NEUROCHEMICAL CONTROL OF GAMETE RELEASE IN STARFISH [1]

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The physiological factor(s) controlling gamete release in starfish had been largely ignored until 1959 when Chaet and McConnaughy first implicated the nervous system by extracting from radial nerves of starfish a hormonal catalyst promoting gamete shedding. Such neural extracts were capable of inducing the release of eggs or sperm when injected into mature starfish of the same species. Since then evidence has been steadily accumulating which suggests that the release of gametes from these echinoderms is, in part at least, controlled by a neurohormone found in their radial nerves.

Materials and Methods

Asterias forbesi from the American Atlantic coast, as well as two species of starfish from the Pacific coast, *Patiria miniata* and *Pisaster giganteus,* were used in the majority of these studies. Large donor starfish were collected for isolating nerves from which extracts were prepared containing gamete-shedding substance. The animals were obtained either by dredging or SCUBA diving and ranged in size from 15 to 20 cm. for *Asterias forbesi,* 7.5 to 15 cm. for *Patiria miniata,* and 20 to 25 cm. for *Pisaster giganteus.* Radial nerves were isolated and assayed from male or female animals in a ripe or spent condition. However, most of the experiments reported herein were performed using extracts obtained from the nerves of both male and female specimens taken during the spent condition.

The nerves were dissected as previously described (Chaet, 1964a) by severing all arms, removing the aboral surface, and making a light incision along the entire length of the ambulacral groove. The left and right halves were then separated, thus exposing the large pigmented radial nerve complex. The nerve was carefully removed with forceps, placed in chilled sea water and pooled with nerves of from 10 to 50 animals. The nerves were washed three times in chilled sea water and sometimes rinsed twice in ether. The nerves were then lyophilized, finely pulverized and stored dry until needed (a simpler, but less quantitative method of lysing the nerves has been described by Chaet and Musick, 1960).

Recipient starfish whose ripe gonads were to be used as assay material were carefully transported in chilled sea water to the laboratory and there maintained in refrigerated running sea water until required. The cold (13–14° C.) sea water not only kept animals from shedding their gametes during their natural shedding

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period, but decreased metabolic rates sufficiently so that little gonadal resorption occurred.

When gonadal fragments were to be used as assay tissue, the ovaries of *Asterias forbesi* or *Pisaster giganteus* were isolated by removing an arm of a ripe female and allowing the paired ovaries to float in a beaker of sea water. The ovaries were carefully dissected from the animal by severing the gonoduct. The gonads of *Patiria miniata* were exposed by performing an aboral mid-arm incision from the center portion of the disc to the tip of each of the 5 arms. The aboral surface was then folded back, and the exposed ovaries freed by cutting the gonoducts and suspending ligaments. In all three species, ovaries were either used intact or were cut into one-millimeter pieces to be employed in a semi-micro assay. In the latter case, cut pieces were washed in cold sea water until all free eggs had been washed away. In most experiments the fragments were used immediately. However, if kept refrigerated, the diced ovaries could be used 24–48 hours after removal from the animal, although re-washing was then necessary. Semi-micro assays were performed in 20 × 30 cm. clear, 96-hole, disposable spot plates containing 0.1 cc. of solution per depression. In all experiments, each individual assay was repeated a minimum of 8 times with ovarian fragments from two to four different animals. All assays contained proper controls, such as sea water, as well as a solution of standardized active shedding substance.

Semi-quantitative results were expressed by assigning a shedding value of from 0 to 5 to each piece of ovary at various times after immersing the tissue in the solutions to be assayed. The "shedding index" then calculated represented the quotient of actual shedding observed by maximum theoretical shedding (5 times the number of fragments).

When nerve extracts containing shedding substance were required, a 2 to 5 mg.% solution was prepared immediately before use by dissolving lyophilized nerve powder in fresh sea water. If storage was necessary, the solution was frozen since it was found that its biological activity was destroyed if salt water extracts were stored at room temperature (18 hours) or even merely chilled (5° C. for 7 days), whereas aliquots of the same extract were active for at least a year if kept frozen (−15° C.).

When fractionating samples of shedding substance, a 10-cc. saturated distilled water solution of lyophilized powder was centrifuged (15 minutes @ 10,000 RPM), and the supernatant collected. The precipitate resulting from the centrifugation was suspended in another 10 cc. of distilled water, centrifuged, and the supernatant pooled with the above. Two addtional washings were collected and pooled. The 40 cc. of supernatant were then lyophilized and redissolved in ½ cc. distilled water and placed on a Sephadex (G-25) column. Both short (17 cm. × 1.5 cm.) and long (60 cm. × 1.5 cm.) columns were used and run in a cold room at 7° C. The samples were eluted with water, collected in 4-cc. fractions, and immediately lyophilized. All fractions were simultaneously assayed on ovarian fragments after re-suspending in 2 cc. of fresh water.

Results

Experiments in vivo

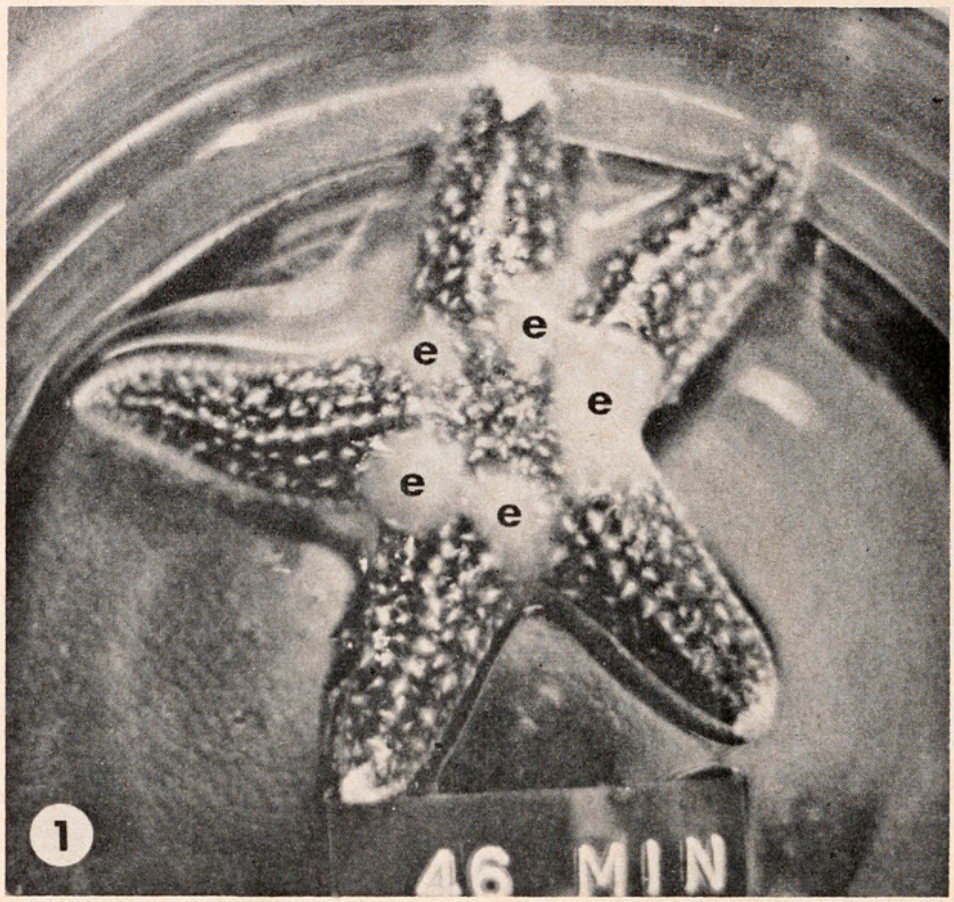
When extracts of *Asterias forbesi* radial nerves, prepared either by (1) heating an isolated nerve in 1 cc. of salt water for 1½ minutes at 76° C.; (2) lysing isolated nerves in distilled water, followed by replacement of salts (Chaet and Rose, 1961); or (3) adding powdered lyophilized nerves to sea water (5 mg.%), were injected (0.15 cc./gm.) into the coelomic cavity of ripe starfish, copious quantities of gametes were exuded through the gonopores (Fig. 1). Similar results were obtained by injecting radial nerve extracts from *Henricia sanguinolenta* into *Asterias forbesi* (Hartman and Chaet, 1962). When 2 mg.% sea water extracts were prepared from radial nerves of *Patiria miniata* and injected into the coelomic cavity of ripe *Patiria miniata,* eggs or sperm were released in large volumes (Fig. 2). Injections of control solutions very seldom elicited a shedding response.

It was noted that a lag period characteristically preceded the initial release of gametes. The precise duration of the interval was more predictable (30–35 minutes) in *Asterias forbesi,* than it was in *Patiria miniata* (25–40 minutes). The eggs released after an injection of shedding substance exhibited virtually complete (99%) germinal vesicle breakdown and were found to be highly fertile. The sperm released by injections of nerve extracts were motile and capable of fertilizing mature eggs.

Experiments in vitro

The presence of a lag period suggested the possibility that an intermediary substance was being formed which in turn stimulated the gonads, and that this over-all reaction required about 30 minutes. In an attempt to determine whether the gamete-shedding substance acted directly on the gonad or whether its action was indirect and involved other tissues of starfish, all 10 of the ovaries from a starfish were removed from the animal, leaving the gonopore, gonoduct, and gonad proper intact and uninjured. A small piece of the aboral surface surrounding the gonopore was left intact when isolating each ovary to insure that the gonoduct and gonopore were not injured. One-half of the gonads were then immersed in lyophilized nerve extracts (2 mg.% in sea water) and the other half in control solution (sea water). Isolated ovaries immersed in shedding substance released eggs which were seen to exude through the gonopore, but none was released from ovaries in sea water. Further, as was the case in all experiments involving intact animals, isolated gonads, or fragments of gonads (see below), shedding of gametes began only after a 30-minute interval. The above experiments were performed only on *Asterias forbesi* since it was surgically impractical to remove whole, intact, uninjured ovaries from *Patiria miniata.*

When ovaries were subjected to a threshold concentration of shedding substance, essentially all of the eggs were exuded through the gonopores within one to two hours. Figure 3 illustrates the magnitude of the reduction in gonad size after 1½ hours in shedding substance. All four gonads in Figure 3 were taken from the same starfish and were originally of equal size.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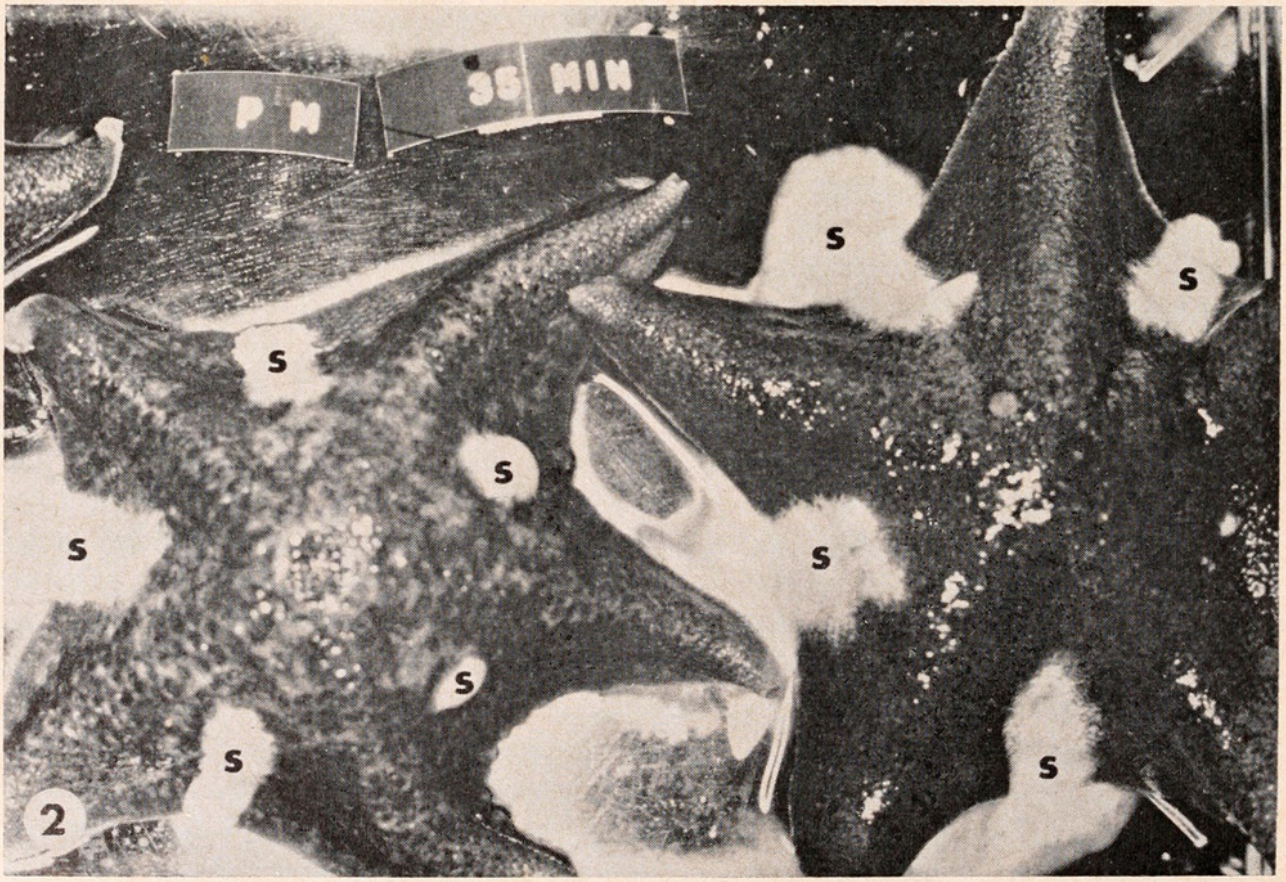
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To determine whether eggs must pass through the gonoduct to insure their complete maturation, ovaries from the same animal were carefully isolated, keeping 5 ovaries intact and puncturing the remaining 5 at various levels from the proximal to the distal ends of the gonads. Both intact and punctured ovaries were then placed in shedding substance, and the results of a typical experiment are shown in Table I. In both cases shedding began 30 minutes after immersion in the neural extract containing shedding substance. Eggs were exuded (shed) from the intact ovaries only through the gonopore, whereas in the punctured ovaries, eggs were exuded mainly through the punctured areas, very few leaving through the gonopore. The disappearance of germinal vesicles (final maturation) was determined microscopically and, as can be seen in Table I, the eggs leaving the ovary through the punctured sites exhibited as high a percentage of germinal vesicle breakdown as did those eggs passing through the gonoducts and gonopores. It should also be noted that not only was shedding completed first in the punctured ovaries, but larger volumes of eggs were released from the punctured gonads than from unpierced specimens when both were immersed in the same concentration of shedding substance.

TABLE I

A typical experiment in which 10 ovaries of Asterias forbesi were immersed for 1½ hours in 10 mg.% solution of nerve extract. Five "intact ovaries" had only their gonoducts severed, whereas the 5 "punctured ovaries" were also punctured in several areas from the proximal to distal end of the ovary. Each figure represents an average of 5 gonads.

Sister gonads immersed in shedding substance			
Intact ovaries		Punctured ovaries	
Quantity of eggs shed (max. value = 5)	Germinal vesicle breakdown	Quantity of eggs shed (max. value = 5)	Germinal vesicle breakdown
2.2	98%	4.8	100%

Since it was found that passage of eggs through the gonoduct was not necessary for complete maturation, and since a micro-assay for determining the presence and characteristics of shedding substance was desirable, the next series of experiments involved cutting isolated ovaries into small fragments (Chaet, Andrews and Smith, 1964). Ovarian fragments, thoroughly washed in sea water, were immersed in 0.1 cc. of experimental or control solutions. Shedding began only in those solutions containing shedding substance and as was observed in all previous experiments, only after a 30-minute interval. Figure 4 illustrates a portion of the picture seen in a typical ovarian fragment experiment. Note the accumulation of eggs around the ovarian fragments immersed in gamete-shedding substance (s.s.) and the lack of eggs around fragments immersed in control solutions (s.w.).

FIGURE 1. Eggs (e) exuding from gonopores of a female *Asterias forbesi* 46 minutes after receiving an intracoelomic injection (5 mg.%—0.15 cc./gm.) of radial nerve extract containing shedding substance.

FIGURE 2. Sperm (s) exuding from gonopores of *Patiria miniata* 35 minutes after injection (2 mg.%) of nerve extract containing shedding substance.

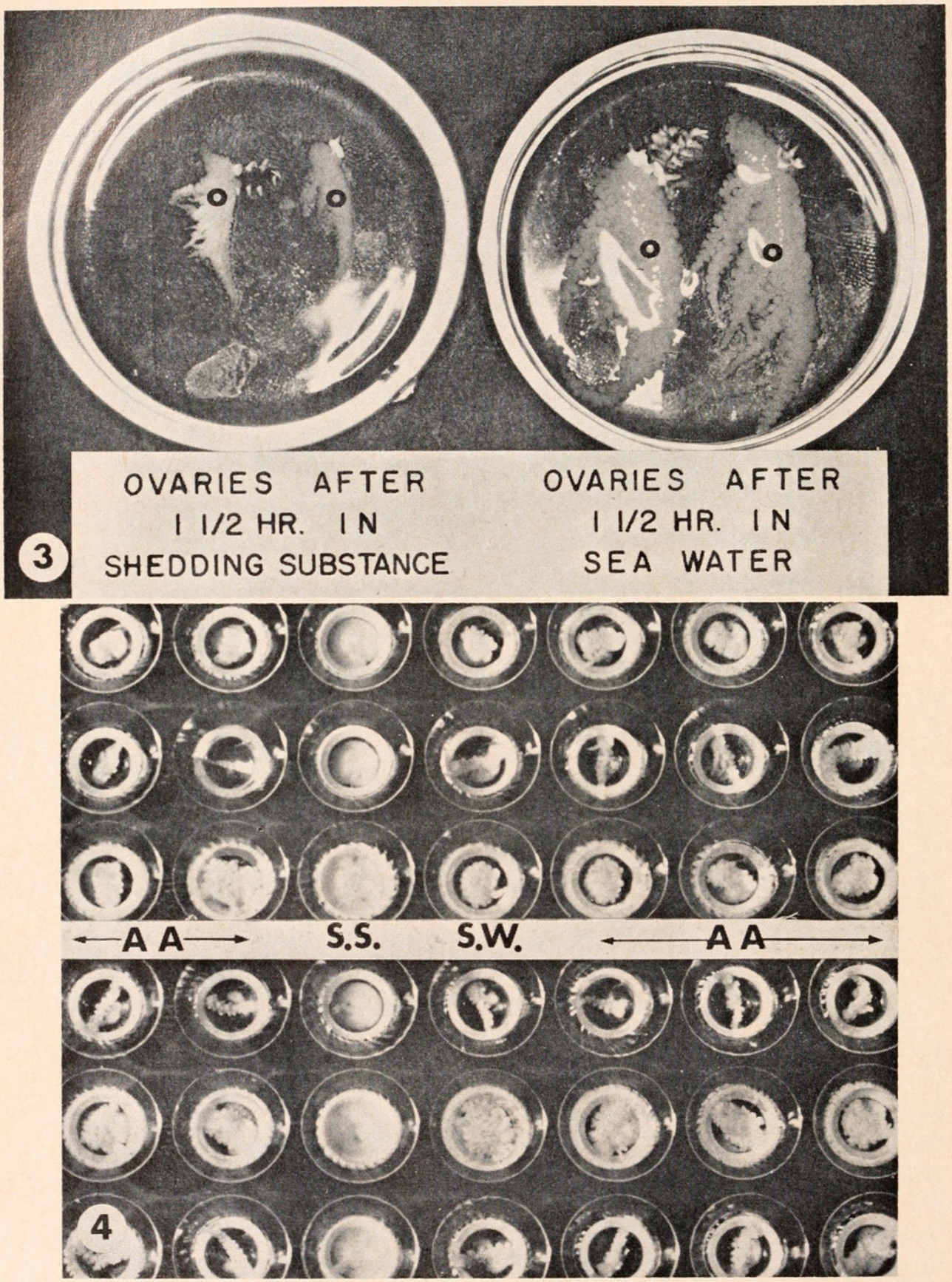

FIGURE 3. Comparison of four sister-ovaries (o) after two were immersed in nerve extract containing shedding substance and had shed their eggs. Ovaries transferred to fresh sea water after 1½ hours in original solutions.

Concentration of shedding substance

By serial dilution assay of lyophilized nerves from both sexes of *Asterias forbesi,* it was found not only that shedding substance was present in nerves of both sexes, but that nerves from male and female contained identical concentrations of shedding substance. Experiments using intact starfish showed that shedding substance taken from nerves of either sex stimulated shedding in both sexes.

Radial nerves taken from *Asterias forbesi* before natural shedding had occurred (May and June) were assayed and the concentration of shedding substance was then compared with that found in nerves taken from starfish after they had naturally shed (July and August). Again serial dilution assays demonstrated that the level of shedding substance was the same in both instances. To further examine this point, monthly collections of starfish (*Asterias forbesi*) were made for a period of two years. Chilled animals were flown (8–20 hours) to the laboratory and the nerves removed, lyophilized and stored until all of the samples could be assayed simultaneously. The results of these experiments illustrated that the level of shedding substance remained constant throughout the year, and that no peak accumulation of gamete shedding substance was found even in those radial nerves that were taken from animals just prior to natural shedding. By chance, a few specimens arrived while they were shedding their gametes. Their assayed nerves revealed the same level of shedding substance as was found in the monthly samples (pre-shed and spent conditions).

Specificity of shedding substance

The shedding substance was found in the radial nerves of 10 echinoderms from both the east and west coasts of the United States. Originally demonstrated in *Asterias forbesi,* the substance was later found in the radial nerves of *Henricia sanguinolenta* (Hartman and Chaet, 1962). Radial nerve extracts from *Patiria miniata* and *Pisaster giganteus,* both found on the west coast, were found to contain shedding substance (Chaet, 1964b); this also applies to *Pisaster ochraceus, Pisaster brevispinus, Astropecten armatus, Pycnopodia helianthoides, Heliaster kubinijii* and *Orthasterias koehleri*). The various chemical characteristics that have been measured suggest similarities between the shedding substances in all 8 species. Although all cross-specificity experiments have not been completed to date, it was found that the shedding substance of *Asterias forbesi* was active on the ovaries of *Pisaster giganteus, Heliaster kubinijii, Pisaster ochraceus,* but not on *Patiria miniata* or *Astropecten armatus. Henricia sanguinolenta* was assayed on *Asterias forbesi* (Hartman and Chaet, 1962) and found to release eggs. The shedding substance from *Patiria miniata* was active on ovaries of *Pisaster giganteus, Heliaster kubinijii, Pisaster ochraceus* and *Astropecten armatus.* The shedding substance from *Pisaster giganteus* and *P. ochraceus* induced the release of eggs from the ovaries of *Heliaster kubinijii, Patiria miniata* and *Astropecten armatus. Pycnopodia helianthoides* shedding substance was found to be active on *Heliaster*

FIGURE 4. A portion of the ovarian fragment assay (*Asterias forbesi*) performed in 96-hole spot plate. Compare columns "shedding substance" (SS) and "sea water" (SW). The remaining columns contain various amino acids (AA), none of which induced shedding.

TABLE II

Relationships of biological specificity between various starfishes. In vitro ovarian fragment assay was used in which 4 mg.% and 10 mg.% solutions were tested. (When shedding did not occur, both higher and lower concentrations were also assayed.)

	Radial nerves from:									
	Asterias forbesi	*Astropectin armatus*	*Heliaster kubinijii*	*Henricia sanguinolenta*	*Orthasterias koehleri*	*Patiria miniata*	*Pisaster brevispinus*	*Pisaster giganteus*	*Pisaster ochraceus*	*Pycnopodia helianthoides*
Assay tissue from:										
Asterias forbesi	Shed			Shed						
Astropectin armatus	None					Shed		Shed		None
Heliaster kubinijii	Shed	Shed	Shed		Shed	Shed	Shed	Shed	Shed	Shed
Patiria miniata	None	None	None		Shed	Shed	Shed	Shed	Shed	None
Pisaster giganteus	Shed							Shed	Shed	
Pisaster ochraceus	Shed					Shed		Shed	Shed	Shed

kubinijii, Pisaster ochraceus, but not on *Patiria miniata* or *Astropecten armatus.* Table II summarizes these findings.

Physiological mechanism

Several types of experiments were designed to help uncover the specific mechanisms believed to be useful in explaining the physiological action of shedding substance on ovaries (for technical reasons, these have not been extensively studied on the testes). The final result of an ovary exposed to shedding substance for one to two hours (see Fig. 3) suggested that (1) the ovary contracted and forced out its gametes from within the gonad, or (2) the eggs were released from the ovary by some other mechanism and as a result of the elasticity of the ovarian wall, the ovary contracted after the eggs had left the gonad. To investigate these alternative possibilities, the over-all length of isolated uninjured ovaries, while suspended in a sea water solution containing shedding substance, was constantly measured through the aid of a myograph-transducer, amplifier, and chart recorder (Chaet, Andrews and Smith, 1964). Figure 5 is a reproduction of a typical result obtained in such an experiment. It should be noted (Fig. 5) that the ovary began to contract at least 25 minutes after the addition of shedding substance—some 2–3 minutes (in some experiments, 5–10 minutes) before eggs were seen to be exuded through the gonopore. These findings suggested that one physiological action of the radial nerve extract containing shedding substance was due to a "contraction factor," which stimulated ovarian contraction, resulting in the active expulsion of eggs. It might be suspected that if contraction played a role in the shedding process, ionic calcium might be implicated in this over-all shedding reaction. It was found (Mecklenburg and Chaet, 1964) that ovarian fragments placed in calcium-free sea water containing shedding substance failed to release their eggs even if kept in such solutions for several hours. However, when these ovarian

fragments were removed, washed, and placed in normal sea water (containing calcium but no shedding substance), the tissues immediately began to shed.

A second type of experiment was designed to explain the fact that 98–100% of the eggs released as a result of shedding substance activity exhibited germinal vesicle breakdown and thus had completed maturation. To study this phenomenon, sister-ovaries were immersed in sea water or shedding substance solutions for one hour. After that time, the gonads were placed in sea water, minced with fine scissors, filtered through cheesecloth, and the eggs in the filtrate were washed in fresh sea water. Microscopic examination illustrated that the "sea water-ovary" contained small immature eggs, large immature eggs and large mature eggs (no eggs

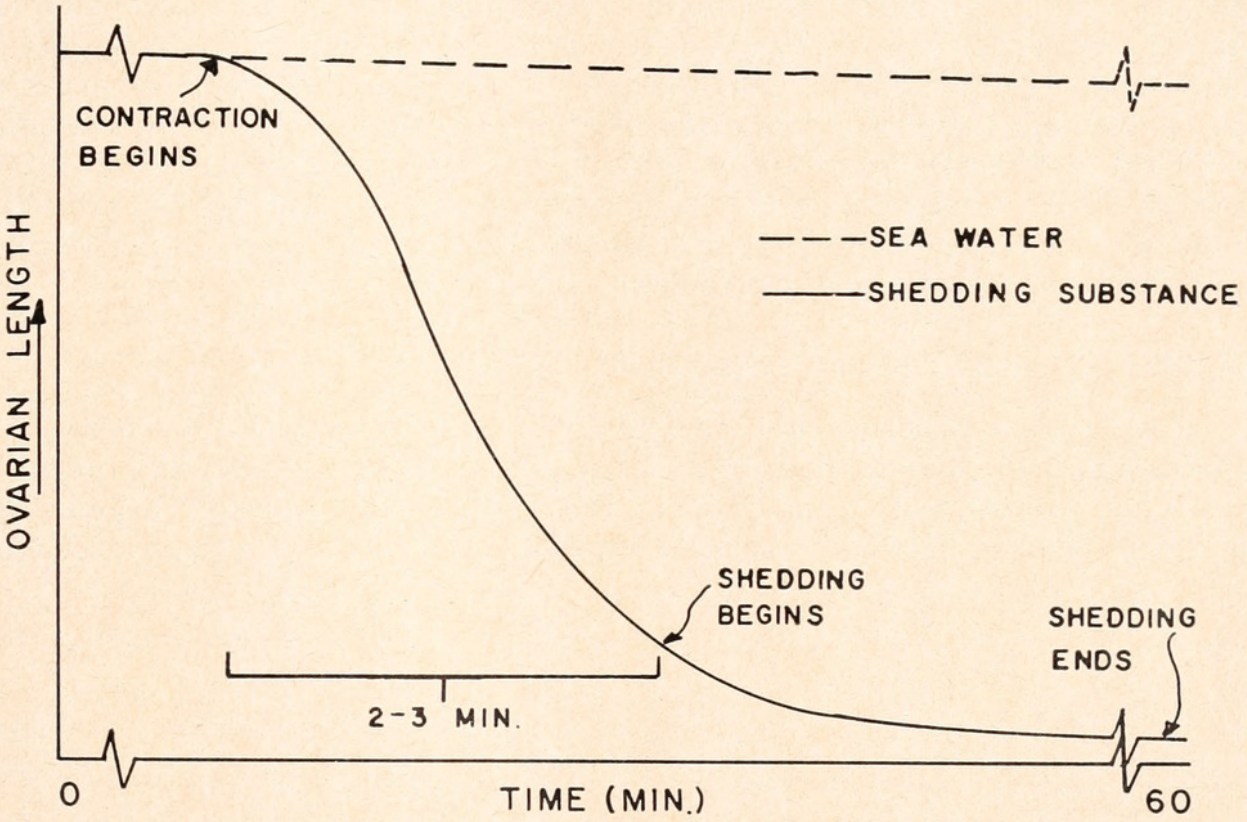

FIGURE 5. Plot of experiment designed to determine if eggs were released from isolated-intact ovaries before or after ovarian contraction. The length of *Asterias forbesi* ovaries was constantly recorded while immersed in 5 mg.% solution of radial nerve extract in sea water, or sea water alone.

were exuded from these ovaries into the sea water). In the remains of the "shedding substance-ovary," only small immature eggs (as well as some malformed large immature eggs) were present, and typical large immature eggs were for the most part absent. As noted above, large mature eggs had been shed into the surrounding medium. The absence of large immature eggs suggested that their conversion to mature eggs was stimulated by the presence of a "maturation factor" in nerve extracts.

To date, all attempts to separate the "contraction factor" from the "maturation factor" have been unsuccessful.

Neurosecretion

Various tissues of *Asterias forbesi* were assayed for the presence of gamete-shedding substance. Hot (76° C.) salt water extracts were prepared from the digestive tract, tube foot, oral and aboral surface, coelomic fluid, gonads, and hepatic caeca. The extracts were assayed on starfish ovaries and found to possess no gamete-shedding activity. Gamete-shedding activity was found only in hot salt water extracts of radial nerve. Since electron micrographs of the radial nerve suggested that the structure from which shedding substance had been extracted was indeed neural tissue, an attempt was made to search for neurosecretory granules in the radial nerve complex of *Asterias forbesi.* Typical neurosecretory stains, such as performic acid alcian blue, paraldehyde-fuchsin, chromalum-hematoxylin-phlozine and azan (Imlay and Chaet, 1965), demonstrated neurosecretory-like granules, 1 μ and 2 μ in diameter, present in the ventral layer of the radial nerve. The neurosecretory substance was positively correlated with the presence of active gamete-shedding substance, thus suggesting the possibility that shedding substance be placed in the category of a neurosecretion. Other experiments (Uter, 1966) in which radial nerves were stripped into layers showed that active gamete-shedding substance was located only in the most aboral region of the nerve—an area known to be rich in neurosecretory-like granules.

Chemical nature of shedding substance

The shedding substance from *Asterias forbesi* and from *Patiria miniata* was subjected to limited chemical analysis. It was found to be adsorbed on Dowex-50, which first suggested the possibility of a basic polypeptide. Further experiments in which shedding substance was incubated in 0.1% trypsin (twice crystallized)

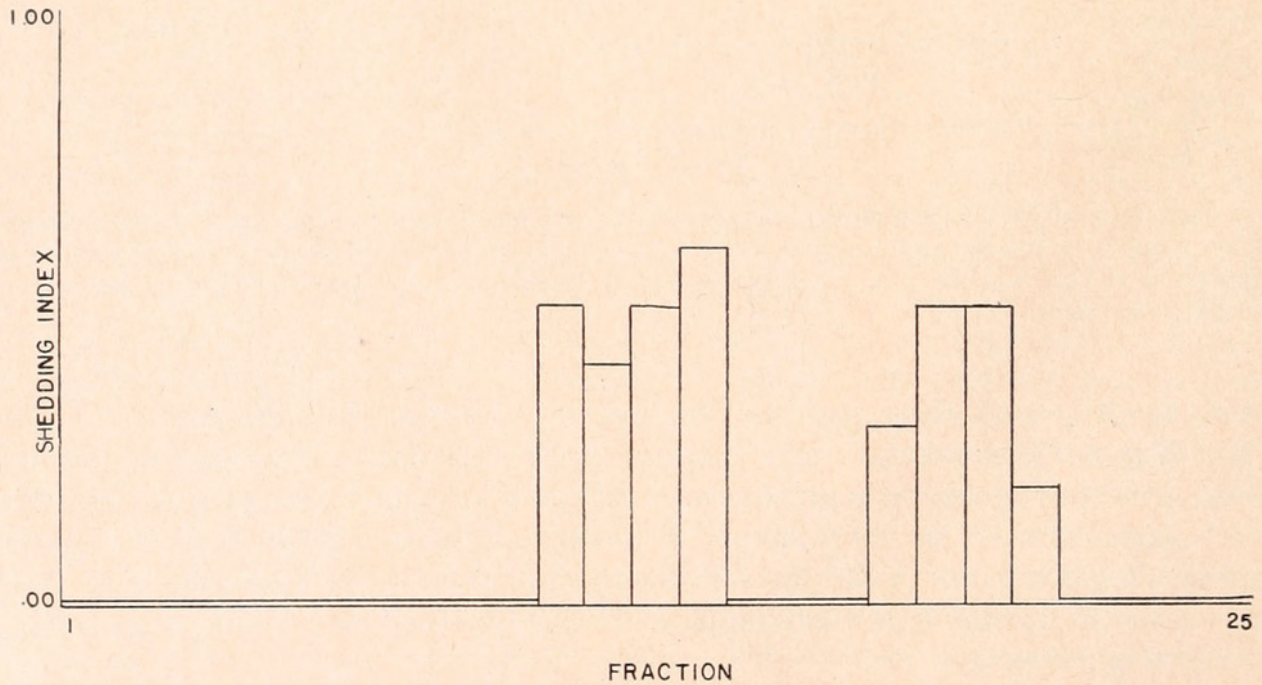

FIGURE 6. Biological activity of *Patiria miniata* shedding substance fractionated with Sephadex G-25 (58 cm. long.) columns. Note the two distinct peaks, a and b.

at pH 7 for 4 hours at 40° C. demonstrated that biological activity of shedding substance was lost after tryptic digestion, suggesting its polypeptide nature (Chaet, 1964c). It was also found that the addition of 0.2% ninhydrin to gamete-shedding substance resulted in a loss of biological activity.

Analysis of distilled water extracts from *Asterias forbesi* nerves by thin layer chromatography suggested the possible presence of 17 different amino acids and/or 19 dipeptides. Thus, various concentrations of known amino acids and dipeptides (chosen according to their R_f values) were assayed using the ovarian fragment spot-plate system. Stock amino acids were assayed for shedding activity, both as individual amino acids and in various combinations. However, none of the amino acids (individually or in combination) resulted in shedding activity. Figure 4 illustrates a typical example of one such assay involving 5 amino acids. An attempt was also made to mimic shedding activity with follicle-stimulating hormone, luteinizing hormone, 4-aminobutyric acid, and dl-2-aminobutyric acid by injection into intact starfish, but none induced the release of gametes.

To further purify the shedding substance, distilled water extracts of lyophilized *Patiria miniata* nerves were fractionated on 58-cm.-long Sephadex (G-25) columns and eluted with distilled water. Each 2-ml. fraction was then lyopholized, dissolved in sea water, and assayed on ovarian fragments. The result of a typical experiment was illustrated in Figure 6, which clearly demonstrated that two separate peaks were obtained, both possessing shedding activity. The above separation was observed, although less pronounced, when using 17-cm.-long columns.

Shedding substance purified on Sephadex was subjected to ninhydrin titer (Moore and Stein, 1954) before and after acid hydrolysis in sealed vials heated to 110° C. for 12 hours. Since hydrolysis of purified shedding substance only resulted in a four-fold increase in absorption (570 mμ) after the addition of ninhydrin and hydrindantin in methyl cellosolve and sodium acetate at pH 5.5, it appeared that the shedding substance was a polypeptide consisting of 10–15 amino acids.

Inhibitory substance

Experiments with *Asterias forbesi* and with *Patiria miniata,* involving various salt water concentrations (0.1 to 100 mg.%) of lyophilized nerves taken from preshed (ripe) starfish, showed that, whereas low concentrations of extract (2–5 mg.%) caused shedding, higher concentrations (10–100 mg.%) failed to cause shedding. If, on the other hand, the nerves were taken from post-shed (unripe) animals, high (10–100 mg.%) as well as low concentrations induced shedding. The results of a typical experiment may be seen in Table III. Since these findings suggested the presence of an inhibitory substance in the nerves taken from ripe animals, which was present at a threshold level only at the higher concentrations, nerve extracts from ripe starfish (which did not induce shedding in high concentrations) were fractionated (4-mg. aliquots) by passing over either long or short Sephadex G-25 columns. Once lyophilized, all fractions were redissolved in 2 ml. sea water, mixed with an equal volume of a fraction previously assayed and known to possess shedding activity, and each fraction was then subjected to ovarian fragment assay. The result of a typical assay experiment (using a short column) is graphically shown in Figure 7, in which one fraction was found (#5)

TABLE III

Assay of nerves obtained from pre- and post-shed Patiria miniata. Figures expressed as shedding index.

	Lyophilized Nerve Extract (in sea water)				
	0 mg.%	5 mg.%	10 mg.%	25 mg.%	100 mg.%
Pre-shed (ripe)	0.00	0.30	0.05	0.00	—
Post-shed (unripe)	0.00	0.53	0.53	0.55	0.25

that inhibited shedding of ovarian fragments even though all fragments were under the influence of active purified shedding substance (#3). Since this fraction inhibited shedding activity, it was called "shedhibin." Similar assays were performed using extremely large ovaries from maximally ripe starfish (*Patiria miniata*). Such ovarian fragments, provided that they were taken from very ripe females, shed spontaneously (even in the absence of added shedding substance) within 15 to 30 minutes after immersion in sea water. However, when these fragments were immersed in each of the above fractions taken from Sephadex columns, all fractions allowed shedding to occur except that fraction (#5) previously found to contain shedhibin. No shedhibin could be detected in those radial nerves taken from post-shed, unripe females whose crude nerve extract induced shedding at higher concentrations.

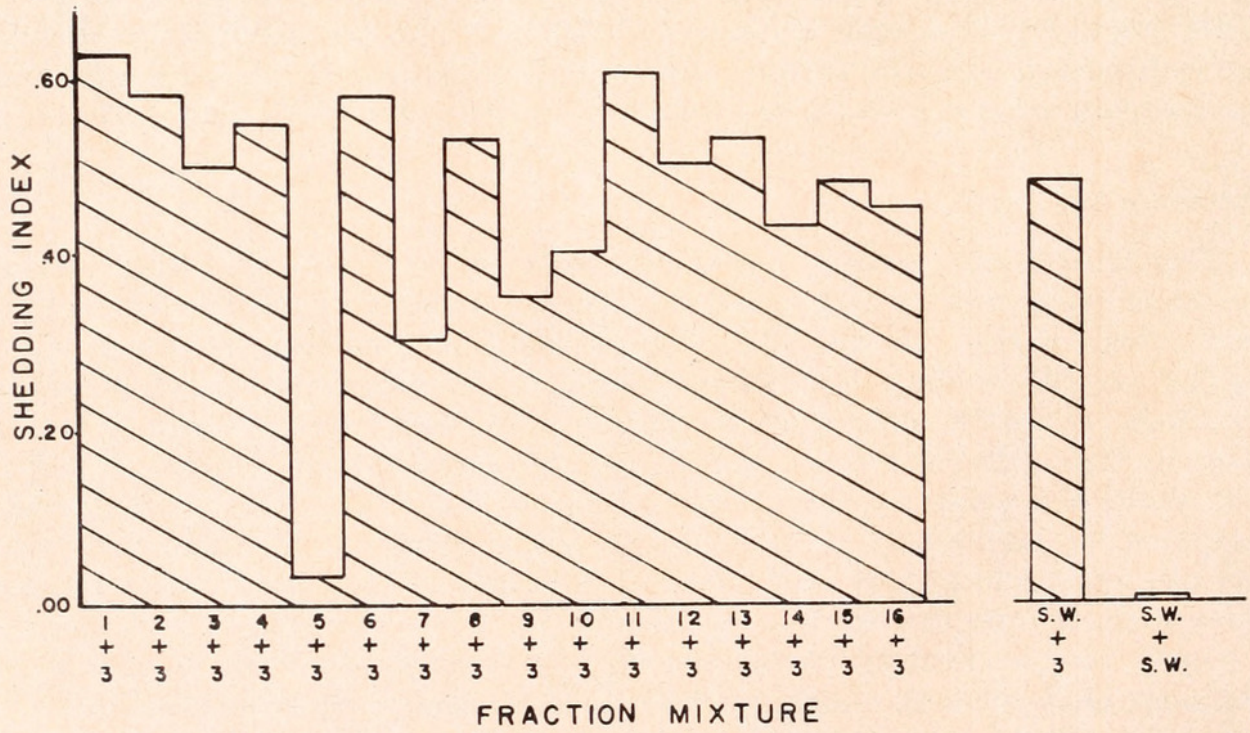

FIGURE 7. Graphic representation of *Patiria miniata* ovarian fragment assay, illustrating presence of shedhibin in fraction #5 of fractionated *Patiria miniata* radial nerves. Five-ml. fractions eluted from short Sephadex G-25 column were assayed after mixing with an equal volume of fraction #3, known to contain shedding substance.

Discussion

The gamete-shedding substance(s) found in the radial nerve of sea-stars was shown to be responsible for inducing the release of mature eggs or sperm when injected into ripe starfish of the same species. Figures 1 and 2 illustrate the release of gametes from two different species, *Asterias forbesi* from the Atlantic coast and *Patiria miniata* from the Pacific coast. Actually a total of 14 different species of sea-stars have now been found to contain gamete-shedding substance. In addition to those listed in Table II shedding activity has been found in *Asterias glacialis* (Unger, 1962), *Asterias amurensis, Astropecten scoparius and Coscinasterias acutispine* (Kanatani and Noumura, 1962). The shedding substance was not species-specific as Hartman and Chaet (1962) first demonstrated when nerve extracts from *Henricia sanguinolenta* were injected into *Asterias forbesi,* resulting in the release of mature gametes. Later, extracts of *Asterina pectinifera* were found to induce spawning in *Astropecten scoparius* or *Coscinasterias acutispina* (Kanatani and Noumura, 1962). On the other hand, a certain degree of species specificity does exist, since, for example, shedding substance from *Asterias forbesi* or *Pycnopodia helianthoides* did not induce shedding when assayed on isolated ovarian fragments of *Patiria miniata* or *Astropecten scoparius,* although extracts from *Pisaster giganteus* and *P. ochraceus* (as well as *Patiria miniata*) proved highly active in *Patiria* ovaries.

It should be noted that in experiments both *in vivo* and *in vitro* it was consistently found that a 30-minute interval was evident—that is, eggs were not shed until 30 minutes after injection of nerve extracts into the coelomic cavity of an intact starfish (Chaet and Musick, 1960), or immersion of an isolated ovary (Chaet and Smith, 1962), or fragment (Chaet, Andrews and Smith, 1964) into a solution of shedding substance. Since experiments *in vitro* involving isolated ovarian fragments demonstrated that the lag period persisted, it was concluded that the shedding substance acted directly on the ovarian tissue itself and might be considered a "first order neuro-endocrine" mechanism as defined by Rothballer, (1957).

Since the onset of ovarian contraction began some 2–3 minutes before shedding actually occurred (Fig. 5), it was theorized that the shedding substance contained a "contraction factor." Further evidence supporting this theory of a contraction factor was seen in those experiments which implicated the role of calcium on the physiological activity of the shedding substance (Mecklenburg and Chaet, 1964). If the shedding substance did indeed act on ovarian muscle, causing an over-all contraction and forcing-out of gametes, it would be expected, according to theories involving muscle contraction (Weber, 1958), that ovarian contraction and, therefore, shedding would not occur in the absence of calcium even though ample quantities of shedding substance were present. It might also be expected that when calcium was again added, shedding would begin. This was found to be the case when using ovaries and shedding substance from both *Asterias forbesi* and *Patiria miniata* (Mecklenburg and Chaet, 1964). The inability of shedding substance to function in the absence of calcium was confirmed for *Asterias amurensis* (Kanatani, 1964).

The presence of a "maturation factor" (Chaet, 1964c; Chaet, Andrews and

Smith, 1964) was suspected by comparing gametes remaining in sister ovaries (*Asterias forbesi*) after immersion in gamete-shedding substance or control solutions. These findings have been confirmed on a second species of sea-star, *Asterias amurensis* (Kanatani, 1964). In addition to contraction and maturation factors previously reported by Chaet and co-workers, Kanatani (1964) has suggested that still another action of shedding substance was to free eggs from cohering to each other and/or from adhering to the ovarian wall.

It was first noted by Chaet and Smith (1962) that the concentration of shedding substance in nerves taken from ripe starfish contained the same concentration of shedding substance as did nerves of an unripe (spent) animal. Further, monthly nerve samples collected over a two-year period, when simultaneously serially assayed, again indicated a constant level of shedding substance in the radial nerve of *Asterias forbesi*. Noumura and Kanatani (1962) reported that a seasonal variation did occur; however, they did not measure the concentration or physiological activity of shedding substance (personal communication). They reached their conclusion after noting a change in transparency of nerves and also pointed out (p. 400) that "histological studies also revealed seasonal changes in the radial nerve, as will be reported elsewhere." Imlay and Chaet (1965) independently noted seasonal variations after studying histological sections of radial nerves stained with "neurosecretory stains," but they could not conclude that these histological variations were in any way correlated with the presence or a change in level of shedding substance. It is possible that the seasonal variation noted may reflect the level of shedhibin, but we have yet to study this point.

Since the level of shedding substance remained constant throughout the year, it was indeed difficult to surmise the role of gamete-shedding substance in natural spawning. However, it was noted when working with *Asterias forbesi* and later with *Patiria miniata* (Table III) that shedding failed to occur when higher concentrations of nerve extracts were used, provided that they were taken from nerves of ripe animals. These findings then stimulated a search for an inhibitor, and at the present time, the inhibitor "shedhibin" was found only in nerves taken from ripe starfish and was absent in nerves taken from spent starfish. Studies are underway to determine the precise level of shedhibin in nerves of *Patiria miniata* by collecting bimonthly specimens. Since high concentrations (10 mg.%) of nerve extracts prepared from nerves of post-shed (unripe) animals continued to induce shedding, and since shedhibin was isolated from nerves of ripe starfish, but could not be found in unripe animals, the obvious suggestion arose that natural control of shedding was regulated both by the level of shedding substance and the presence (or absence) of shedhibin. It appears that a seasonal disappearance of shedhibin may be responsible for the release of gametes in the presence of shedding substance.

The physiological significance of two distinctly different fractions of shedding substance of different molecular weight (Fig. 6) has not been explained and may merely represent an artifact, but it is of interest that both substances remained biologically active. Whether the shedding substance complexed with other molecules of different size or whether the shedding substance has been split into a smaller molecule which maintained its physiological activity when degraded has yet to be determined. Kanatani and Noumura (1962) independently reported that they concentrated shedding substance on Sephadex columns, but found only

one peak rather than the two reported by Chaet (1964b). This was understandable since they used columns only 35 cm. in height, which, according to our own experiments, would not adequately separate the two peaks. They also reported an ability to concentrate shedding substance, a phenomenon which may also be explained merely on the basis that they unknowingly had removed shedhibin which inhibited their shedding substance activity.

It should be noted that granules were observed (Imlay and Chaet, 1965) which stained positively with four different neurosecretory stains. Some, but not all, of the granules were present during pre- and post-shedding stages. Granules staining with the neurosecretory stains were present in the most ventral layer of radial nerves of *Asterias forbesi* (Imlay and Chaet, 1965), which was the only area of the radial nerve complex in which physiologically active shedding substance was found (Uter, 1966). Using the most critical criteria of neurosecretion (Bern, 1963), it is difficult to place conclusively the shedding substance in the realm of a neurosecretory substance, and the above findings merely suggest that the granules are neurosecretory in nature. The studies of Unger (1962) as well as studies of Philpott and Chaet (unpublished), involving the electron microscopy of radial nerves of two asteroids, as well as those light microscopy studies of Noumura & Kanatani (1962), all of which illustrated the presence of "secretory granules," continue to suggest that the shedding substance represents neurosecretory activity.

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Summary

1. A neurochemical substance from the radial nerve of *Asterias forbesi* and *Patiria miniata* has been studied *in vivo* and *in vitro* and found to initiate both gamete release and maturation. The significance of a 30-minute lag period before gamete release, which was evident in all experiments, was discussed.

2. Gamete release and/or maturation did not depend upon passage through the gonoduct or gonopore.

3. The level of shedding substance in radial nerves of starfish was found to be constant throughout the year, and was found in a total of 10 different species. Cross-experiments indicated that a limited degree of species specificity existed.

4. The possibility that the shedding substance is a neurosecretory product was discussed.

5. The shedding substance was found to be a polypeptide, consisting of 10–15 amino acids, which was purified for future amino acid analysis.

6. An inhibitor to the shedding substance, shedhibin, was also found in the radial nerve. Its presence varied with the reproductive state of the animal.

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