the theoretical Nernst equation which satisfactorily describes results from nerves and vertebrate skeletal muscles (*6*). When choline chloride, in the presence of sufficient atropine to counteract its "cholinergic" effect, was used to replace NaCl there was little change in the height of the action potential even with Na concentrations as low as 25 percent normal, the overshoot usually increasing somewhat on application of these fluids. In contrast with these relatively small effects on amplitude, the maximum rate of depolarization changed quite strikingly as it does in other excitable cells, the rate decreasing in low $[Na]_o$, and decreasing to a similar extent when either sucrose or choline chloride was used as a substitute for NaCl. This finding confirms previous work with cardiac cells (see *7*) in suggesting that Na ions are the main carriers for inward current during the initial phase of the action potential.

For the interpretation of these results it is important to note that the maximum of the action potential is reached some 20 to 30 msec after its moment of fastest rise—that is, at a time when probably, as in other heart cells (*8*), the initial increase in permeability to Na ions has subsided toward a much lower but longer-maintained level. It is thus possible that at this time ions other than Na participate in determining the height of the overshoot. The possibility that Ca is involved is suggested by the finding that Ca influx is facilitated in the presence of either Ca-rich or Na-depleted fluids (*9*). However, the extra influx of ionic calcium during an action potential determined in frog ventricles with Ca^{45}-labeled fluids was only ≤ 0.2 picomole/cm^2 surface membrane, which would not contribute much to the height of the potential. It seems more probable that associated with the movement of Ca ions there is an inward current of Na which is also facilitated by the action of Ca on the hypothetical membrane sites. The degree of the permeability change responsible for this delayed Na-current would thus depend on the external Ca and Na concentrations (or on the activities of the Ca and Na ions). To test this hypothesis the dependence of the overshoot potential on external Na activities has been examined, keeping the ratio $\gamma_{Ca}[Ca]_o/(\gamma_{Na}[Na]_o)^2$ (that is, the concentration of Ca-occupied sites) constant. Under these conditions, the results are fairly close to the theoretical line (Fig. 3) and thus support the idea that the inward current which causes the overshoot is mainly carried by sodium ions.

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Spawning of Starfish: Action of Gamete-Shedding Substance Obtained from Radial Nerves

Abstract. *An extract of the radial nerves of the starfish,* Asterias amurensis, *acts on the ovary in two ways: it induces meiosis and brings on spawning. Contraction of the gonadal wall, the driving force for spawning, does not happen until this gamete-shedding substance acts to liberate the eggs that adhere to each other or to the inner surface of the gonadal wall.*

When injected into the coelomic cavity, a water extract of starfish radial nerves induces the shedding of eggs or sperm from the mature gonads (*1*). The active extract can be obtained from both male and female starfish and seems to be identical in the two sexes. Although there may be some species differences in detail, the active substances seem to be chemically analogous among starfishes; the nerve extract obtained from one species acts similarly in several species (*2*). The active substance obtained from *Asterias amurensis* was reported to be a polypeptide with a relatively small molecular weight (*3*). Moreover, neurosecretory cells were revealed in the radial nerves of *A. glacialis* (*4*). This was confirmed in Japanese starfishes, including the species used in this study (*5*). These facts suggest that the active element responsible for the spawning of starfishes is a product of neurosecretion. Described in this report are some experimental effects of the spawning factor, obtained from the radial nerves, on the ovary and ovarian eggs; a possible mechanism of spawning in *A. amurensis* is suggested.

In the first series of experiments, an ovary was removed and torn with fine forceps so that the eggs were released into sea water. The germinal vesicles broke down after about 20 to 30 minutes, and the first polar bodies appeared after 70 to 80 minutes at 18°C. Discharge of the second polar bodies began after 110 to 120 minutes. Maturation of the starfish egg in sea water is said to be promoted by calcium ions (*6*). Isolated ovarian fragments were thoroughly washed with calcium-free sea water, and eggs thus obtained were suspended in calcium-free sea water before being transferred to artificial sea water containing calcium in various concentrations. Figure 1 shows that breakdown of the germinal vesicles became more frequent as the concentration of calcium rose. Maturation did occur, however, even in the absence of calcium, when these eggs were treated with calcium-free sea water containing nerve extract (*7*) desalted on a Sephadex G25 column (Fig. 2).

Under natural conditions, the initiation of meiosis seems to be due to the action of the gamete-shedding substance, since the eggs within isolated ovaries do not undergo maturation in sea water, and since most of the germinal vesicles of eggs spawned from the gonopores disappear by the time of spawning. Meiosis proceeded in isolated ovarian fragments when they were ligated and then placed in nerve extract. After 2 hours of such treatment, polar bodies were observed in most of the eggs within the ovary. Without the ligature, an isolated gonad fragment immersed in nerve extract shed its eggs vigorously from the cut surface after a certain period, and discharge of polar bodies occurred in sea water.

In the next experiment, the nerve extract was applied locally. The distal part of an isolated ovary was immersed in sea water containing the nerve extract; its proximal part, in sea water alone (Fig. 3*A*). After 40 minutes,

eggs were removed from various parts of the isolated gonad and examined. Germinal vesicles were never observed in eggs taken from that part of the gonad immersed in nerve extract, whereas they remained intact in the eggs taken from the proximal portion. Breakdown of the germinal vesicles was thus clearly confined to the region treated with nerve extract and its close vicinity, indicating that the active principle acts directly on the eggs through the gonadal wall, and that diffusion of the active principle within the ovary is rather slow.

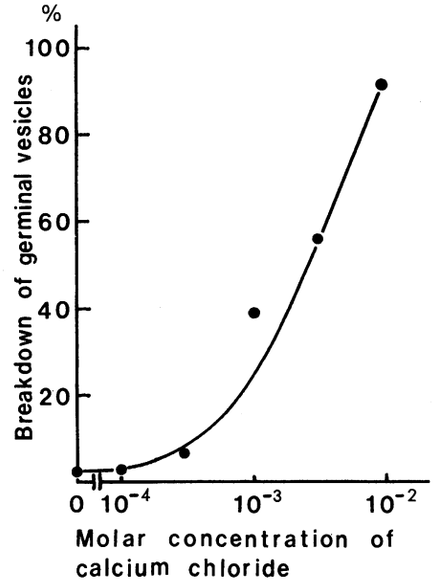

Fig. 1. Effect of calcium on the breakdown of germinal vesicles. Eggs were suspended in calcium-free sea water containing various concentrations of calcium chloride. Concentration of calcium in artificial sea water is $9.2 \times 10^{-3}M$.

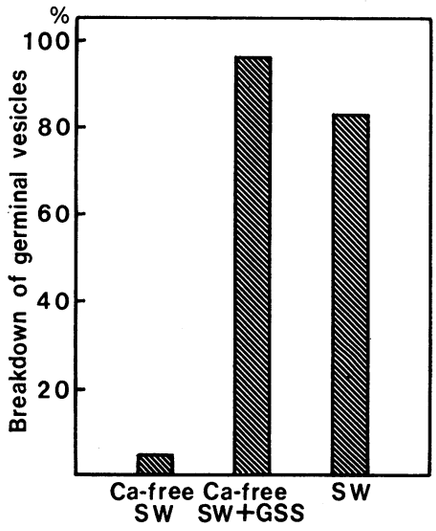

Fig. 2. Effect of desalted nerve extract on the breakdown of germinal vesicles in calcium-free sea water. *Ca-free SW*, calcium-free sea water; *Ca-free SW + GSS*, calcium-free sea water containing nerve extract (0.4 mg lyophilized nerve per milliliter); *SW*, artificial sea water.

In a third experiment, the action on the ovary of gamete-shedding substance was shown in another way. After local treatment with nerve extract for 1 hour, the isolated ovary was transferred to sea water and its gonadal wall was torn with fine forceps to make three small slits: proximally, centrally, and at the proximal boundary of the treated distal region.

Although eggs were discharged immediately from these openings, discharge from the regions not treated with nerve extract soon ceased. On the other hand, the shedding of eggs from the slit made in the portion treated with nerve extract was intense and continued. This part contracted to a large degree and was clearly distinguishable from the non-treated portion (Fig. 3*B*). This suggests that contraction of the gonadal wall is essential for spawning.

The degree of maturation in various parts of the ovary was also determined by using the isolated arm of an individual in which spawning began 34 minutes after the injection of nerve extract. All the eggs in the distal tips of the ovary were found to have no germinal vesicles about 45 minutes after the injection, whereas 12 percent of the germinal vesicles in the middle part of the ovary remained intact. However, about 30 percent of the eggs being shed from the gonopore still had intact germinal vesicles. Further, a large proportion of the eggs in the gonadal alveoli near the gonoduct still had germinal vesicles. Thus, the action of the nerve extract is apparently delayed in that part of the gonad near the gonoduct.

The fourth experiment was conducted to determine whether or not the contractile ability is always present in the gonadal wall. When the wall of an isolated alveolus was slit by fine forceps in sea water, the alveolar wall suddenly contracted, with discharge of eggs from the slit, and turned inside-out because of the presence of eggs adhering to its inner surface. The eggs continued to adhere to each other and to the alveolar wall. These observations indicate that considerable tension is always present in the wall of a ripe, distended gonad, and it is believed that this force is sufficient to cause the discharge of eggs. However, the masses of

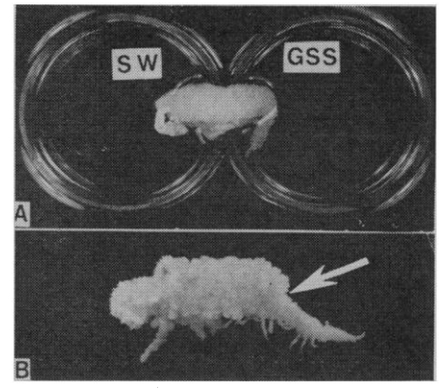

Fig. 3. Local application of gamete-shedding substance to isolated ovary. *A*, An isolated ovary partially exposed to nerve extract and sea water. *SW*, sea water; *GSS*, sea water containing nerve extract. *B*, Same ovary after discharge of eggs from treated portion. Arrow, boundary between treated and untreated regions.

eggs adhering to each other or to the inner surface of the gonadal wall seem to obstruct the inner openings between the individual alveoli and the main canal of the gonad so that they resist the contraction of the alveolar wall. In the nerve extract most of the eggs in the isolated alveolus were freely discharged after a certain period even without a slit being made; they did not adhere to each other. Thus, another action of the gamete-shedding substance, besides bringing about meiosis, seems to be to free the eggs from adherence to each other or to the inner surface of the gonadal wall.

In the fifth experiment conducted later in the spawning season, nerve extract was injected into the coelomic cavity of females which then shed immature eggs about 20 minutes later. Observations of these eggs threw some light on the relation between spawning and meiosis. For example, a female injected with the nerve extract from 1 mg of lyophilized nerve began spawning 22 minutes later. Examination of the discharged eggs revealed that all the germinal vesicles remained intact 25 minutes after injection. However, they began to disappear within several minutes; only 18 percent of the eggs still had germinal vesicles 34 minutes after injection. These eggs formed first polar bodies after about 85 minutes, and second polar bodies after 115 minutes. Breakdown of germinal vesicle is not, therefore, a prerequisite for gamete-shedding through the gonopore. In addition, considering the time needed for the breakdown of germinal

vesicles and the formation of polar bodies in eggs taken from intact ovaries and suspended in sea water, or in sea water containing nerve extract, it appears that the gamete-shedding substance acts on the ovarian eggs soon after injection.

The sixth experiment determined the changes in pressure inside the ovary after the application of nerve extract. The distal portion of an ovary (about 2 cm long) was isolated. A capillary tube (0.8 mm in diameter and 200 mm long) was sealed at one end and its open end was inserted deeply into the main canal of the isolated ovary fragment in sea water; the proximal part of the ovary fragment was tied tightly around it. The gonadal portion, including a part of the capillary tube, was immersed in a glass tube containing 7 ml of sea water, with the capillary tube in a vertical position with a scale attached to it. The upper, sealed tip of the capillary was then broken.

After an appropriate period (usually about 30 minutes) for stabilizing the height of the concentrated egg suspension in the capillary, the level of which was somewhat lower than that of the medium, 0.5 ml of the sea water was replaced with the same volume of isotonic sodium chloride solution containing nerve extract (2.5 mg of lyophilized nerve). The level of the egg suspension began to rise immediately after addition of the extract. The level ceased rising at a certain height, indicating that it was balanced. Fig. 4 shows the results. Incidentally, the ovarian fluid thus balanced would rise again when the medium was replaced by hypotonic sea water.

The increase in pressure inside the ovary may be hypothetically attributed to liquefaction of the connective substances between the eggs caused by the extract diffusing into the ovary, or to absorption of water and swelling of the eggs induced by the extract. Although the problem remains unsolved, the results clearly show that the gamete-shedding substance begins to act on the ovary immediately after its application. In my sixth experiment, the capillary was inserted deeply into the main canal of the gonad and its diameter was large enough to permit the passage of groups of eggs, so that the eggs seemed to be able to ascend in the capillary immediately without clogging it.

It therefore appears that, although

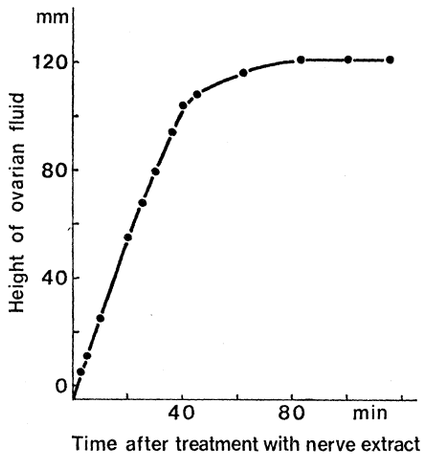

Fig. 4. Ascent of ovarian fluid in a capillary tube on treatment with the nerve extract.

the normal tension present in the gonadal wall is adequate to force the gametes out of the ovary, spawning cannot occur until the obstructing egg masses are freed by the gamete-shedding substance. This may explain why the injection of isotonic potassium chloride solution, which stimulates contraction of the ovarian wall in sea urchins, did not induce spawning in several starfishes tested (*2*). Under natural conditions, liberation of the eggs within the ovary, apparently a prerequisite for spawning, may be brought about by liquefaction or dissolution by the gamete-shedding substance of the substances causing the eggs to adhere to each other or to the gonadal wall. Isolated gonad fragments released their eggs when they were placed in isotonic sodium chloride solution or artificial sea water lacking divalent cations. In calcium-free sea water no such discharge was observed; in these cases, however, the germinal vesicles remained intact. It is well known that divalent cations such as calcium and magnesium act to stabilize the connectives between tissue cells, and in the experiments described here the lack of divalent cations may have induced liberation of the eggs. Nevertheless, the possibility that lack of such cations may induce contraction of the gonadal wall cannot be completely excluded.

It is of interest that the gamete-shedding substance has an effect similar to the effect of calcium ions on maturation, but in the liberation of the eggs it has an effect similar to that of the absence of divalent cations. Whether the gamete-shedding substance directly dissolves the connecting material or liberates a specific enzyme from the eggs themselves is not yet known. The substance seems to cause some cortical change in the ovarian eggs, which in turn induces meiosis. The follicles around the eggs, which are observed when any ovary is dissected in sea water, completely disappeared in the ligated ovary treated with nerve extract for an appropriate period.

On the basis of these results, the latent period between treatment and the onset of spawning may be explained as the time needed to release the eggs near the gonaduct. The fact that the release of sperm generally begins after a shorter latent period than the release of eggs may be ascribed to the lower resistance which they offer in passing through the gonaduct. Furthermore, later in the breeding season it was found that small fragments of a ripe, distended testis often contracted when isolated in sea water, shedding a cylindrical mass of sperm, even without the nerve extract. The mechanism of starfish spawning as induced by the gamete-shedding substance may be as follows. The tension always present in the wall of a ripe, distended gonad provides the driving force for spawning, but this is not effective until the gamete-shedding substance liberates the ovarian eggs adhering to each other or to the gonadal wall. Whether or not the substance acts directly to increase the tension of the gonadal wall is not yet known.

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7. To make nerve extract, lyophilized radial nerves were homogenized in deionized water and centrifuged at 27,000*g* for 1 hour. Sodium chloride solution or hypertonic sea water, or calcium-free hypertonic sea water, was added to the supernatant directly, or after desalting on a Sephadex column, to make it isotonic with sea water. The concentration of nerve extract was equivalent to 0.5 or 1 mg of lyophilized nerve per milliliter.
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