PONTIFICIA
ACADEMIA
SCIENTIARVM

COMMENTARII

VOL. II N. 27

HARUO KANATANI

ON THE SUBSTANCES CONTROLLING CERTAIN REPRODUCTIVE PHENOMENA IN STARFISHES

EX AEDIBVS ACADEMICIS IN CIVITATE VATICANA

PONTIFICIA
ACADEMIA
SCIENTIARVM

COMMENTARII
Vol. II - N. 27
pag. 1-36

ON THE SUBSTANCES CONTROLLING CERTAIN REPRODUCTIVE PHENOMENA IN STARFISHES

HARUO KANATANI
Laboratory of Physiology, Ocean Research Institute, University of Tokyo, Japan

In most animals, oocytes which are still in the ovary usually have a single large nucleus, known as the « germinal vesicle », with the double complement of chromosomes. In the course of being released from the ovary, these oocytes undergo the reduction divisions, discarding half of the chromosomes as polar bodies, and becoming mature, fertilizable ova with a single set of chromosomes. These reproductive phenomena, like many other physiological events, seem to be under hormonal control in invertebrates as well as in vertebrates.

Since starfishes are plentiful and relatively simple in body structure, they provide good material for biochemical as well

---

Die 18 mensis aprilis in Academiae Sessione Plenaria clarissimus vir Professore HARUO KANATANI de Academicorum consensu meruit ut coronam auream, vulgo « Medaglia d'oro Pio XI », e manibus Summi Pontificis PAULI Papae VI acciperet.

Idem post habitas laudes, coram Academicorum Coetum eodem die hanc orationem dixit de studiis suis

as physiological studies. Investigations on oocyte maturation and gamete release with this animal have provided clues to some of the mechanisms underlying these phenomena such as the biochemical mechanism of the action of a certain hormonal peptide released from the nervous tissue. These studies also contribute to elucidating the fundamental principle of the reproductive phenomena from biochemical as well as physiological aspects, and in a more restricted sense, to establishing a theory of the mechanism of meiotic division. These are undoubtedly among the biological problems of fundamental importance which have yet to be solved. It is historically of interest that a century ago sperm entrance into an animal egg was first observed by the Swiss scientist HERMANN FOL [1] who worked with the starfish *Marthasterias glacialis*.

In 1959, the American scientists CHAET and MCCONNAUGHY [2] first reported that a water extract of the radial nerves of *Asterias forbesi* induces gamete-shedding when injected into ripe starfish. Since that time, evidence has been accumulated, suggesting that a neural substance plays an important role in starfish spawning. The presence of such active substance has been shown in more than twenty starfish species, and cross experiments among different starfishes show that the substance, with some exceptions, acts non-species specifically [3, 4, 5, 6, 7, 8, 9, 10, 11, 12].

Another action of this substance was at first thought to be induction of oocyte maturation, since oocytes within the ovary are immature, whereas eggs released from the gonopore are on the way to the first meiotic division [7, 13, 14]. However, in 1967 SCHUETZ and BIGGERS in the United States [15] and KANATANI and SHIRAI [16] in Japan independently found that the neural substance acts on the ovary to produce a second substance which induces oocyte maturation. We have further demonstrated that the purified fraction of the second substance by itself brings about gamete release, even in the absence of the neural substance. We have therefore concluded that this

« meiosis-inducing substance », MIS, is also the spawning-inducing factor, and that the so-called « gamete-shedding substance », GSS, obtained from radial nerves, should rather be called « gonad-stimulating substance » [12, 17]. The present report is a survey of investigations carried out mainly in our laboratory to elucidate the mechanism of oocyte maturation and spawning in starfishes.

First, I should like to deal with the action of the gonad-stimulating substance, GSS, obtained from radial nerves (Fig. 1). The nerve extract is obtained by homogenizing the lyophilized nerve in a small amount of water. This is centrifuged and the supernatant is diluted with sea water. When isolated ovarian fragments are immersed in this solution, they begin to discharge their eggs from the cut surfaces after about 30 minutes (Fig. 2) [7, 8, 9]. Nerve extract is still active at concentrations of about 6-20 µg of dry nerve/ml. Hereafter we will refer to the sea water containing nerve extract as GSS-SW.

When ovarian fragments are ligated to prevent the discharge of eggs, and treated with GSS-SW for one hour, histological sections clearly show that the oocytes within the ovary are undergoing the first meiotic division, while the germinal vesicles of oocytes in untreated ovaries remain intact (Fig. 3 [12, 18]. It thus appears that a second effect of GSS is to bring about maturation of the oocytes within the ovary.

GSS was found in all seasons [8], and also in the coelomic fluid, but only when the starfish were undergoing natural spawning. This suggests that GSS is a secretory substance, that is, a hormone [9, 19].

When the distal part of an isolated whole ovary is treated locally with GSS-SW for one hour, and then its wall is torn with forceps at various parts, intense discharge of eggs occurs only in the treated portion. GSS, therefore, seems to act directly on the gonadal surface from outside [7, 11, 18].

The question immediately arising from these observations in connection with the mechanism of starfish spawning is whether

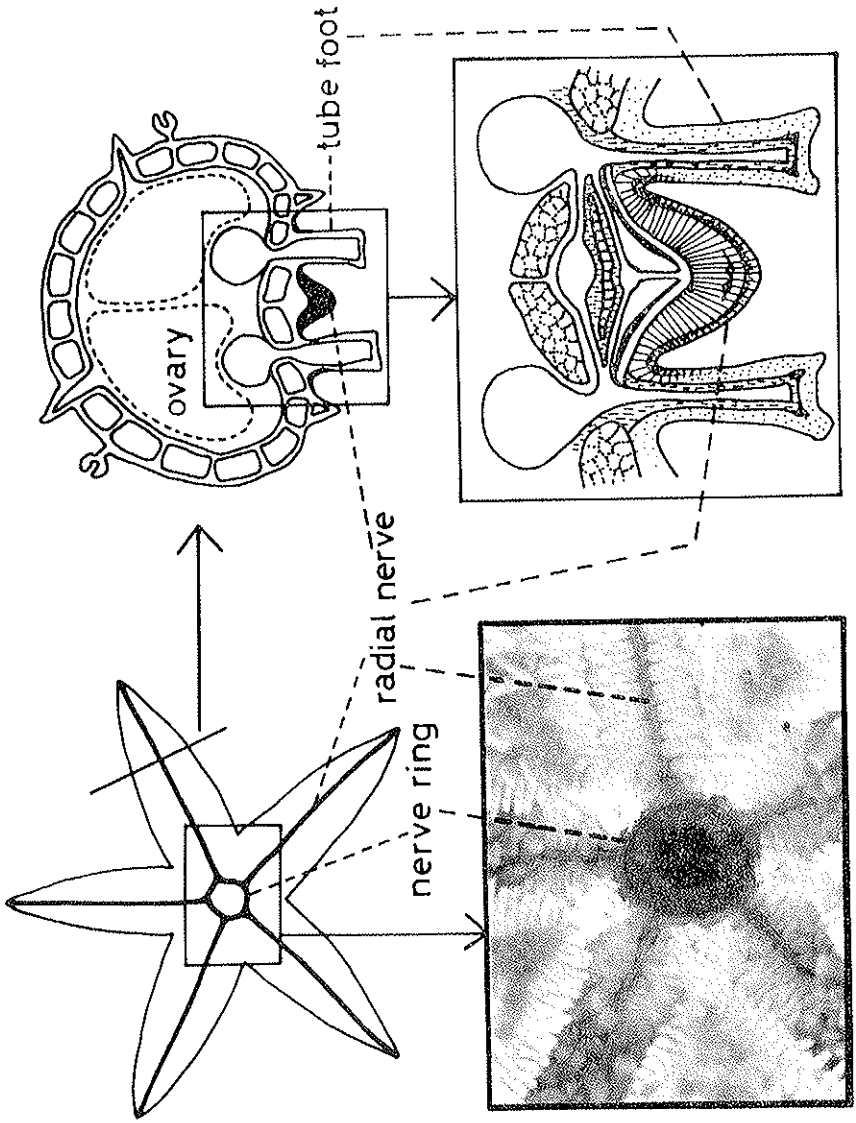

FIG. 1 — Nerve ring and radial nerve of *Asterias amurensis*. Left; seen from oral side. Right; cross section.

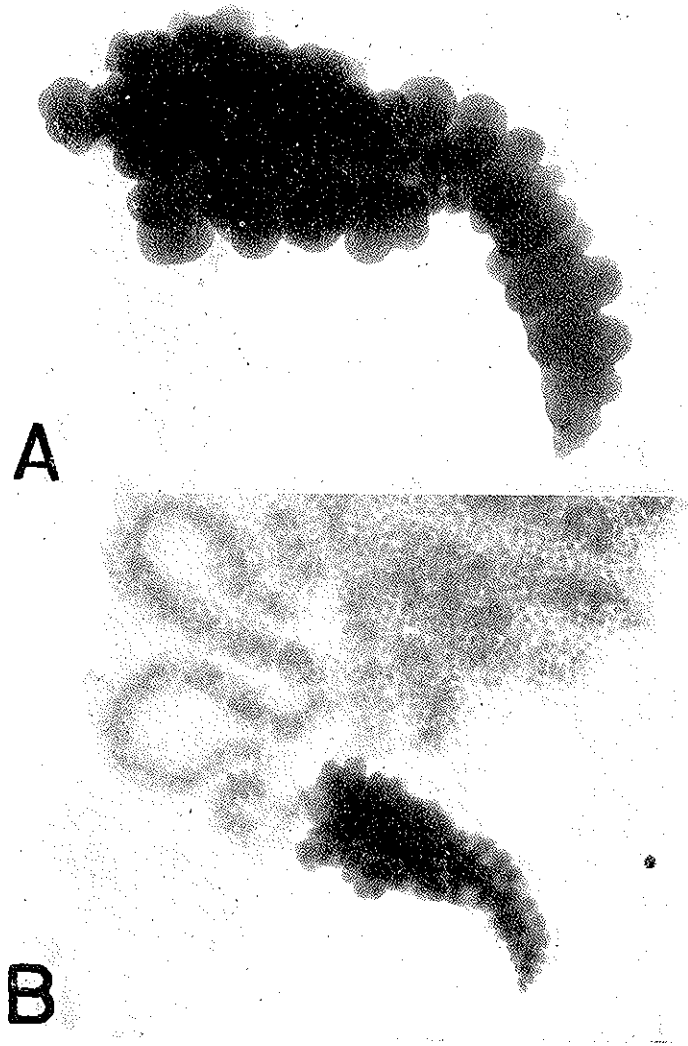

FIG 2 — *In vitro* assay of GSS. (A) Ovarian fragment (*ca.* 12 mm) of *Asterias amurensis*. (B) Same ovarian fragment discharging eggs after treatment with nerve extract. Same magnification.

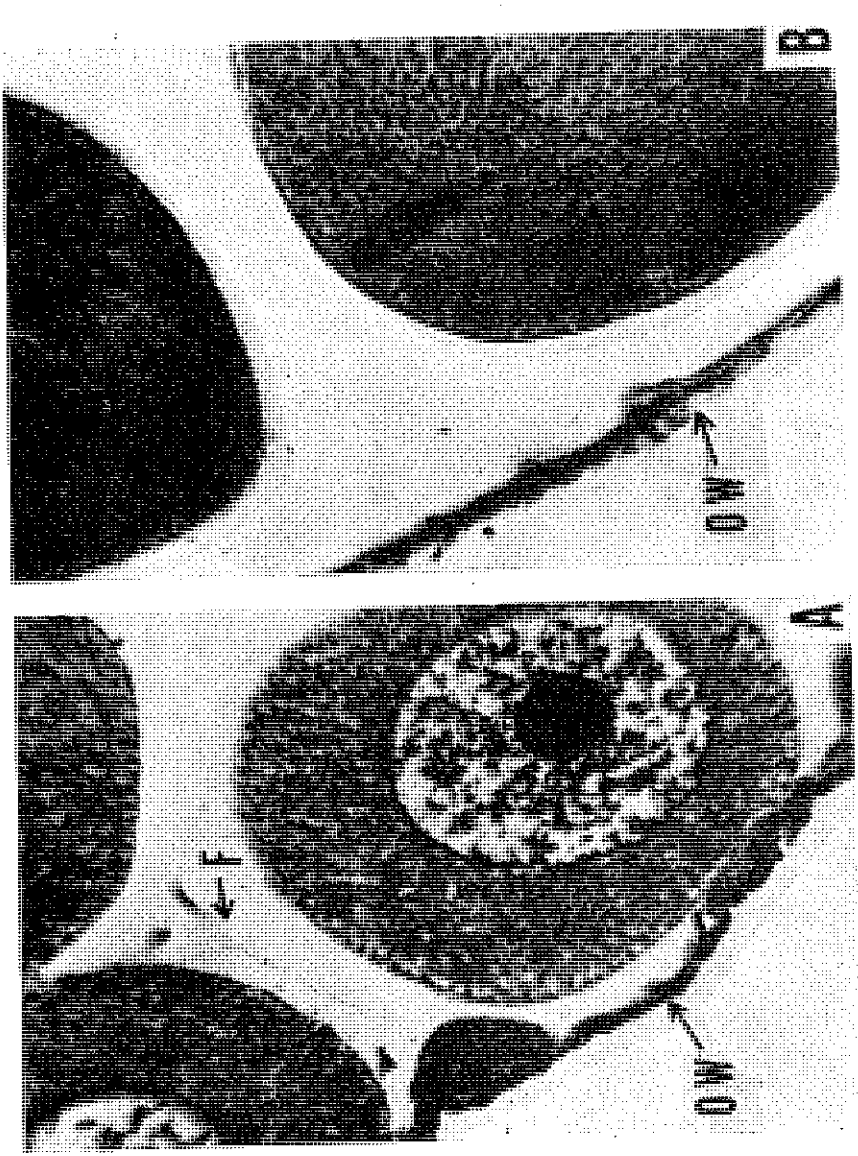

FIG. 3 — Maturation of oocytes within ovary brougt about by nerve extract. (A) Section of ovarian fragment immersed for 1 hour in sea water (control); OW, ovarian wall; F, follicle. (B) Section of ovarian fragment immersed in sea water containing nerve extract for 1 hour. Note that oocytes are detached from the ovarian wall and undergoing first maturation division. Follicles surrounding oocytes have disappeared.

GSS causes the ovarian wall to contract actively, and thus expel the eggs from the gonad. This was the original theory of GSS action [8, 10, 20]. If this is the case, spawning should be induced by known contraction-inducing agents such as potassium chloride, acetylcholine and electric stimulation. However, none of these agents induces significant spawning in starfishes [11, 39]. Some experiments conducted in our laboratory revealed, however, that isolated ovarian wall itself contracts vigorously when treated with potassium chloride or acetylcholine. As shown in Figure 4 the distal part of an ovary is cut out, ligated, and attached to the kymographic apparatus. When purified GSS without contaminating small molecules is applied to this preparation, little or no contraction occurs, whereas either potassium chloride or acetylcholine brings about marked contraction of the ovarian wall [11]. From these experiments, GSS must be thought to act on the ovary in some way other than by causing the contraction of its wall. That contraction-inducing substances such as potassium chloride and acetylcholine do not induce starfish spawning seems to be due to the presence of firm follicles around the oocytes within the ovary. The release of individual oocytes from the ovary seems to be prevented by the mutural adherence of the follicles. When an ovarian alveolus was isolated from an ovary and its wall slit by fine forceps in sea water, the alveolar wall contracted suddenly discharging a mass of eggs from the slit, and turning inside-out because of the presence of eggs adhering to its inner surface and to each other (Fig. 5). Also, most of the eggs in isolated alveoli treated with GSS were freely discharged after a certain period even without a slit being made. Therefore, we can assume that GSS acts on the ovary to dissolve the cementing substance between adjacent follicle cells and make the eggs within the ovary freely movable, so that they are forced out by the contraction of the ovarian wall. This contraction seems to result from the tension always present in the wall of a ripe, distended ovary [7, 18].

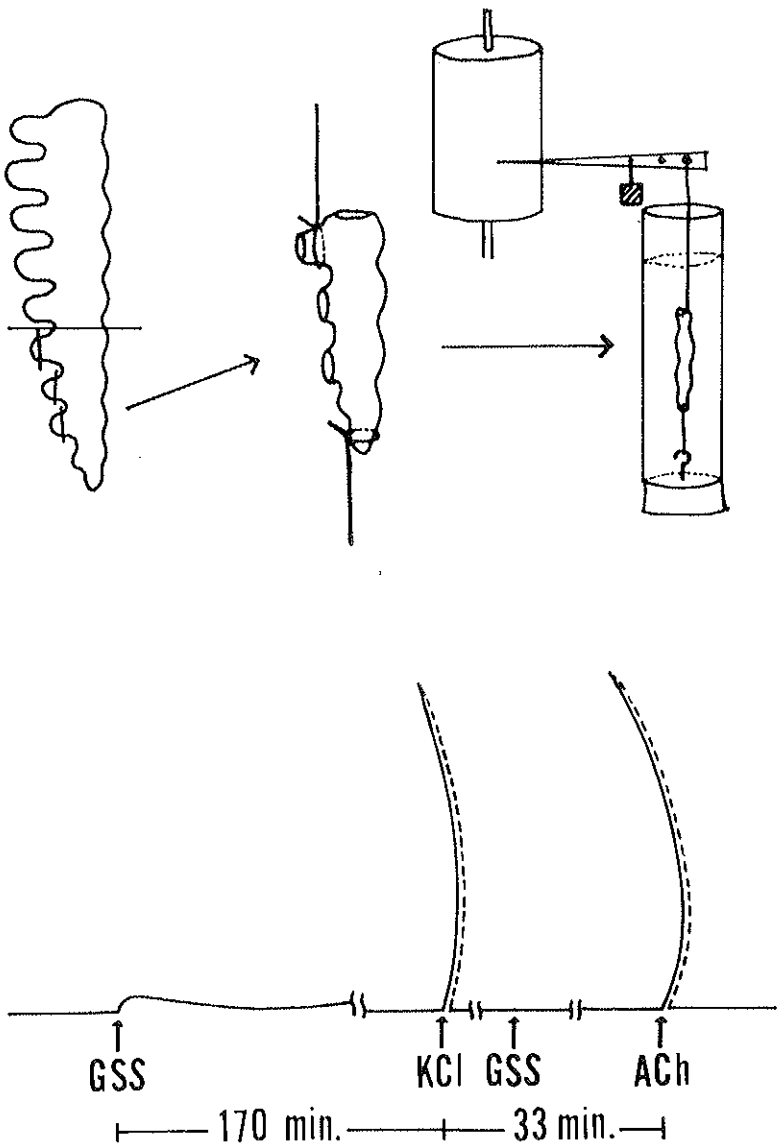

FIG. 4 — Contraction of isolated ovarian fragment induced by KCl and Acetylcholine. Upper figure shows the ovarian preparation used in this contraction experiment. Lower figure is reproduced from kymographic drawing.

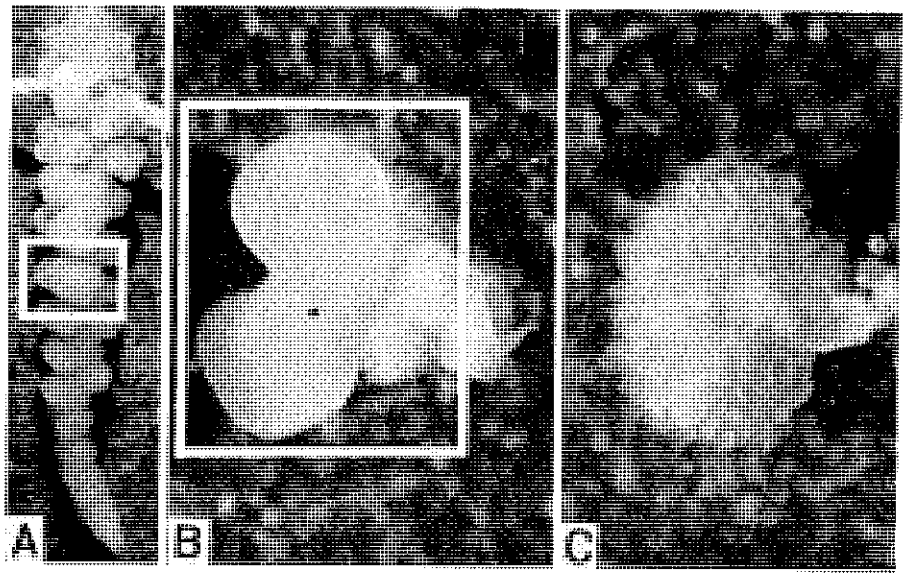

FIG. 5 — Adherence of eggs to each other and to the inner surface of the gonadal wall. (A) Ligated ovarian fragment of *Asterias amurensis;* white quadrangle shows an ovarian alveolus; (B) isolated ovarian alveolus; (C) same alveolus turns inside-out after its wall is slit. Note adherence of eggs to each other.

Since it is well known that bivalent cations such as calcium and magnesium act to stabilize intercellular cementing substances, ovarian fragments were treated with calcium-free sea water or magnesium-free sea water in an attempt to induce artificial spawning in the absence of GSS. An ovarian fragment placed in magnesium-free sea water begins to discharge the eggs after about 30 minutes. Also ovarian fragments placed in calcium-free sea water for 30 minutes spawn their eggs immediately when they are transferred into normal sea water. Failure of spawning in calcium-free sea water suggested that the contraction of the ovarian wall requires calcium [7, 11, 14, 20]. These experiments clearly show that dissolving the cementing substance and stripping off the follicles from the

eggs within the ovary are prerequisites of spawning, and GSS has been thought to have such action.

Now, I should like to discuss the chemical nature of this spawning-inducing substance obtained from radial nerves. This substance is rather heat-stable and insoluble in organic solvents such as ether, chloroform, acetone and ethanol [4]. Treatment with proteolytic enzymes such as trypsin, pepsin and pronase destroys its activity [4, 6, 11]. This suggests that GSS is a polypeptide.

Purification of this substance has been done in our laboratory, using the radial nerves of *Asterias amurensis* [12]. Sixty-eight grams of acetone powder of radial nerves taken from about 7,400 starfish were extracted with 0.05M sodium chloride and GSS was isolated through several steps of purification procedures consisting of gel-filtrations on Sephadex G-50 and G-25 columns of various sizes and iron-exchange column chromatography on DEAE-Sephadex columns by gradient as well as step-wise elution (Fig. 6). The 1.3 mg of purified GSS finally obtained induced spawning at a concentration of 0.0096 μg/ml. Using this sample, amino acid analysis was carried out with an amino acid autoanalyzer [21], and the molecular weight of GSS was determined by the sedimentation equilibrium method [22] with an analytical ultracentrifuge, as well as by simple gel-filtration on a Sephadex G-50 column. These results are shown in Table 1. GSS of *Asterias amurensis* was found to consist of about 23 amino acids, and to have a molecular weight of about 2,000. GSS seems to be a chain-like molecule since it has neither cysteine nor cystine.

Now, back to the morphological view point, I should like to deal briefly with the localization of GSS within the radial nerve [23]. When radial nerves of *Asterina pectinifera* were homogenized in 0.25M sucrose containing 0.1M KCl, 0.1M NaCl and 5mM EDTA (pH 7.8) and differentially centrifuged from 1,300 x *g* to 109,800 x *g*, the highest specific activity for inducing spawning was found in the sediment obtained from centri-

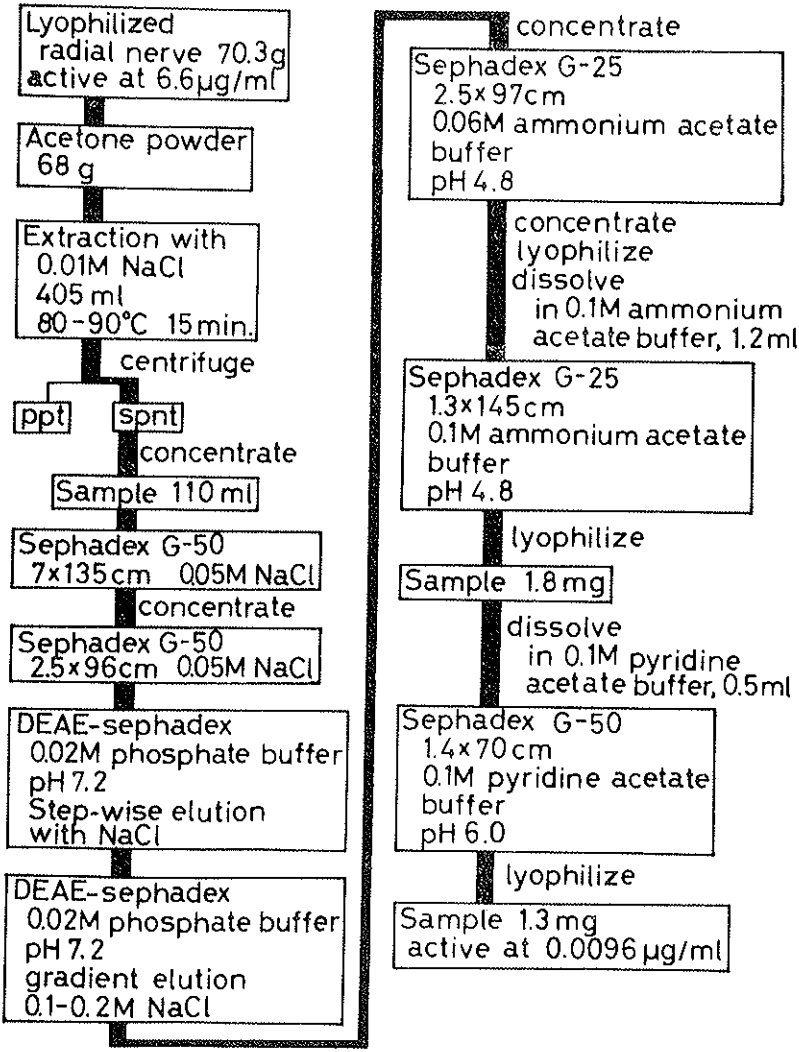

FIG. 6 — Main steps of procedure for purifying gonad-stimulating substance of *Asterias amurensis*.

TABLE I — *Molecular weight and tentative amino acid composition of « gonad-stimulating substance » (GSS) obtained from radial nerves of* Asterias amurensis

Aspartic acid	2
Threonine	1
Serine	7
Glutamic acid	1
Proline	1
Alanine	2
Glycine	4
Valine	1
Isoleucine	1
Leucine	1
Histidine	1
Ornithine	1
	23 residues

assuming that GSS contains a single prolyl residue

Molecular weight	2153
from amino acid composition	
	2100
from sedimentation equilibrium method	
	2100
from gel-filtration (Sephadex G-50)	

fugation at 10,200 X g. This active sediment was next subjected to sucrose density gradient ultracentrifugation at 40,000 rpm for 3 hours. The concentration of sucrose was 1M to 2M and the active fraction was obtained at about 1.5M. Electron micrography of the fraction having the highest activity showed that this fraction consisted of granules of various sizes. They are very similar to the preparations of granules containing vertebrate hormones such as vasopressin and oxytocin obtained from bovine posterior pituitary by BINDLER and others [24]. In electron micrographs of radial nerves these granules were found in the supporting cell layer as well as in the central part of the radial nerve (Fig. 7). When small samples of fresh supporting cell tissue were carefully separated from the radial nerve treated with sea water lacking magnesium and calcium ions, strong spawning-inducing activity was found in thess samples [30].

Next, I should like to deal with the production of the meiosis-inducing substance, MIS, under the influence of GSS

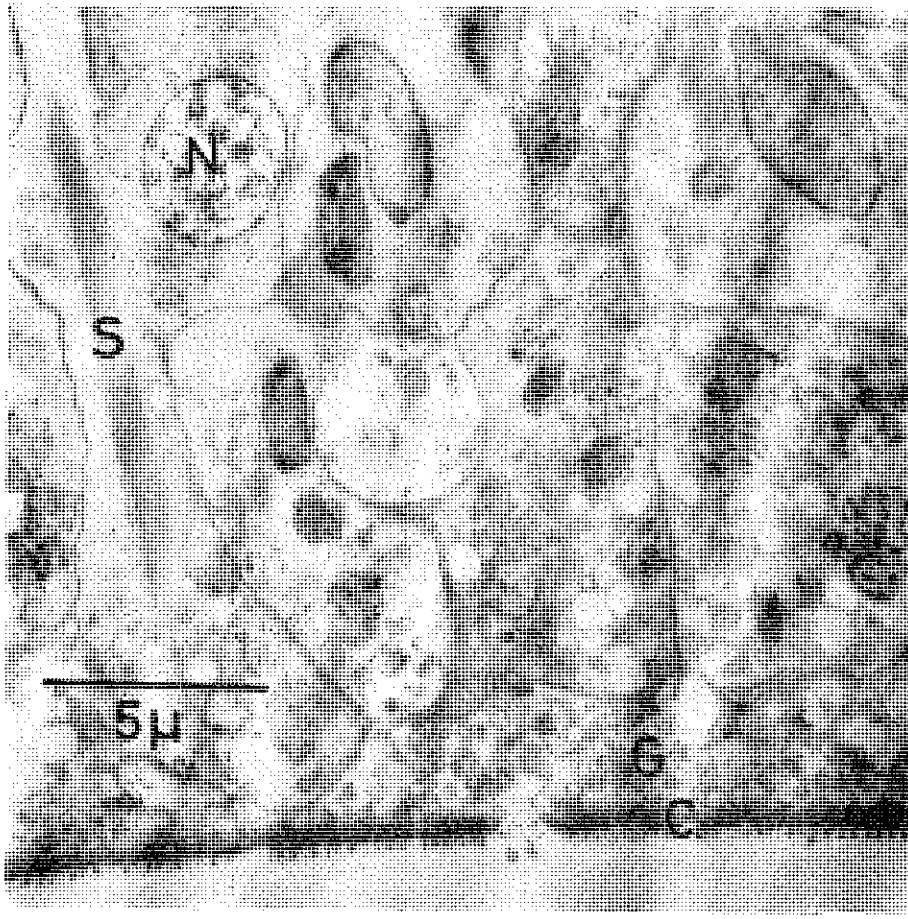

FIG. 7 — Electron micrograph of supporting cells of radial nerve of *Asterina pectinifera*. Small granules (G) found just inside of the cuticle (C) are thought to contain gonad-stimulating substance. (S) Supporting fiber; (N) nucleus.

in the starfish gonad [16]. The oocytes of *Asterina pectinifera* undergo no change when they are removed from the ovary to sea water. Moreover, treatment with GSS-SW does not induce such isolated oocytes to begin meiosis or break down

their follicles. On the other hand, when *Asterina* ovaries are treated with GSS-SW, they discharge eggs without germinal vesicles or follicles. These observations led us to assume that in *Asterina*, GSS acts on the ovary to produce another substance which is responsible for dissolving the cementing substance and for inducing meiosis. To test this possibility, ovarian fragments were isolated and placed in GSS-SW for one to three hours. The spawned eggs and ovarian tissues were removed by centrifugation. When isolated oocytes with germinal vesicles and follicles were transferred into the supernatant, they stripped off their follicles and underwent meiosis. These results suggested that some active substance is liberated into the medium from the ovary under the influence of GSS. This substance is referred to as MIS (meiosis-inducing substance). Since this active medium containing MIS also contained GSS, we tried to separate the two substances by gel-filtration on a Sephadex G-15 column. *Asterina* ovaries were placed in artificial sea water containing GSS for 2.5 hours, and the supernatant was applied to a Sephadex G-15 column equilibrated with artificial sea water. After gel-filtration, fractions were assayed for maturation- and spawning-inducing activities, using isolated oocytes and isolated ovarian fragments, respectively. Figure 8A shows the results of such gel-filtration. The meiosis-inducing substance, MIS, appeared in quite different fractions from those containing GSS. It was very interesting to find out that the fractions showing meiosis-inducing activity also showed spawning-inducing activity [16, 26, 31]. For comparison, nerve extract and ovarian extract were also fractionated in the same way. However, as shown in Figures 8B and C, no fractions having meiosis-inducing activity were obtained from such gel-filtrations.

The process of breakdown of follicles and germinal vesicles on treatment with MIS is shown in Figure 9 [17]. Breakdown of follicles begins with the rupture of some weak region of the follicular envelope. The follicular envelope material shrinks

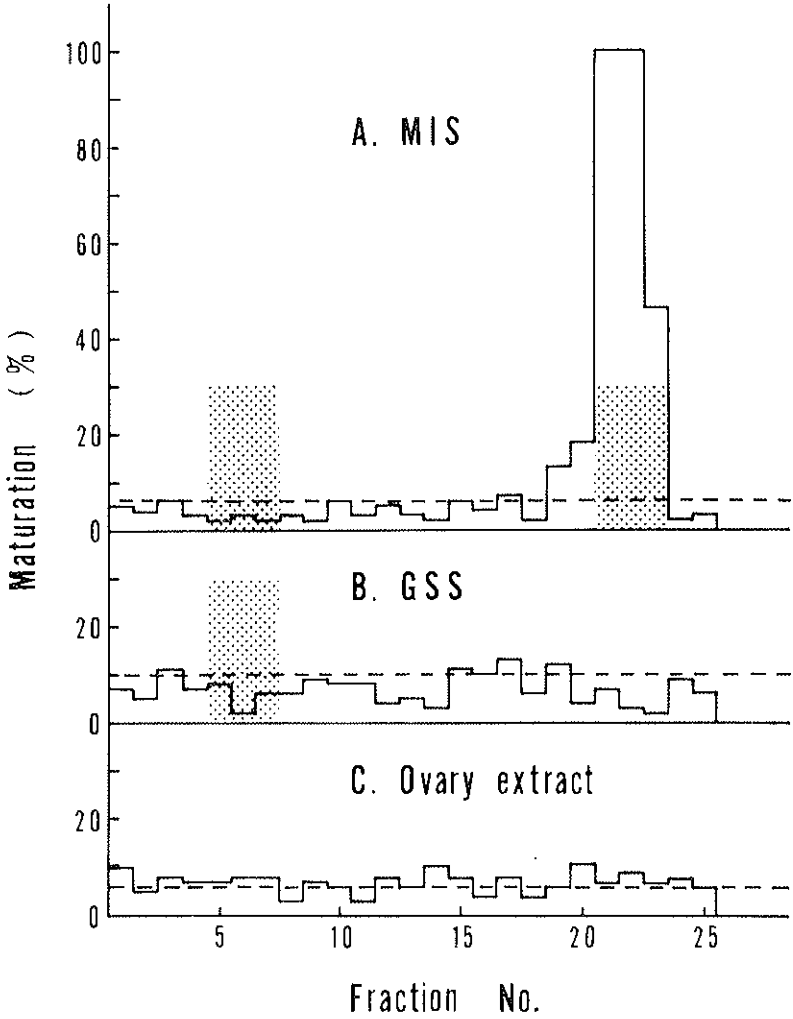

FIG. 8 — Meiosis-inducing activities of fractions obtained by gel-filtration of (A) supernatant of mixture of ovary and nerve extract; (B) nerve extract (GSS); and (C) ovarian extract on Sephadex G-15. Broken lines represent percentages of maturation in sea water (control). Dotted area represent the fraction having gamete-shedding activity. Column size, 1.4 × 42 cm; sample size, 3 ml; fraction size, 5 ml; eluant, artificial sea water (pH 8.2-8.3); flore rate, 30 ml/hr. Observations were made after 1 hour.

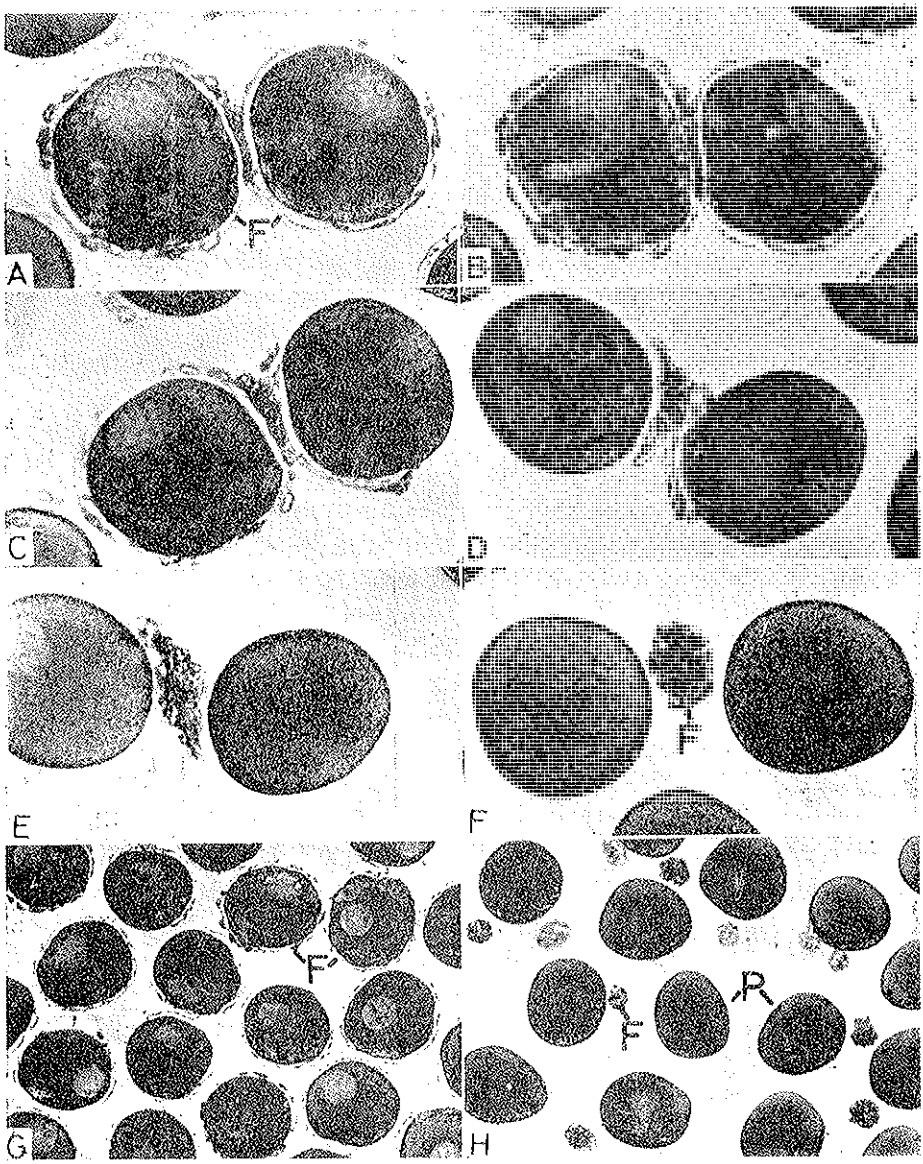

FIG. 9 — Effect of MIS in causing breakdown of follicles and germinal vesicles in isolated oocytes of *Asterina pectinifera*. Isolated eggs were transferred to sea water containing MIS. Breakdown of follicles begins with rupture of some weak region of follicular envelope. Distended follicular envelope shrinks with the dissolution of cementing substance between follicles and egg surface, finally forming small clumps. *F*, Follicular envelope; *P*, polar body. (A) 2 min; (B) 18 min; (G) 20 min; (D) 21.5 min; (E) 23 min; (F) 60 min; (G) control in sea water after 130 min; (H) 130 min after treatment with MIS.

and finally forms a small clump within 30 minutes and germinal vesicle breakdown is complete within the same period [17].

These facts suggested that the gonad-stimulating substance, GSS, of neural origin, which is a hormonal peptide, acts on the ovary as the target organ, and there produces a second active substance responsible for initiating meiosis and spawning. This active substance, MIS, seems to be an intermediate substance of hormone action. The site of production of MIS seems to be the ovarian wall, since GSS did not induce MIS-production in the absence of ovarian wall tissue.

Comparative studies showed that six Japanese starfishes produce MIS [17]. Figure 10 shows the results of separating the MIS of various starfishes on a Sephadex G-15 column. From this, it is clear that the substance appears in the same fractions at the same pH. Further, cross-experiments among several starfish genera showed that GSS is, to some extent, species-specific, while MIS appears to be non-specific, since although *Asterias* GSS fails to induce spawning of *Asterina*, *Asterias* MIS is very effective in inducing spawning of *Asterina*. *Asterina* ovary does not produce MIS when treated with *Asterias* GSS. Therefore, the failure of injected GSS of *Asterias* to induce spawning in *Asterina* [3] seems to be due to this failure of GSS of *Asterias* to cause the production of MIS in the ovary of *Asterina* [19]. It is thought that species specificity persists at the level of GSS, which is a polypeptide, but is no longer present at the small-molecular level of MIS.

Starfish testis tissue also produces MIS under the influence of GSS, although in smaller amount than ovary [19]. It has been shown by the gel-filtration study that testis MIS seems to be identical with ovarian MIS. The role of MIS in males is supposed to be to dissolve some substance between the spermatozoa and facilitate their release.

Further, it was found that production of MIS in the starfish ovary begins immediately after the addition of GSS [19]. The amount of MIS produced increases as the concentration of GSS

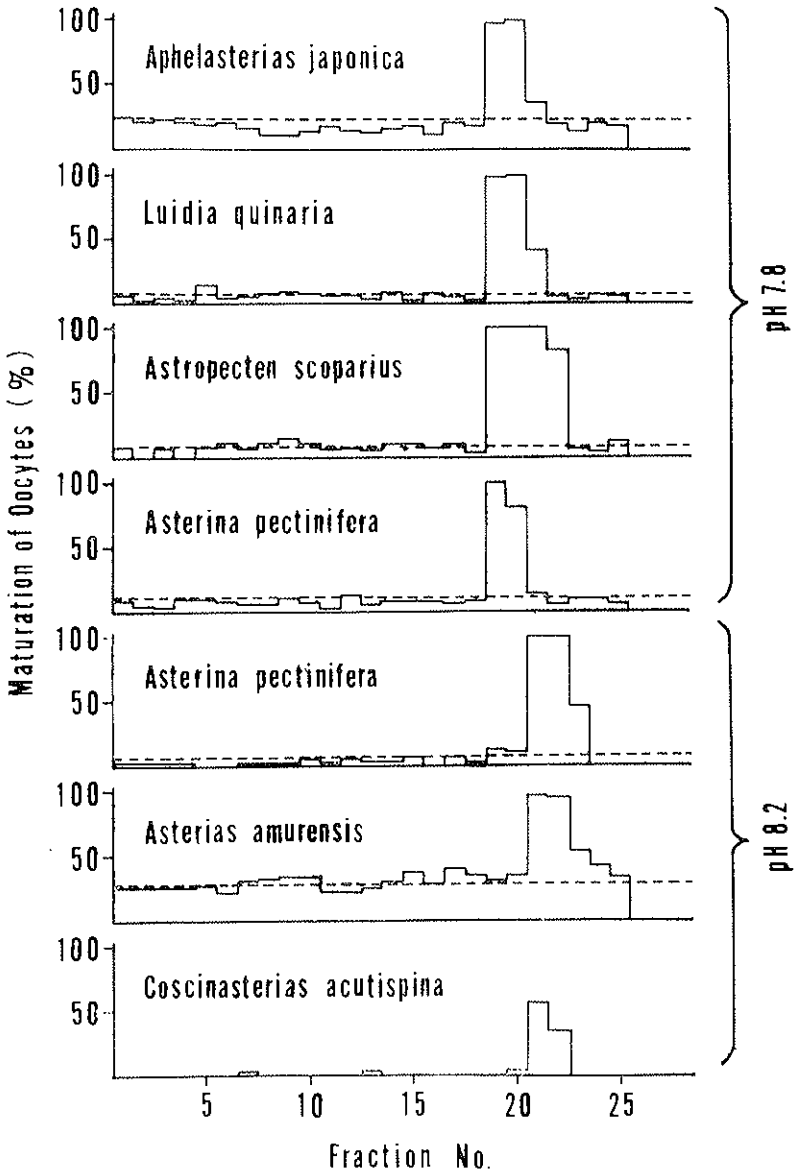

FIG. 10 — Meiosis-inducing activities of fractions obtained by gel-filtration of supernatant of mixture of ovary and nerve extracts in various starfishes. Essay was performed using oocytes of *Asterina pectinifera*. Broken lines represent percentages of maturation in sea water (control). Method of gel filtratrion was the same as that employed in the experiment shown in Fig. 8. Note that meiosis-inducing activity appears in the same fraction at the same pH.

is raised [19]. In addition, the longer the time of treatment with GSS, the greater is the amount of MIS produced, suggesting that production of MIS occurs continuously in the ovary under the influence of GSS [19]. With respect to the properties of MIS of *Asterina pectinifera*, it has been found that it is rather heat-stable, insoluble in ether, benzene and petroleum ether but soluble in 95% ethanol. Its activity is not destroyed by proteolytic enzymes such as trypsin, pepsin, chymotrypsin and pronase [19, 26].

Next, I should like to deal with the purification of MIS of *Asterias amurensis* carried out in our laboratory two years ago [27]. A preliminary experiment with about 600 *g* of ovaries of *Asterina pectinifera* showed that about 20 to 30 kg of ovaries would be necessary for obtaining 10 mg of purified sample. A total of 20 kg of fresh ovaries were therefore taken from about 3,000 females of *Asterias amurensis* and incubated in 100 liters of artificial sea water containing GSS derived from 20 g of dry nerve at 20°C for 6 hours. The incubation mixture was centifuged and stocked in a deep freezer. Thus about 14 liters of the concentrated crude MIS solution were obtained as starting material. The main procedures employed in the isolation of MIS are given in the flow diagram shown in Figure 11.

Since the starting material contained a large amount of inorganic salts derived from artificial sea water, a main effort was first made to decrease the salt content. This was done by precipitating calcium and magnesium with potassium phosphate. Sodium and potassium salts were precipitated by adding ethanol. MIS activity was assayed with isolated oocytes of *Asterina pectinifera*. After concentrating it to 1 liter, the sample was washed with chloroform and ether to remove lipids. The active water phase was further concentrated and gel-filtrated on a large Sephadex G-15 column equilibrated with 0.5M borate buffer (pH 8.5) containing 0.1M sodium chloride. Figure 12 shows the MIS activity and the elution curve expressed by optical density at 254 mμ obtained with an ultraviolet absorp-

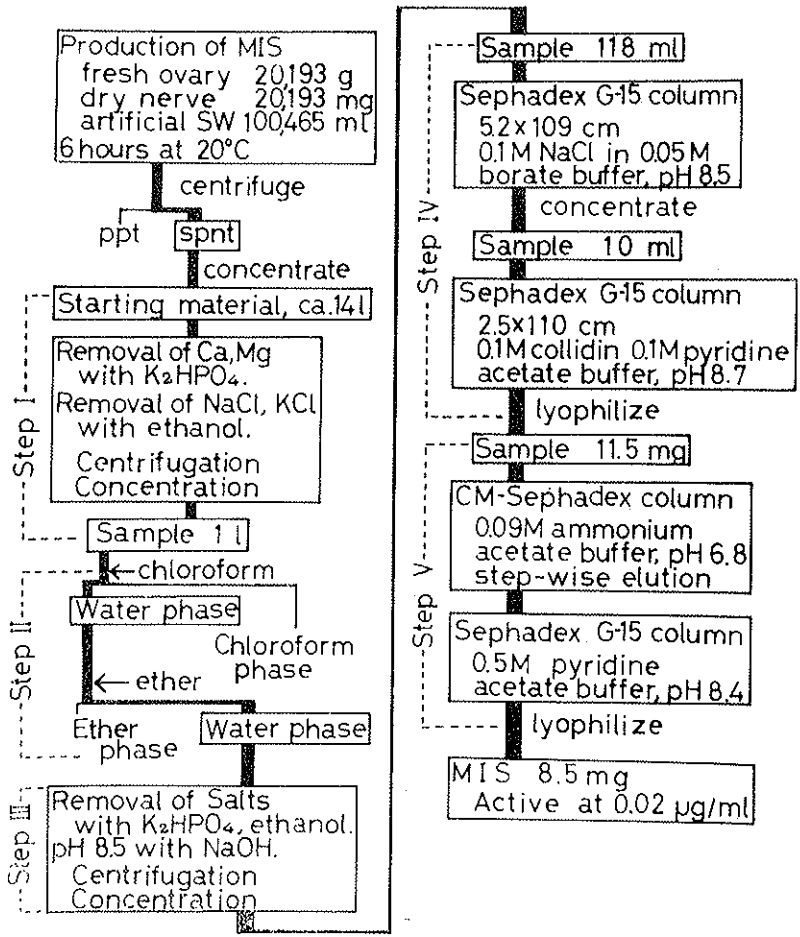

FIG. 11 — Main procedures employed in the isolation of meiosis-inducing substance (MIS) of *Asterias amurensis*.

tion meter. The active fractions were collected and concentrated. After adjusting the pH to about 8.7, the sample was further fractionated on a Sephadex G-15 column equilibrated with 0.1M γ-collidine, 0.1M pyridine acetate buffer (pH 8.7)

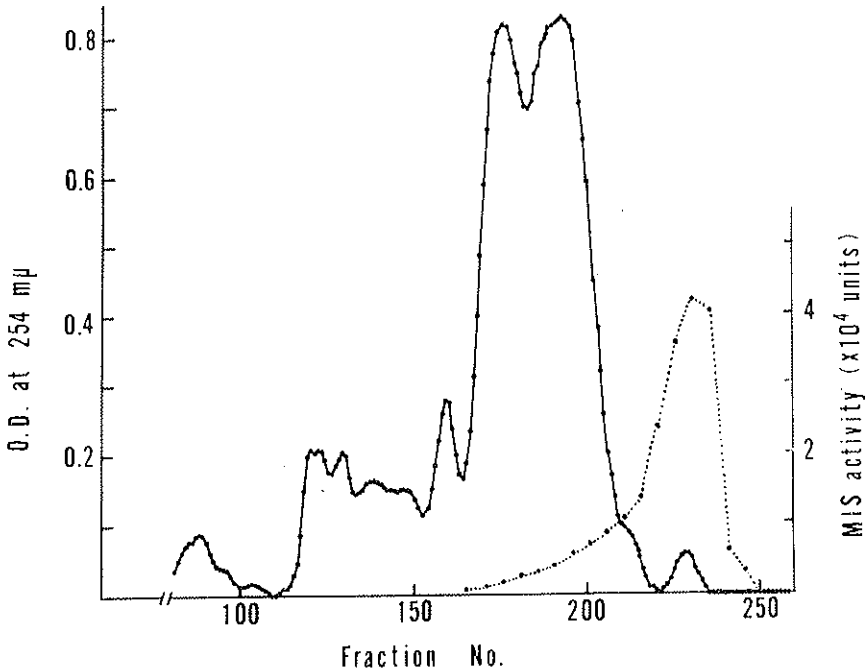

FIG. 12 — Gel filtration of *Asterias* crude MIS on a Sephadex G-15 column (5.2 x 109 cm) in 0.1M NaCl containing 0.05M borate buffer, pH 8.5 at 3° C. Sample size, 117 ml (pH 8.5); Flow rate 138 ml/hr; Fraction size, 15 ml. MIS activity is expressed in terms of MIS units. One MIS unit corresponds to the minimum dose contained in 1 ml of active sample required to induce 100% breakdown of germinal vesicles in *Asterina oocytes*. .——. Optical density; ·- - - - MIS activity.

(Fig. 13). The active fractions were pooled and lyophilized after washing with acetone. Thus, about 11.5 mg of purified sample was obtained. The sample was dissolved in 0.09M ammonium acetate buffer (pH 6.8) and fractionated on a CM-Sephadex C-25 column, equilibrated with the same buffer, by step-wise elution with 0.25M and 0.35M ammonium acetate buffer (pH 6.8). Figure 14 shows the elution curve expressed by the optical density at 261 mμ. At this wavelength the active fraction showed the maximal absorption. MIS was eluted as 0.25M

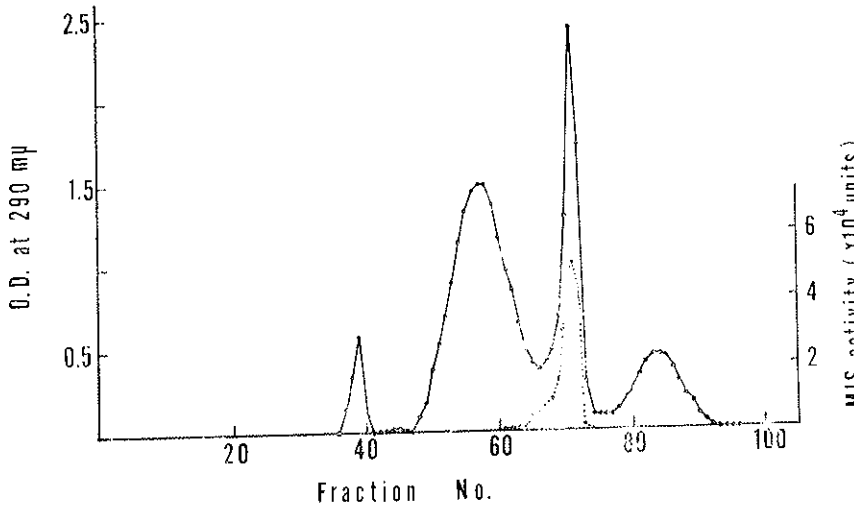

FIG. 13 — Further gel filtration of the MIS sample obtained from previous gel filtration (Fig. 12). Sephadex G-15 column (2.5 x 110 cm) in 0.1M γ-collidine-0.1M pyridine acetate buffer (pH 8.7) was used at 3°C. Sample size, 10 ml; Flow rate, 45 ml/hr. Elution curve was obtained by measuring the optical density of fractions at 290 mμ using the buffer as a blank.

ammonium acetate. The active fractions were concentrated and gel-filtrated on a Sephadex G-15 column in order to remove ammonium acetate. The active fractions were concentrated and lyophilized. Finally, about 8.5 mg of the purified sample of MIS was obtained. This sample was active at 0.02 μg/ml in inducing 100% breakdown of the germinal vesicle in *Asterina* oocytes. Fine needle-like crystals of MIS were obtained when acetone was added to the purified sample dissolved in a small amount of water.

The purified MIS was then investigated, to determine its chemical structure, in collaboration with Prof. NAKANISHI of the Department of Chemistry of Tohoku University [27]. Since purified MIS showed strong ultraviolet absorption at 261 mμ in ammonium acetate (pH 6.8), we suspected that it was

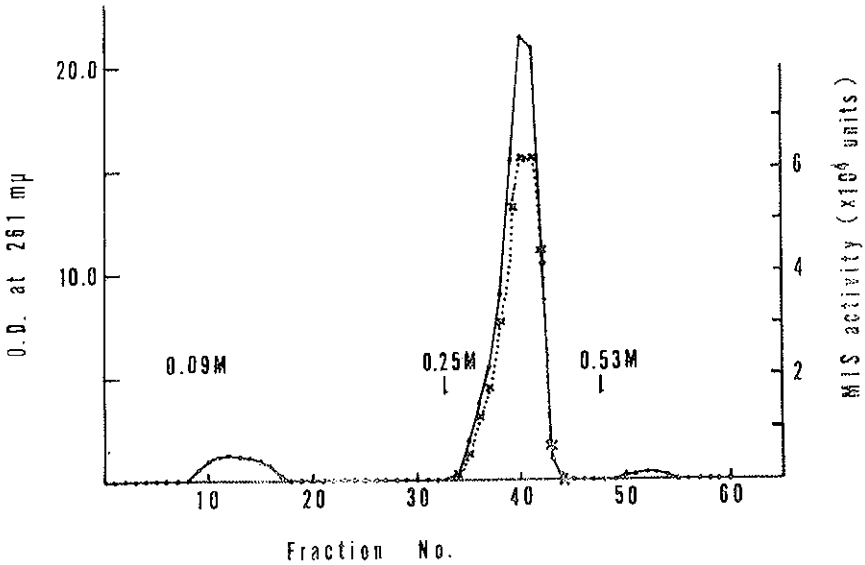

FIG. 14 — Chromatography of sample of MIS, purified by previous gel filtrations, on a CM-Sephadex C-25 column (1.5 × 25 cm) equilibrated with 0.09 M ammonium acetate buffer (pH 6.8). Sample size 11.5 mg; Fraction size 5 ml; Flow rate 18 ml/hour; Temperature 3°C.

one of the nucleic acid bases. Its ultraviolet absorption was therefore reinvestigated at different pHs. Table 2 shows the results of such determination of the wavelengths of maximal ultraviolet absorption of MIS. These values correspond well with those of 1-methyladenine, which had been synthesized according to the method of JONES and ROBINS [28]. Adenosine dissolved in demethylacetoamide was methylated with methyl-iodide, and the resulting 1-methyladenosine was then hydrolyzed to 1-methyladenine.

The infrared-absorption spectrum of the purified MIS was in good agreement with that of 1-methyladenine, as shown in Figure 15. In the high resolution mass spectrum of MIS, a peak assignable to a molecular ion was present at m/e 149.0725

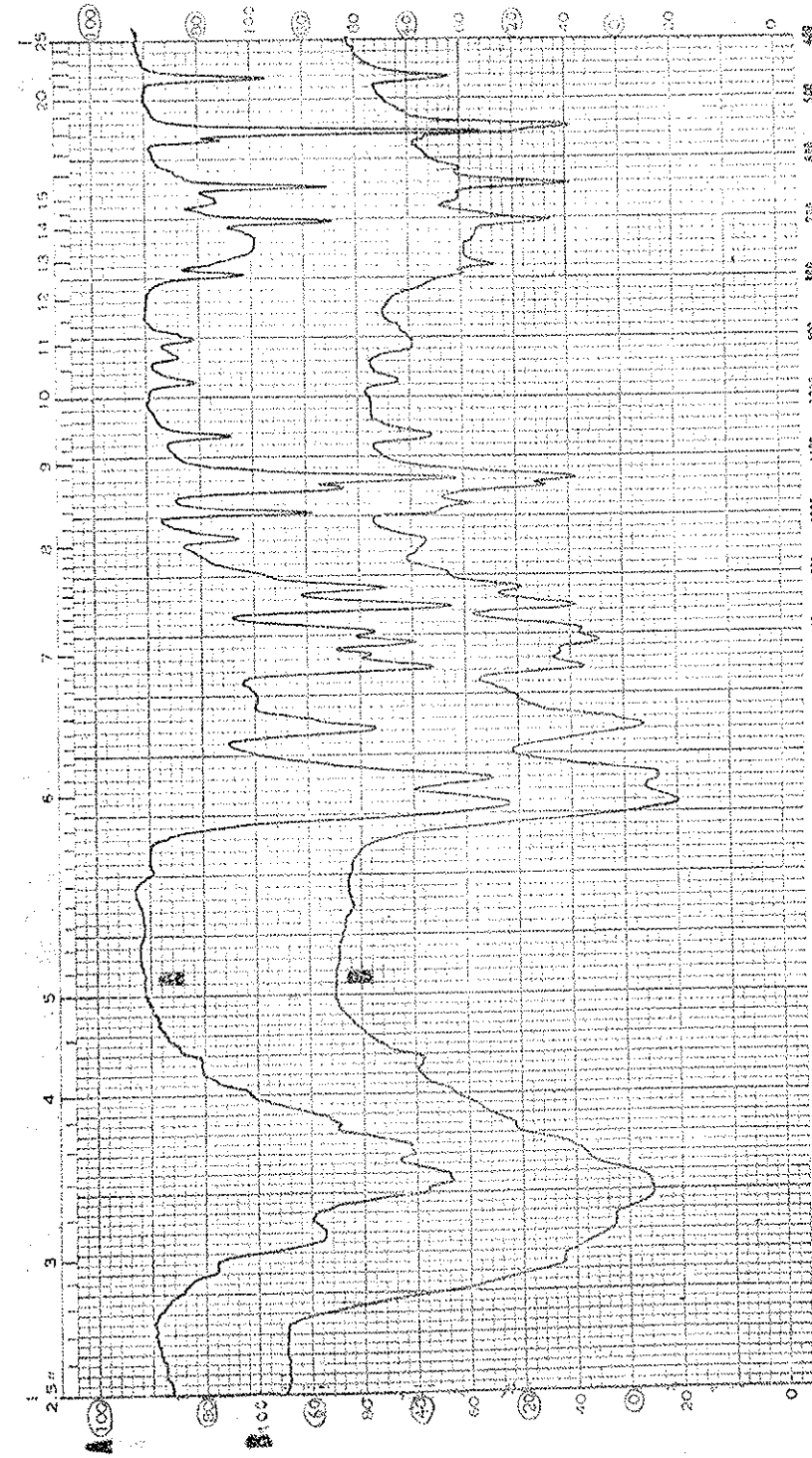

FIG. 15 — Infrared absorption spectra of 1-methyladenine (A) and MIS (B).

TABLE 2 — *Maximum absorption of ultraviolet of MIS and 1-methyladenine at various pHs.*

solvent	pH	MIS	1-methyladenine
H_2O	1.4	259	259
	7.1	265.5	264
	10.0	271	272
	12.3	271	271

($C_6H_7N_5$), suggesting that MIS is 1-methyladenine itself. There were also strong peaks at m/e 151.0806 (M+2), 150.0792 (M+1), 122.0593 (M-HCN) and 95.0452 (M-2CHN). The melting point of MIS was identical with that of 1-methyladenine (301° - 303°C). Furthermore, synthetic 1-methyladenine has a strong capacity to induce oocyte maturation, and its minimum effective dose was the same as that of purified MIS. From these results we can conclude that the meiosis-inducing substance of starfish is 1-methyladenine. 1-Methyladenine seems to represent a second messenger in hormone action, like 3′,5′-cyclic adenosinemonophosphate, which is the only substance known to play such a role among vertebrates [33].

Besides inducing oocyte maturation, 1-methyladenine also induces spawning [29]. Ovarian fragments of seven starfish species examined invariably spawn following treatment with 1-methyladenine (3 European, 2 American and 2 Japanese species). Injection of 1-methyladenine into the coelomic cavity also induces spawning *in vivo* [29]. These experiments using the synthetic substance clearly prove that MIS acts on the ovary in two ways: to induce meiosis in the oocytes and to bring about their release from the ovary.

Next, we examined the effect of various adenine derivatives on oocyte maturation and spawning, using isolated ovarian fragments of the American starfish, *Asterias forbesi,* in order

to determine the chemical structure required for such biological activities [32]. Among 19 derivatives tested, only three were effective when ovarian fragments were treated with the test solutions and observed. 1-Methyladenine was effective at 3×10^{-7}M, 1-ethyladenine at 10^{-6}M, and 1-methyladenosine at 10^{-5}M to 3×10^{-5}M. Sixteen other derivatives, including cyclic adenosinemonophosphate, had no such effect (Table 3). From these experiments it is thought that a short alkyl radical such as the methyl or ethyl radical in the N 1 site and an imino-radical combined with a double bond at the site of C6 of the purine nucleus are important for the induction of oocyte maturation and spawning (Fig. 16).

TABLE 3 — *Effect of Adenine Derivatives on Oocyte Maturation and Spawning in* Asterias forbesi

Compound	Activity	Compound	Activity
Adenine	—	Adenosine triphosphate	—
9-Methyladenine	—	6-Methylpurine	—
1-Methyladenine	+ + +	Inosine	—
1-Ethyladenine	+ +	5′-Inosinic acid	—
N_6-Benzyladenine	—	Guanine	—
Adenosine	—	1-Methylguanine	—
1-Methyladenosine	+		
Kinetin	—	5′-Guanylic acid	—
5′-Adenylic acid	—	Hypoxanthine	—
3′,5′-Adenylic acid (Cyclic AMP)	—		

Next, the site of action of 1-methyladenine within a single oocyte was studied in an attempt to determine whether the substance acts directly on the oocyte nucleus, or indirectly, by affecting the surface of the oocyte, which in turn exerts a meioisis-inducing effect on the nucleus. For this purpose, tbe

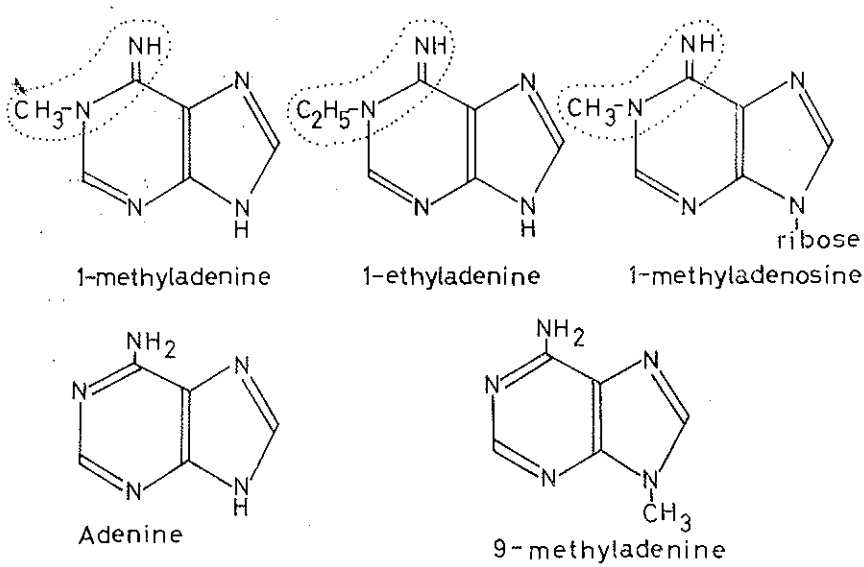

FIG. 16 — 1-Methyladenine and its related substances.

microinjection technique [34] was employed in collaboration with Dr. HIRAMOTO of University of Tokyo [35]. When $1.5 \times \times 10^{-5}$ µg of 1-methyladenine was injected into single *Asterina* oocytes, their germinal vesicles and follicles remained intact and meiosis failed to occur even after 2 hours, whereas external application of a one-tenth dose of 1-methyladenine invariably induced oocyte maturation and breakdown of follicles within 35 minutes. These experiments suggest that the site of action of 1-methyladenine is the surface of the oocyte. This leads to the hypothesis that 1-methyladenine acts from outside of the oocyte to induce the production of a third messenger in the oocyte cortex, which in turn, diffusing inward, induces the breakdown of the germinal vesicle [19]. This hipothetical substance may possibly be the true inducer of oocyte meiosis.

Next, I should like to discuss the biochemical mechanism of production of 1-methyladenine in the gonad under the influence of the gonad-stimulating hormonal peptide. Although the precise chemical pathway of 1-methyladenine production is not yet clear, we can assume that at least two steps of reactions are involved in its production. The first is the methylation of the purine nucleus of some compound such as adenosine, and the second is the breakdown of 1-methyladenosine or some related compound into 1-methyladenine and ribose. The fact that heating the incubation mixture of GSS and ovary arrests the production of 1-methyladenine [19] strongly suggests that some enzyme activity is involved in the reactions. Recently we have found that the activity of an enzyme, present in the gonadal wall of *Patiria miniuta, Asterina pectinifera* and *Asterias amurensis,* splits 1-methyladenosine into 1-methyladenine and ribose [36]. Furthermore, it was found that this enzyme is not an ordinary adenosine ribohydrolase [37] which splits adenosine into adenine and ribose, but a new specific enzyme called 1-methyladenosine ribohydrolase which does not act on adenosine. The enzyme can be obtained as precipitate by adding ammonium sulfate at 0.45 saturation to the supernatant of the ovarian wall homogenate, while adenosine ribohydrolase is precipitated at 0.75 saturation. Moreover, this enzyme acts, under physiological conditions, in the absence of the gonad-stimulating hormone. Isolated oocytes of *Asterina pectinifera* or *Patiria miniata* incubated with 1-methyladenosine undergo spawning, and oocytes within ligated ovaries treated in the same way lose their germinal vesicles and undergo maturation. This suggests that 1-methyladenosine ribohydrolase present in the ovary splits 1-methyladenosine into 1-methyladenine and ribose, and the resulting 1-methyladenine acts on the oocytes and ovary to bring about meiosis and spawning. Since this reaction does not require the presence of the hormone, the hormonal role in the production of 1-methyladenine seems

to be to activate the enzyme involved in the methylation of adenosine or some related substance.

Next, I should like to talk briefly about the action of 1-methyladenine on the reproductive behavior of whole animals. Figure 17 shows a female of the American starfish, *Asterias*

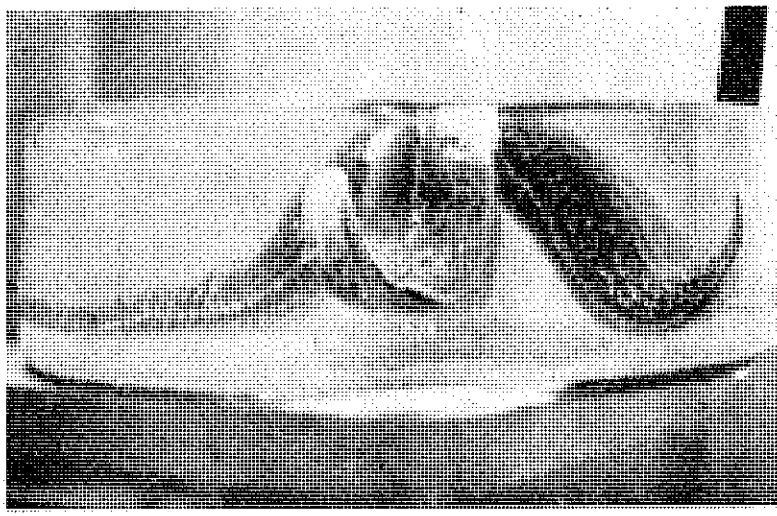

FIG. 17 — Spawning posture of *Asterias forbesi*.

forbesi, injected with 1-methyladenine. This animal takes a special posture. The central disk is raised high without locomotive movement. Instead, the arms undergo rhythmic waves of circular contraction from the tips to the proximal parts. These constrictional waves undoubtedly push the gonad to expel the eggs or sperm. Starfish undergoing natural spawning and starfish injected with the nerve extract (GSS) also behave in the very same way. It seems possible that 1-methyladenine may act on some higher center of the nervous system, since this movement seems to be highly coordinated.

It is well known that the females of some starfishes such as *Leptasterias hexactis* take a special posture by which they hold the fertilized eggs in a mass under the oral disk to brood their larvae through metamorphosis [38]. When these starfishes are injected with 1-methyladenine, they take this brooding posture as well as the spawning posture. Furthermore, they collect the eggs released and scattered on the bottom of the container and continue to brood them under the oral disk [40].

Although analysis of the mechanism of these phenomena induced by 1-methyladenine as well as the neural hormone has not yet been conducted, it seems possible that further studies of this kind will provide clues to the understanding of one of the most puzzling aspects of neuroendocrinology, that is, control of animal behavior by hormonal action. In addition, these observations suggest that this part of the reproductive process in starfishes is a chain reaction beginning with the release of a peptide hormone, GSS, from the nervous system. It is to be noted here that GSS as well as MIS has no effect in inducing spawning when applied from the outside of the body, that is, neither acts as a pheromone [41].

In conclusion, the mechanism of starfish spawning and oocyte maturation is summarized in Figure 18. The mechanism of release of GSS from the nervous system is still unknown. One may assume that it is caused by a rise in temperature or something like that, that is, some external stimulus [42, 43] (Fig. 18 *A*). It may be that an as yet unknown hormonal substance derived from the ripe gonads stimulates the nervous system to release GSS (Fig. 18 *A'*), although there is still no evidence to support such a view.

Whatever the stimulus for the release of GSS may be, once liberated from the nerves (Fig. 18 *B*) it reaches the coelomic cavity and there acts on the ovarian wall to produce a second substance, MIS (Fig. 18 *C*). MIS seems to diffuse through the ovarian tissue and dissolve the cementing substance between the cells, causing the rupture and stripping off of the follicles.

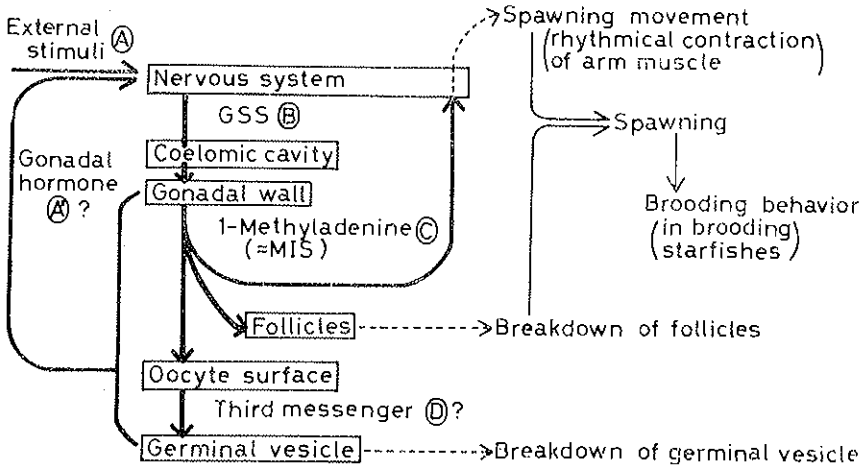

FIG. 18.— Possible mechanism of starfish spawning and oocyte maturation.

This in turn makes the individual oocytes within the ovary freely movable. Contraction of the ovarian wall is undoubtedly a motive force leading to discharge of the eggs, that is, spawning. This, however, cannot occur until MIS dissolves the intercellular cementing substance and frees the eggs from the follicles, since the mass of eggs firmly adhering to each other and to the gonadal wall by means of the follicles restricts its contracting capacity.

MIS produced in the ovary seems to act also on some higher center of the nervous system in a feedback fashion, resulting in a command by the nervous system to bring about a special spawning movement, that is, a rhythmical contraction of the arm muscles, which undoubtedly pushes the gonads and assists in expelling the eggs from the gonopores. Further, in some brooding starfishes, MIS also brings about the brooding behavior through the action of the nervous system. Besides inducing spawning, MIS acts on the surface of the oocytes to

stimulate the initiation of meiosis, perhaps by producing a third messenger substance in the egg cortex (Fig. 18 *D*).

These results suggest that spawning and oocyte maturation in starfishes are brought about as the results of a chain reaction which begins with release of GSS from the animal's nervous tissue. Undoubtedly a number of further experiments are required to elucidate the mechanism of each event involved in such a chain reaction, in order to obtain a better understanding of these problems of biological importance.

In closing, it is hoped that this demonstration of the role of GSS and 1-methyladenine in causing the breakdown of ovarian follicles in starfishes may help to explain the mechanism of spawning in lower vertebrates such as fishes, and of ovulation among higher vertebrates, and also that this new understanding of the mechanism of the action of 1-methyladenine in starfish meiosis may contribute to establishing a more general principle of the induction of oocyte maturation.

REFERENCES

[1] FOL H., *Sur le commencement de l'hénogénie chez divers animaux* « Arch. zool. exp. et gén. », *6*, 145-169, 1887.

[2] CHAET A.B. and R.A. MCCONNAUGHY, *Physiologic activity of nerve extracts*. « Biol. Bull. », *117*, 407-408, 1959.

[3] NOUMURA T. and H. KANATANI, *Induction of spawning by radial nerve extracts in some starfishes*. « J. Fac. Sci. Univ. Tokyo », Series IV, *9*, 397-402, 1962.

[4] KANATANI H. and T. NOUMURA, *On the nature of active principles responsible for gamete-shedding in the radial nerves of starfishes*. « J. Fac. Sci. Univ. Tokyo », Series IV, *9*, 403-416- 1962.

[5] CHAET A.B., *A mechanism for obtaining mature gametes from starfish*. « Biol. Bull. », *126*, 8-13, 1964.

[6] CHAET A.B., *The shedding substance activity of starfish nerves*. « Tex. Rep. Biol. Med. », *22*, 204, 1964.

[7] KANATANI H., *Spawning of starfish: action of gamete-shedding substance obtained from radial nerves*. « Science », *146*, 1177-1179, 1964.

[8] CHAET A.B., *Neurochemical control of gamete release in starfish*. « Biol. Bull », *130*, 43-58, 1966.

[9] KANATANI H. and M. OHGURI, *Mechanism of starfish spawning I. Distribution of active substance responsible for maturation of oocytes and shedding of gametes*. « Biol. Bull. », *131*, 104-114, 1966.

[10] CHAET A.B., *The gamete-shedding substance of starfishes: a physiological-biochemical study*. « Amer. Zool. », *6*, 263-271, 1966.

[11] KANATANI H., *Neural substance responsible for maturation of oocytes and shedding of gametes in starfish*. « Gunma Symposia on Endocrinology », *4*, 65-78, 1967.

[12] KANATANI H., *Mechanism of starfish spawning with special reference to gonad-stimulating substance (GSS) of nerve and meiosis-inducing substance (MIS) of gonad*. « Japan. J. Exptl. Morphol. », *21*, 61-78, 1967 (in Japanese).

[13] CHAET A.B., P.M. ANDREWS and R.H. SMITH, *The shedding substance of starfish nerve; its function and micro-assay.* « Fed. Proc. », *23*, 204, 1964.

[14] SCHUETZ A.W. and J.D. BIGGERS, *Effect of calcium on the structure and functional response of the starfish ovary to radial nerve factor.* « J. Exp. Zool. », *168*, 1-10, 1968.

[15] SCHUETZ A.W. and J.D. BIGGERS, *Regulation of germinal vesicle breakdown in starfish oocytes.* « Exp. Cell Res. », *46*, 624-628, 1967.

[16] KANATANI H. and H. SHIRAI, In vitro *production of meiosis-inducing substance by nerve extract in ovary of starfish.* « Nature », *216*, 284-286, 1967.

[17] KANATANI H., *Mechanism of starfish spawning: action of neural substance on the isolated ovary.* « Gen. Comp. Endocrinol. Suppl. », *2*, 582-589, 1969.

[18] KANATANI H. and H. SHIRAI, *Mechanism of starfish spawning II. Some aspects of action of a neural substance obtained from radial nerve.* « Biol. Bull. », *137*, 297-311, 1969.

[19] KANATANI H. and H. SHIRAI, *Mechanism of starfish spawning III. Properties and action of meiosis-inducing substance produced in gonad under influence of gonad-stimulating substance.* « Development, growth and differentation » in the presse, 1970.

[20] MECKLENBURG T.A. and A.B. CHAET, *Calcium and the shedding substance of* Patiria miniata. « Amer. Zool. », *4*, 414, 1964.

[21] SPACKMAN D.H., W.H. STEIN and S. MOORE, *Automatic recording apparatus for use in the chromatography of amino acids.* « Anal. Chem. », *30*, 1190-1206, 1958.

[22] YPHANTIS D.H., *Equilibrium ultracentrifugation of dilute solutions.* « Biochemistry », *3*, 297-313, 1964.

[23] KANATANI H., T. WATANABE and H. SHIRAI, *Unpublished.*

[24] BINDLER E., F.S. LABELLA and M. SANWAL, *Isolated nerve endings (neurosecretosomes) from the posterior pituitary — Partial separation of vasopressin and oxytocin and the isolation of microvesicles.* « J. Cell Biol. », *34*, 185-205, 1967.

[25] SCHUETZ A.W., *Induction of oocyte shedding and meiotic maturation in* Pisaster ochraceus: *kinetic aspects of radial nerve factor and ovarian factor induced changes.* « Biol. Bull. », *137*, 524-534, 1969.

[26] SCHUETZ A.W., *Chemical properties and physiological actions of a starfish radial nerve factor and ovarian factor.* « Gen. Com. Endocrinol. », *12*, 209-221, 1969.

[27] KANATANI H., H. SHIRAI, K. NAKANISHI and T. KUROKAWA, *Isolation and identification of meiosis inducing substance in starfish* Asterias amurensis. « Nature », *221*, 273-274, 1969.

[28] JONES J.W. and R.K. ROBINS, *Purine nucleosides III. Methylation studies of certain naturally occurring purine nucleosides.* « J. Amer. Chem. Soc. », *85*, 193-201, 1963.

[29] KANATANI H., *Induction of spawning and oocyte maturation by 1-methyladenine in starfishes.* « Exp. Cell Res. », *57*, 333-337, 1969.

[30] VIGLIA, A., E. DE ANGELIS *and* H. KANATANI, Unpublished.

[31] SHIRAI H., H. KANATANI, T. KUROKAWA and K. NAKANISHI, *Isolation and identification of meiosis- inducing substance (MIS) and activity of its related substances in starfish.* (in Japanese), « Japan J. Develop. Biol. », *23*, 18, 1969.

[32] KANATANI H., T. KUROKAWA and K. NAKANISHI, *Effects of various adenine derivatives on oocyte maturation and spawning in the starfish.* « Biol. Bull. », *137*, 384-285, 1969.

[33] SUTHERLAND E.W., I. ØYE and K.W. BUTCHER, *The action of epinephrine and the role of the adenyl cyclase system in hormone action.* « Recent Progress in Hormone Research », *21*, 632-646, 1965.

[34] HIRAMOTO Y., *Microinjection of the live spermatozoa into sea urchin eggs,* « Exp. Cell Res. », *27* 416-426, 1962.

[35] KANATANI H. and Y. HIRAMOTO, *Site of action of 1-methyladenine in inducing oocyte maturation in starfish.* « Exp. Cell Res. », In press.

[36] KANATANI H. and H. SHIRAI, Unpublished.

[37] WANG T.P., *Hydrolytic nucleosidases.* In « Methods in Enzymology », S.P. Colowick and N.O. Kaplan, Ed. (Academic Press, New York), Vol. II, 456-464, 1955.

[38] CHIA FU-SHIANG, *Brooding behavior of a six-rayed starfish,* Leptasterias hexactis. « Biol. Bull », *130*, 304-315, 1966.

[39] MOORE D. and J.D. BIGGERS, In vitro *studies on the shedding activity found in* Asterias *nerve extracts.* « Biol. Bull. », *127*, 381-382, 1964.

[40] KANATANI H., Unpublished.

[41] KANATANI H. and H. SHIRAI, *Does GSS or MIS act as a pheromone? Problems concerning the participation of a pheromone in starfish spawning.* « Zool. Mag. », *77*, 207-212, 1968.

[42] GALTSOFF P. and V. LOOSANOFF, *Natural history of* Asterias forbesi. « Bull. U. S. Bur. Fisheries », *49*. (Cited from Hyman, L.H. 1955. « The Invertebrates: Echinodermata », *4*. McGraw-Hill, New York).

[43] NEWMAN H.H., *An experimental analysis of asymmetry in the starfish.* « Biol. Bull. », *49*, 111-138, 1925.