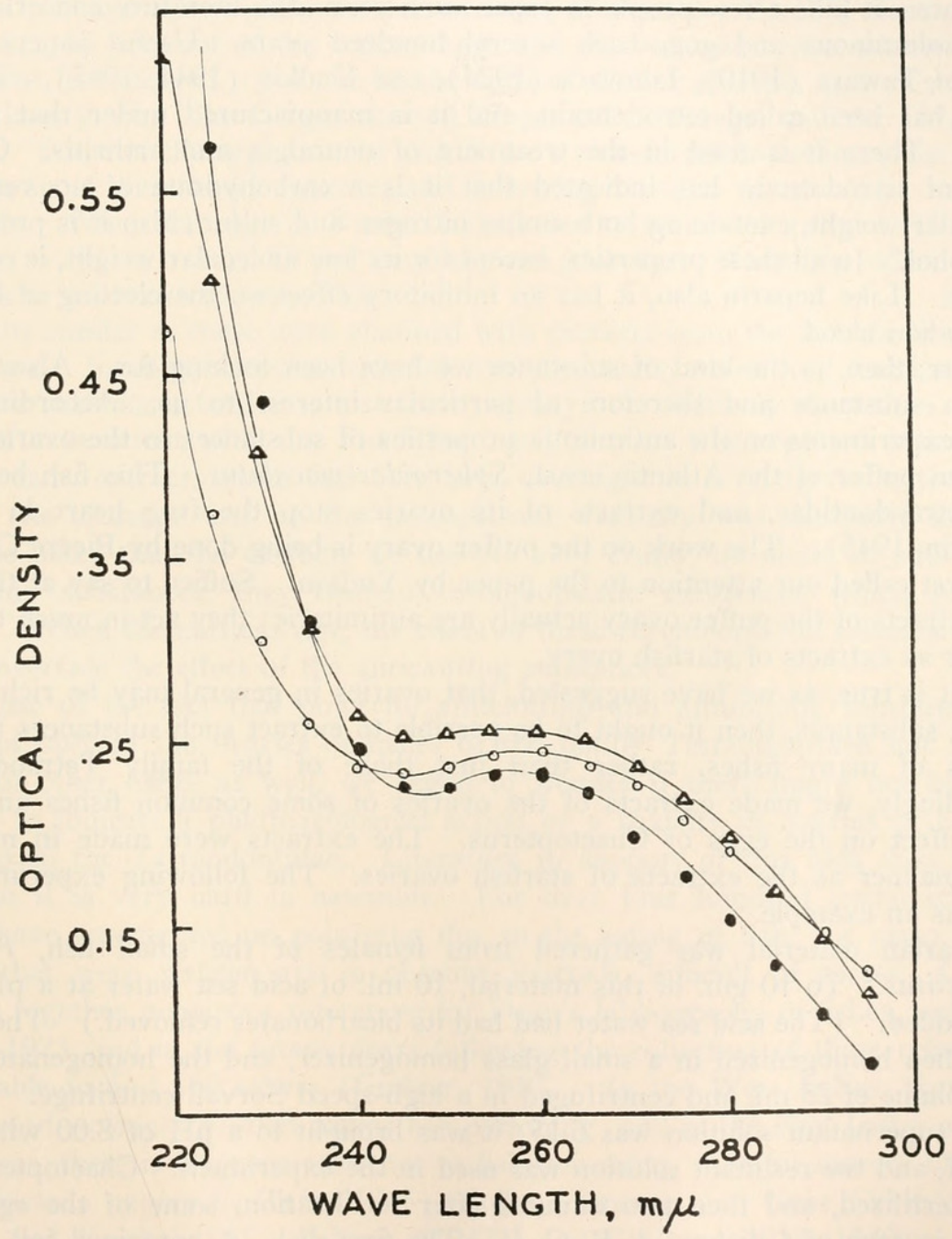

FIGURE 1. Comparison of the absorption spectra of heparin and two samples of starfish ovary extract. The open circles show the absorption spectrum of sodium heparinate. For further details, see text.

product of it, stops the frog heart in diastole (Kraus, Fuchs and Merländer, 1931; Chaet, 1952), starfish ovary extract has no effect on frog heart, although it does stop the clam heart in diastole. Also, in a few preliminary experiments, we found that starfish ovary extract had no obvious toxic action when injected subcutaneously into mice.

Fortunately, our attention was called to the fact that some fish of the family Tetraodontidae have in their ovaries a substance which has a very potent pharmacological and toxic action. This substance stops the heart of a toad or frog in diastole, and it thus acts like heparin, or rather like the breakdown product of heparin studied by Chaet (1952). Partly because of the toxic nature of this substance—it kills a few people in Japan each year—the literature concerning it is quite voluminous and goes back several hundred years. Useful papers include those of Tawara (1910), Ishiwara (1924), and Yudkin (1944, 1945). The substance has been called tetrodotoxin, and it is manufactured under that name in Japan. There it is used in the treatment of neuralgia and arthritis. Chemical study of tetrodotoxin has indicated that it is a carbohydrate of no very great molecular weight, containing both amino nitrogen and sulfur; also it is precipitated by alcohol. In all these properties, except for its low molecular weight, it resembles heparin. Like heparin also, it has an inhibitory effect on the clotting of bird and mammalian blood.

Here, then, is the kind of substance we have been looking for. Also, it is an ovarian substance and therefore of particular interest to us. Accordingly, we began experiments on the antimitotic properties of substances in the ovaries of the common puffer of the Atlantic coast, *Spheroides maculatus*. This fish belongs to the Tetraodontidae, and extracts of its ovaries stop the frog heart in diastole (Yudkin, 1945). The work on the puffer ovary is being done by Pierre Couillard, who first called our attention to the paper by Yudkin. Suffice to say at this point that extracts of the puffer ovary actually are antimitotic; they act in much the same manner as extracts of starfish ovary.

If it is true, as we have suggested, that ovaries in general may be rich in antimitotic substances, then it ought to be possible to extract such substances from the ovaries of many fishes, rather than just those of the family Tetraodontidae. Accordingly, we made extracts of the ovaries of some common fishes and tested their effect on the eggs of Chaetopterus. The extracts were made in much the same manner as the extracts of starfish ovaries. The following experiment will serve as an example.

Ovarian material was gathered from females of the small fish, *Fundulus heteroclitus*. To 10 gm. of this material, 10 ml. of acid sea water at a pH of 5.3 were added. (The acid sea water had had its bicarbonates removed.) The ovaries were then homogenized in a small glass homogenizer, and the homogenate diluted to a volume of 25 ml. and centrifuged in a high-speed Sorvall centrifuge. The pH of the supernatant solution was 6.15; it was brought to a pH of 8.00 with 0.1 N NaOH, and the resultant solution was used in the experiment. Chaetopterus eggs were fertilized, and then two minutes after fertilization, some of the eggs were placed in each of 4 dishes, *A, B, C, D*. The first dish, *A*, contained full strength extract; in *B* the extract was diluted with an equal volume of sea water, so that the resultant mixture was half strength. *C* had three parts of sea water for each

part of extract, and *D* had seven parts of sea water for each part of extract. Of the control eggs in sea water, 100 per cent gave off polar-bodies, but in *A*, polar-body formation was completely inhibited. In *B*, there was 12 per cent polar-body formation; in *C*, 27 per cent, and in *D*, 42 per cent. Thirty minutes after fertilization, at a time when it is known that the gelation has developed (Heilbrunn and Wilson, 1948), centrifuge tests were made rapidly with a hand centrifuge, as in previous studies from this laboratory. These tests showed that whereas the protoplasm of normal eggs had a viscosity well above the arbitrary value of 8 (and presumably, in accordance with earlier studies, about 14), the eggs in *A* exposed to the full strength Fundulus ovary extract had a protoplasmic viscosity of about 4. The eggs in *B* had a protoplasmic viscosity value of about 6. At 55½ minutes after fertilization, 50 per cent of the control eggs had cleaved, and a few minutes later, 97 per cent had cleaved. No one of the eggs in *A* ever cleaved, and only 25 per cent of those in *B*. The *C* eggs showed 27 per cent cleavage, and the *D* eggs 31 per cent. Some of the eggs from *A* and *B* were transferred to ordinary sea water. The eggs in *A* were badly injured, and following transfer to sea water they did not divide. When the eggs in *B* were transferred to sea water (after a 63-minute exposure to the extract), 53 per cent showed cleavage. Thus the effect of the extract is to some extent reversible.

Results similar to these were obtained with extracts from the ovaries of various other fishes. In all our studies with fish ovary extracts, we noticed that the active antimitotic substances tended to lose their potency in a relatively short time. Thus when the experiment with the Fundulus ovary extract was repeated a day later with the same extract, which had been kept overnight in a refrigerator, the effect both on the cleavage and on the protoplasmic viscosity was decidedly less. It should be noted that the extracts we use are very crude; no doubt in addition to anticlotting substances, they contain thromboplastic substances which promote clotting. When the extracts age, the effect of these thromboplastic substances may tend to override the effect of the anticlotting substances.

Because of the fact that powerful anticlotting and antimitotic substances are found not only in the ovaries of fishes of the family Tetraodontidae but also in ovaries of other fishes as well, we began to wonder if there might not be some evidence to indicate a pharmacological action or a toxicity of ovaries of fish not belonging to the Tetraodontidae. Literature in support of this idea does indeed exist, but it is very hard to assemble. For over four hundred years, scientific writers have commented on poisoning due to the eating of fish, but many of the articles that were written are in obscure journals, difficult of access. Gudger gathered together numerous references for Dean's bibliography of fishes, published in 1916–1923, and in the fifteen years following the collection of these references, he was able to find 180 others (Gudger, 1930). In the West Indies, there is a special word—*ciguatera*—that means fish poisoning; and in the East Indies and the South Seas, there is frequent reference to fish poisoning. Useful sources of information include papers by Taft (1945), Cohen, Emmert and Goss (1946), Vonfraenkel and Krick (1945), Gilman (1942), and Gudger (1918, 1930). Books by Phisalix (1922) and Pawlowsky (1927) may also be consulted. When men are poisoned by eating fish in the tropics, there is often uncertainty as to the

cause. Always there is a possibility that the fish may have spoiled; also there is an old superstition that fish become poisonous because they have eaten poisonous fruit. In the case of the barracuda, often found to be poisonous, it seems clear that only larger fish contain poison and then only at certain seasons of the year (Chisholm, 1808). This seems to indicate that the gonads may be involved. According to Coker (1930), the roe of garpikes (genus Lepisosteus) is said to be toxic, and in Germany it is well known that the ovaries of the barbel, a large cyprinid fish (*Barbus vulgaris*) are poisonous. According to McCrudden (1921), the ovaries of the pike are even more toxic than those of the barbel. Köhler, in 1933, writing in a magazine for practicing physicians, states: (p. 292) "Manche an sich ungiftige Fische geben zur Laichzeit unter nicht näher bekannten Verhaltnissen Ursache zur Vergiftungen." There is thus clear indication that fish ovaries may contain potent substances, substances which under certain conditions have a serious effect when ingested.

In fishes, the ovaries are not alone in containing substances that prevent the clotting of protoplasm. This is only to be expected if, as we believe, all types of living material contain anticlotting as well as clotting factors (see Heilbrunn, 1952b). We were not surprised, therefore, that when we extracted the testis of the toadfish (*Opsanus tau*) in the same manner that we extracted the ovary, we were able to obtain a substance that kept the protoplasm fluid and prevented cell division. As a matter of fact, in the Tetraodontidae, the testis is toxic as well as the ovary (Remy, 1883), and apparently contains the same type of substance that the ovary does. Also it will be remembered that the starfish testis contains much antimitotic substance (Heilbrunn, Wilson and Harding, 1951). In animals that breed only once a year, at times when little or no mitosis is occurring in the testis, this organ may presumably be rather rich in substances which prevent cell division. We found too that fish liver might also contain easily recognizable amounts of anticlotting and antimitotic substances—we used the liver of the angler or goosefish (*Lophius piscatorius*). This is to be correlated with the fact that the liver is a ready source of heparin and also with the fact that in Tetraodon the liver may be poisonous as well as the ovary (Tani, 1940). It is possible that the tetrodotoxin of the ovary is secreted in the liver.

The results reported in this paper provide additional evidence to show that many diverse types of living tissue, and indeed possibly all types of living cells, contain substances that prevent the clotting of protoplasm and exert a powerful antimitotic action. The ovaries of many animals are especially rich in such substances. Many of the anticlotting substances are either heparins or heparin-like substances. There is good reason to believe that the various substances vary widely both in molecular size and molecular composition.

At the present time the search for antimitotic substances in various organs and tissues of various organisms is being continued. There is undoubtedly a large and diverse group of naturally occurring heparin-like compounds which can act as anticlotting and antimitotic agents. Out of this large group of compounds, it should be possible to discover some which may be of real value in the treatment of tumors. More work is urgently needed. We need to know more about the chemistry of these heparin-like compounds, and their effect should be tested not only on simple isolated cells, but also on tumors.

Summary

1. Starfish ovaries contain a substance which prevents maturation divisions in the eggs contained in these ovaries.

2. By homogenizing starfish ovaries before extracting them with acid sea water, we have been able to prepare antimitotic extracts much more powerful in their action on Chaetopterus eggs than the extracts reported on previously.

3. There is additional many-sided evidence to indicate that the potent substance in these extracts is a heparin-like compound. Some of this evidence comes from chromatographic studies; also from studies of the absorption spectrum of the extract. Moreover, the potency of the extract disappears after treatment with periodate.

4. Ovaries of various species of fishes contain antimitotic substances which resemble in their action the substance or substances in starfish ovary extracts. In at least one family of fishes, the ovaries are known to contain a potent substance of heparin-like chemical composition and heparin-like properties.

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