YOLK GLYCOPROTEIN PRECURSOR TRANSLOCATION IN THE ECHINOID

By

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ABSTRACT

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The circulation of a plasma glycoprotein by the echinoid perivisceral coelomic fluid appears to be the main mechanism by which the yolk glycoprotein precursor is translocated into the ovary from its site of production outside of the ovary. For Dendraster excentricus, the plasma glycoprotein predominantly consists of a 200K dalton glycopeptide complexed into a particle with the sedimentation coefficient of 22S. Similarly, for Strongylocentrotus purpuratus, the plasma glycoprotein mainly consists of a 200K dalton glycoprotein complexed into a 24S particle. These characteristics of the plasma glycoprotein for both species match the properties of the yolk glycoprotein precursor previously described in the ovarian accessory cells. The majority of the yolk glycoprotein precursor in D. excentricus is synthesized by the coelomocytes of the perivisceral coelom. Through the

use of coelomocyte culture techniques, the coelomocytes of S. purpuratus were found to secrete the plasma glycoprotein. Therefore, after the glycoprotein is secreted into the plasma, it could be translocated into the ovary via the coelomic fluid circulation.

The effect of estrogen on protein synthesis in echinoid coelomocytes was also studied. While estrogen had no effect on total protein or yolk glycoprotein precursor synthesis, a novel protein was stimulated to be synthesized by the hormone. This estrogen-induced protein could be involved in regulating echinoid vitellogenesis. In the related member of the phylum Echinodermata, asteroids, estrogen regulation of translocation of yolk protein precursor from a temporary storage site outside of the ovary to the oocytes has been reported. Since the phylum Echinodermata is the most recent group to diverge from the chordate line of evolution, and since vertebrate chordates such as amphibians, birds, reptiles and fishes are known to have the synthesis of yolk precursor controlled by estrogen, the work presented here suggests that estrogen's role in vitellogenesis might have evolved prior to the divergence of the two phyla, Echinodermata and Chordata.

DEDICATION

To my wife Susan, and my daughter Melissa.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION AND LITERATURE SURVEY

The general introduction:

Vitellogenesis is the stage in oogenesis when the oocyte accumulates yolk. In many animals, both vertebrates (Clemens, 1974; Wallace, 1978; Wahli et al., 1981) and invertebrates (Wyatt and Pan, 1978; Engelman, 1980; Postlethwait and Jowett, 1980; Voogt et al., 1985), vitellogenesis involves, at least in part, the production of yolk precursors at sites outside of the ovary, and the precursors are translocated by the circulatory system to the ovary where they are specifically taken up by the oocytes. In insects, the yolk precursors are synthesized in the fat body under the control of the steroid hormone ecdysterone. The precursors, called vitellogenins, are secreted from the fat body, circulated through the hemolymph, and finally taken up by pinocytosis at the oocyte surface. In vertebrates, such as amphibians, reptiles, fishes and birds, the liver is the site of vitellogenin synthesis, and the steroid hormone estrogen regulates its synthesis by increasing transcription of the messenger RNA coding for vitellogenin. After the secretion from the liver, the vertebrate vitellogenins, which are high molecular weight glycolipophosphoproteins, are carried by the blood to the

oocyte surface where they are taken up by pinocytosis. While for mammals, very little is known about vitellogenesis, a recent report has found evidence for the accumulation of yolk proteins via pinocytosis in guinea pig oocytes (Verma and Ishikawa, 1984). The present study, along with the material reviewed in the subsequent parts of the introduction, suggests that synthesis and secretion of yolk protein precursors at sites outside of the ovary, translocation via the circulation, and specific uptake mechanisms at the oocyte surface appear to be common to the vitellogenic process in echinoids.

The echinoid yolk proteins:

Three general types of prominent yolk granule proteins have been identified: glycoproteins (Ii et al., 1978; Ozaki, 1980; Harrington and Easton, 1982; Kari and Rottman, 1985), lipoglycoproteins (Ii et al., 1978) and simple proteins without any apparent post-translational modifications (Ozaki, 1980). In addition to the abundant proteins which are stored in the yolk granules, several investigations revealed that enzymes which hydrolyze protein (Krischer and Chambers, 1976) and carbohydrate (Schuel et al., 1975) were enriched in the yolk granule fractions. The yolk glycoprotein is composed of high molecular weight glycopeptides in the range of 100-200K daltons which are complexed into a particle with a sedimentation coefficient in the range of 22-24S (Ii et al., 1978; Ozaki et al.,

1986). These glycoproteins are major protein components of many echinoid eggs (Malkin et al., 1965; Goldshmidt-Vasen, 1967; Infante and Nemer, 1968; Miki-Nomura, 1968; Bibring and Baxandall, 1969; Kondo, 1972; Kondo and Koshihara, 1972; Ruzdijic et al., 1973; Ii et al., 1978; Ozaki, 1980; Harrington and Easton, 1982; Giga and Ikai, 1985b; Kari and Rottman, 1985; Ozaki et al., 1986).

In echinoid eggs, the yolk is in membrane-bound granules (Afzelius, 1956). Nørrevang (1968) reviewed five general ways that yolk granules could form in animal eggs: 1) freely in the cytoplasm, 2) in the cisternae of the endoplasmic reticulum, 3) in preexisting vacuoles such as in pinocytotic vesicles or Golgi apparati, 4) inside mitochondria and 5) in a complex manner involving two or more of the first four ways. A modern view of glycoprotein production has established that glycoprotein biosynthesis is closely associated with membrane systems, particularly the rough endoplasmic reticulum and the Golgi apparatus (Schachter, 1984). Since glycoproteins are a prominent constituent of echinoid yolk, these membrane systems would be prerequisite for yolk synthesis within the echinoid egg. Afzelius (1956) suggested that in echinoids, yolk granules form in regions of the egg where the Golgi apparati are located. Other electron microscopic studies have shown the presence of both the rough endoploasmic reticulum and the Golgi apparatus in echinoid eggs (Verhey and Moyer, 1967b; Millonig et al., 1968), but their role in yolk synthesis is

not clear. While specific proteins such as tubulin and histones have been shown to be synthesized by oocytes isolated from the ovary, (Cognetti et al., 1967a, 1967b; Ruderman and Schmidt, 1981), the synthesis of the major yolk glycoprotein could not be detected within the oocytes (Harrington, 1980).

Vitellogenesis in relation to the echinoid ovarian cycle:

Echinoids commonly undergo a yearly, synchronous, reproductive cycle which culminates in a mass spawning by the individual males and females (Boolootian, 1966). In the ovary, the cycle consists of defined stages of oocyte development: (1) recovering spent, which is after the spawning period when there are few primary oocytes; (2) growing, when the primary oocytes become more abundant and begin to increase in size; (3) premature, when large oocytes are present; (4) mature, when the full-grown oocytes are ready for spawning; and (5) spent, when spawning is completed (Fuji, 1960). The overall mass of the ovary also changes, being smallest after spawning and largest when the oocytes are full-grown. However, as the ovary begins to increase in size, its growth is not due to the growth of the primary oocytes, but due to the increase in the number and content of the ovarian accessory cells (Fuji, 1960, Gonor, 1973). It has been hypothesized that the accessory cells accumulate materials destined to be transfered into the growing oocytes (Miller and Smith, 1931; Liebman, 1947;

Holland and Giese, 1965; Chatlynne, 1969; Ozaki et al., 1986). Therefore, prior to yolk accumulation within the oocytes, yolk precursors might first need to accumulate in the ovarian accessory cells which would then be transferred into the oocytes and deposited as yolk.

Immers (1960) reported that Periodic Acid-Schiff reagent (PAS), which reacts with carbohydrate, stained the yolk granules of a number of different echinoid species. Furthermore, studies on the whole ovary revealed that the PAS-staining yolk did not accumulate in the oocytes until the terminal stage of the oocyte growth (Cowden, 1962; Esper, 1965). Therefore, PAS staining could be used to study the transfer of glycoprotein from the accessory cells to the oocytes (Chatlynne, 1969; Ozaki et al., 1986). Before the oocytes begin to accumulate the yolk (previtellogenic stage), the oocytes are small and devoid of PAS-stained material, while the accessory cells stain heavily with PAS. Then with the onset of vitellogenesis in the later stages of oocyte growth, the oocytes become strongly stained with PAS. Concomitant with vitellogenesis, the mass of accessory cells which had packed much of the ovary now regresses. Through electron microscopic examination of the ovaries, evidence for the transfer of nutrient material from the accessory cells to the oocytes has been obtained as pinocytotic pits and vesicles are present at the oocyte surface (Takashima and Takashima, 1966; Tsukahara, 1969; Tsukahara and Sugiyama, 1969; Geary,

1978).

A yolk glycoprotein precursor in the accessory cell has been recently identified in two species of echinoids (Ozaki et al., 1986). The precursor in the accessory cells is a fast-sedimenting particle, which has a sedimentation coefficient of 22S in Dendraster excentricus and 24S Strongylocentrotus purpuratus. In D. excentricus, the precursor particle in the accessory cell has similar physical properties to the yolk glycoprotein of the egg, including the molecular weights of the constitiuent glycopeptides, immunological determinants, high mannose content, and amino acid composition. In S. purpuratus, the yolk granules of the egg were previously reported to contain a glycoprotein particle of about 24S (Goldschmidt-Vasen, 1967). In addition the accessory cell precursor particle was similar to the egg yolk glycoprotein in that they both contained predominantly a 200K-dalton glycopeptide, and the proteins appeared to have the high-mannose type glycosylation (Ozaki et al., 1986).

While the accessory cells appear to temporarily store the yolk precursor, it appears that these cells may not be capable of producing the large amounts of yolk precursor needed during oogenesis. Based on autoradiographical experiments examining RNA and protein synthesis in the ovaries, the accessory cells have very low synthetic activities (Immers, 1961; Verhey and Moyer, 1967a). Therefore, the yolk glycoprotein precursor is likely to be

synthesized outside of the ovary. The evidence for the extra-ovarian origin of the yolk glycoprotein precursor will be discussed in the following section.

Role of echinoid coelomic fluid in vitellogenesis:

Nutrient transport in the echinoid is carried out primarily by the perivisceral coelomic fluid (Farmanfarmaian and Phillips, 1962; Ferguson, 1982; Walker, 1982). Based on radioactive tracer analysis, nutrients which are digested in the gut quickly pass into the perivisceral coelomic fluid and are transferred to the various tissues (Farmanfarmaian and Phillips, 1962). In addition, hemal canals interconnect the intestines and the gonads (Hyman, 1955), and could also play a role in nutrient transport. The perivisceral coelomic fluid and the fluid of the hemal canals are continuous with each other due to connections at the axial organ (Millott and Vevers, 1964). It has been suggested that the axial organ pumps fluid through the hemal canals (Boolootian and Campbell, 1964, 1966), but extensive flow of nutrients through the hemal system into the ovary can not be demonstarated. Cutting the hemal connections to the ovaries does not interfere with accumulation in the ovary of radioactive nutrients which the animal injested (Farmanfarmaian and Phillips, 1962). Therefore, the hemal system does not appear to be extensively involved in nutrient transport as does the circulation of the perivisceral coelomic fluid.

Within the perivisceral coelom, there is a cellular (the coelomocytes) and a non-cellular (the plasma) component (Endean, 1966). Either component could carry nutrients destined for the developing oocytes. The coelomocytes are free-wandering cells which are found throughout the bodies of both sexes, in the gonads, intestines, perivisceral coelom and hemal system (Kindred, 1926; Hyman, 1955; Boolootian, 1966; Endean, 1966). The coelomocytes have a variety of morphologies and functions. They have been shown to be involved in defense mechanisms including clotting and repair in response to injury (Endean, 1966; Höbaus, 1980), bactericidal activities (Johnson and Chapman, 1970; Wardlaw and Unkles, 1978; Yui and Bayne, 1983), phagocytosis of foreign material (Bertheussen and Selgelid, 1978), and they contain factors which are similar to some of the components of the complement cascade in the vertebrate immune system (Bertheussen, 1983). They have even been found outside the body where they have been shown to secrete digestive enzymes so that the epidermis can absorb nutrients directly from the external environment (Pequignat, 1966; 1967). The coelomocytes also function in transport of respiratory gases and waste-products for excretion (Endean, 1966; Farmanfarmaian, 1966; Binyon, 1972). A specific type of coelomocyte, the trephocyte, has long been suspected to function in supplying nutrients to the developing gonads (Liebman, 1947, 1950). Liebman considered that the accessory cells of the ovaries were trephocytes in origin,

and that materials located in the trephocytes were transferred directly into the growing oocytes due to the close spatial relationship between the two cell types. Egg lipids have also been reported to be translocated into the ovary by the coelomocytes during the reproductive build up of the gonad (Allen, 1974). Recently, a glycoprotein which could be a yolk precursor has been found in coelomocytes from the perivisceral coelom of D. excentricus (Ozaki and Harrington, 1986). It is a 200K-dalton glycopeptide which is synthesized by these cells at a very high rate. Not only is the molecular weight of this glycopeptide similar to the major yolk glycopepetide of the egg, but it also possesses some of the immunological determinants of the yolk glycoprotein.

How is the precursor glycoprotein in the coelomocytes of the perivisceral coelom translocated into the ovary? It could be carried directly by cells migrating from the coelom to the ovary. Another possibility is that the coelomic plasma is involved in precursor transport; a high molecular weight glycoprotein with properties similar to the yolk glycoprotein of the egg has been found to be the major component of the plasma (Harrington, 1980; Harrington and Easton, 1982; Ozaki and Harrington, 1986). At present , no conclusions can be made as to the role of the plasma and coelomocyte glycoproteins in the mechanisms of precursor translocation into the ovary. In the present research, an investigation was be carried out to further characterize the

plasma glycoprotein, to determine the origin of this glycoprotein, and to identify its possible route of translocation into the ovary.

The hormonal regulation of echinoderm vitellogenesis:

For echinoids, which belong to the phylum Echinodermata, there is no information at present on how vitellogenesis is regulated. For asteroids, another group in the phylum, the hormonal regulation of vitellogenesis is being elucidated. The pyloric caecum appears to be the site of yolk precursor synthesis and temporary storage (Voogt et al., 1985). The yolk precursor is a lipoglycoprotein composed of at least three subunits (Broertjes et al., 1984a and b). During the reproductive cycle, the pyloric caecum first enlarges and stores yolk precursors. Then, as the pyloric caecum decreases in size, the ovary simultaneously undergoes a size increase as the oocytes grow and accumulate yolk. The hemal canals which interconnect the pyloric caecum and the gonad have been found to be the route for the yolk precursor transfer (Beijnink et al., 1984). There is a great deal of evidence for the involvement of the steroid hormone estrogen. The levels of estrogen in the asteroid fluctuate in relation to the reproductive cycle (Schoenmakers and Dieleman, 1981), and the treatment of an asteroid in vivo (Schoenmakers et al., 1981; Takahashi, 1982) or ovaries in vitro (Takahashi and Kanatani, 1981) with estrogen seems to stimulate vitellogenesis. The

pyloric caecum where the yolk precursor is temporarily stored is an estrogen target tissue since it contains estrogen receptors (De Waal et al., 1982). From this evidence, the hypothesis has been advanced that in asteroids, the hormone regulates the mobilization of stored yolk precursor and its translocation into the oocyte (Voogt and Dieleman, 1984; Voogt et al, 1985).

In vertebrates such as amphibians, birds, fishes and reptiles, estrogen regulates yolk protein precursor synthesis by stimulating the transcription of yolk protein precursor messenger RNA (Tata, 1976; Gruber et al., 1976; Chen, 1983; Bast and Gibson, 1985). At present it is not known what effect the hormone might have on precursor synthesis in asteroids. Besides asteroids which contain both 17β-estradiol and estrone (Schoenmakers and Dieleman, 1981), estrogenic subastances have also been found in echinoids (Donahue and Jennings, 1937; Donahue, 1940; Hagerman et al., 1957; Botticelli et al., 1961), but their physiological roles are not known. Since echinoid coelomocytes synthesize the yolk glycoprotein precursor, the coelomocytes can be used to test in vitro whether estrogen stimulates yolk protein precursor synthesis in echinoderms as in vertebrates. 17β-estradiol will be used in this experiment at a does slightly less than the amounts used to obtain biological responses in asteroids. The phylum Echinodermata was the most recent group to have diverged from the chordate line of evolution (Jefferies, 1979), and

the role of estrogen in vitellogenesis could have arisen before the divergence of the echinoderms and the chordates. It is of interest to see if the regulation of vitellogenesis by estrogen was conserved or changed following this divergence of the echinoderms and the chordates.

STATEMENT OF THE PROBLEM

Recent investigations on the analysis of echinoid yolk glycoproteins, reviewed in the previous section, have shown that proteins similar to the yolk proteins are located in places outside of the oocyte, such as in ovarian accessory cells, coelomocytes and coelomic plasma. These proteins outside of the oocyte appear to be precursors to the yolk, but the pathway of accumulation, either temporal or spatial, is not fully understood at the present time. While it is thought that pinocytosis is involved in the uptake of yolk glycoprotein precursor by the oocyte from the ovarian accessory cells during vitellogenesis, it is not known by what mechanism precursor glycoprotein is transferred from a known site of its synthesis, the coelomocytes in the perivisceral coelom, to the ovary during the previtellogenic stage. It is possible that the precursor being transferred into the ovary is carried from the coelom to the ovary directly by coelomocyte migration, or by fluid circulation after the precursor is secreted from the coelomocytes. This study will focus on the role of the coelomic fluid in yolk glycoprotein precursor transport.

The first problem to be examined is to see whether the plasma of the perivisceral coelomic fluid contains a

glycoprotein particle similar to the yolk glycoprotein particles previously described in the ovary. Since the yolk glycopeptides of the eggs and ovarian accessory cells of both D. excentricus and S. purpuratus are complexed in fast-sedimenting particles, and since the major plasma glycopeptide is similar to the major yolk glycopeptide of the egg in S. purpuratus, sucrose density gradient centrifugation and SDS-polyacrylamide gel electrophoresis will be used to determine if the plasma glycopeptides are complexed in a fast-sedimenting particle.

The second problem to be addressed is the origin of the plasma yolk glycoprotein precursor. For D. excentricus, it is known that the coelomocytes of the perivisceral coelom actively synthesize the yolk glycoprotein precursor, thus the plasma glycoprotein could be a coelomocyte secretory product. A coelomocyte culture technique will be developed and will be used to test if yolk glycoprotein precursor accumulates in the culture medium. The conditioned medium will be separated from the cells, and its contents then analyzed by SDS-polyacrylamide gel electrophoresis. If the cells secrete the plasma glycoprotein, then its transport into the ovary is likely to occur via fluid circulation. In addition, since coelomocytes are ubiquitous in the echinoid body, the relative contributions of coelomocytes to total yolk glycoprotein precursor production will be assessed in the three major body compartments, the perivisceral coelom, the ovary and the intestines. This will be done by

radioactively labeling the cells and tissues in vitro, and then analyzing the labeled proteins by SDS-polyacrylamide gel electrophoresis and fluorography.

The last problem to be studied in this dissertation is whether the synthesis of the yolk glycoprotein precursor in echinoids is regulated by estrogen as it is in many other organisms. This will be tested by adding estrogen to coelomocyte cultures. At the end of the hormone exposure, the cells will be labeled briefly with radioactive amino acids. Then, scintillation counting techniques, SDS-polyacrylamide gel electrophoresis and fluorography will be used to compare the estrogen treated and control cells to learn if the hormone induces changes in protein synthetic rates or protein synthetic patterns.

MATERIALS AND METHODS

Animals:

Dendraster excentricus were collected at monthly intervals from Alki Beach, Seattle, Washington, shipped to Michigan State University and held in salt water aquaria for short periods until used. These echinoids were mainly used for the whole-animal experiments and those experiments which required the usage of animals at a particular time of the reproductive cycle. A single collection of D. excentricus was also made off the San Juan Islands in Puget Sound, Washington (August, 1982), and were maintained in a salt aquarium for up to a year. They were fed Tetramin (Tetrawerke, Melle, West Germany) fish food, and sexed by a gonadal biopsy which was done with a needle and syringe. The females were used in the hormone experiments, and it was found that these females produced mature eggs in May 1983, which is about the same time as would be expected if the animals had been in their native habitat (Strathmann, 1974). Strongylocentrotus purpuratus were obtained from Pacific Bio-Marine Co. (Venice, California) and kept in salt water aquaria for short periods. They were obtained at two different times, in October when the females were previtellogenic, and in February, when the eggs were ready

for spawning. The asteroid Pisaster giganteus was also obtained from Pacific Bio-Marine Co., at the time when the females had mature eggs.

Collection of Coelomic Fluid Components:

Coelomic fluid from D. excentricus was withdrawn by a needle and syringe inserted through the peristomial membrane into the perivisceral coelom. The syringe used for withdrawal contained 9 parts Herbst's artificial sea water, and 1 part 0.2 M EDTA/NaOH pH 7.5 of a volume equal to the amount of coelomic fluid to be withdrawn. The fluid was immediately centrifuged at 200 x g for 5 min at 4°C. The pelleted cells (coelomocytes) were resuspended in sea water medium and used for various experiments. The supernatant was recentrifuged at 3000 x g for 10 min at 4°C, and supernatant from this second centrifugation was used as the plasma fraction.

The coelomic fluid from S. purpuratus was obtained by cutting the peristomial membrane and pouring the coelomic fluid into an ice cold beaker and immediately diluting with an equal volume of the withdrawal fluid described above. The mixed fluid was then passed through a 135 μm nylon mesh to remove any debris. The filtrate was centrifuged as above to obtain the coelomocyte and plasma fractions.

Prior to sucrose gradient centrifugation or SDS-polyacrylamide gel electrophoresis of plasma proteins, the plasma fractions were either dialyzed and concentrated

by the Micro-ProDiCon apparatus with a ProDiMem PA-15 membrane (Bio-Molecular Dynamics, Beaverton, Oregon) which has a molecular weight cutoff of 15K daltons, or enriched for glycoproteins by concanavalin A-chromatography as described below. Total protein content of the plasma was determined by the Lowry method (Lowry et al., 1951) after acid precipitation.

Collection of Tissues:

The tissues of the echinoids were excised following the removal of the coelomic fluid. For D. excentricus, a pair of needle-nose pliers was used to grasp the test at the oral opening, and pieces of the test were carefully chipped away to expose the internal organs. Starting just aboral to the latern apparatus, the foregut and midgut could be removed in one piece by cutting the mesenteries. The hindgut was dissected out by cutting away the gonad which it passed through, and then forceps were used to pull off the adhering gonadal pieces. The ovary mass on the side free of the hindgut could be carefully scooped out with a spatula. Several pieces of the intestinal tract were also collected for study: the foregut (the first coil leading out of the lantern apparatus), the midgut (the short segment after the gut coil passes over the rectum), and the hindgut (the portion embedded in the gonad). The animals being dissected and the tissue pieces were kept on ice until the start of the experiments.

Since the S. purpuratus were already opened up to collect the coelomic fluid, the ovaries could be quickly obtained. Each of the five separate ovaries were scooped out intact with a spatula.

Histology:

Samples of tissue pieces were fixed overnight in Bouin's solution, then put through a series of ethanol dehydrations to prepare the samples for histological examination. The samples were embedded in parafin and cut in 8 µm slices. The sections were attached to slides with albumen, and stained with Harris' hematoxylin and eosin. To determine the sex of the animals, squashes of freshly obtained gonad pieces were checked under a light microscope, and additionally by examination of tissue samples after preparation for histology.

Culture of Coelomocytes:

Coelomocytes were obtained from the two echinoids as described above. These cells were cultured for up to 48 h; they remained viable as judged by light microscopy and their ability to incorporate radioactively labeled amino acids into protein. The culture medium consisted of 9 parts artificial sea water buffered with 10 mM HEPES/sodium carbonate pH 7.5, and 1 part distilled water with a final concentration of 0.25 mg/ml streptomycin sulfate, and 150 U/ml of penicillin G. For the hormone experiments,

17β -estradiol (Sigma, St. Louis, Missouri) or progesterone (Parke, Davis and Co., Detroit, Michigan) was dissolved in 95% ethanol and added to the medium at a final concentration of 10^{-6} M estradiol or progesterone, and 0.05% ethanol. Control cultures contained 0.05% ethanol. Some cultures also contained 1 mg/ml proteose peptone (Difco, Detroit, Michigan) as a nutrient source. Cells were cultured in 20 ml of medium in 25 ml spinner culture flasks (Wheaton, Millville, New Jersey) at 15^{o}C at concentrations of 0.3-1.0x10^{5} cells per ml.

After various periods of time, the spinner culture fluid was collected and centrifuged at low speed to obtain the cell and cell-free fractions. The pelleted cells were labeled with radioactive amino acids as described below. The cell-free fraction of the spinner culture fluid was recentrifuged to be sure all cells were removed. This supernatant material was the conditioned medium which was analyzed for secretory products. Prior to the analysis of the conditioned medium, it was either concentrated in the Micro-ProDiCon apparatus or enriched for glycoproteins by concanavalin A-chromatogaphy. The proteins in these preparations were dissolved in 2X sample buffer (Laemmli, 1970) for analysis by SDS-polyacrylamide gel electrophoresis.

In Vitro Labeling of Proteins Synthesized by Cells and Tissues:

Coelomocytes (freshly collected or after spinner culture), and tissue pieces of ovary and digestive tract were incubated in artificial sea water containing 0.25 mg/ml streptomycin sulfate and radioactively labeled amino acids, either L-[4,5-^{3}H]-leucine or L-[^{14}C]-valine, for 1 h. The cells and tissues were then washed with sea water containing an excess of the nonradioactive amino acid, and homogenized in boiling sample buffer (Laemmli, 1970). The samples were subjected to electrophoresis and analyzed by fluorography as described below. Aliquots of coelomocyte lysates (hormone treated and control) were quantitated for protein (Lowry et al., 1951), and analyzed to determine the relative rate of amino acid incorporation into protein. For the latter determination, cell lysates were applied to thin-layer chromatographic strips (Gelman ITLC-SG, Ann Arbor, Michigan), and the chromatograms were developed using the solvent 10% acetic acid, 10% trichloroacetic acid and 30% methanol (Instructions for New England Nuclear Translation Systems, Method II, May 1981, Boston, Massachusetts) to separate free amino acids from protein. The developed chromatographic strips were cut into pieces, digested in Protosol (NEN), and then counted in Econofluor (NEN). After correcting the data for counting efficiencies by a channels ratio method (Packard, 1965), the relative rates of amino acid incorporation were computed as the amount of protein

counts divided by the total number of counts. The final results were expressed as ratios of relative rate of amino acid incorporation in the hormone treated cells over the rate in the control cells.

Sucrose Density Gradient Centrifugation:

Linear 5 to 20% sucrose density gradients were constructed with buffer consisting of 0.01 M KCl, 0.015 M $MgSO_4$, and 0.015 M Tris/HCl pH 7.5. Samples of protein which were layered on top of the gradients were obtained in the following ways: 1) previtellogenic ovaries or mature eggs were homogenized in 10 volumes of the same buffer, the post-ribosomal supernatants of the homogenates prepared by centrifugation at 226,000 x g for 45 min, and protein to be loaded on the gradients sedimented from this supernatant by an additional centrifugation for 2-3 h; and 2) plasma proteins were dialyzed and concentrated in the Micro-ProDiCon apparatus against the same buffer. The loaded gradients were centrifuged at 160,000 x g for 5-6 h at $4^{o}C$. The gradients were fractionated from the bottoms of the tubes and the amount of protein in the fractions was determined by the Lowry method (Lowry et al., 1951). Aliquots of peak fractions were pooled and dissolved in 2X sample buffer (Laemmli, 1970) for analysis by electrophoresis. The sedimentation coefficient was calculated using porcine thyroglobulin (Sigma) as a standard of 19.4S.

Concanavalin A-chromatography:

A column (9 mm in diameter and 22 mm in length) of concanavalin A-Sepharose (Sigma) was equilibrated with starting buffer consisting of 0.5 M NaCl and 20 mM Tris/HCl pH 7.5. Samples for chromatography were as follows: 1) post-ribosomal supernatants of previtellogenic ovaries homogenized in starting buffer; and 2) plasma diluted with an equal volume of starting buffer (some plasma samples were concentrated against starting buffer in the Micro-ProDiCon apparatus prior to the chromatography, but this was not essential since there was a high affinity between the lectin and the ligand). After loading, the unbound material was washed through with 5 bed volumes of starting buffer, and then the bound glycoprotein was eluted with starting buffer with 0.5 M α-methyl-D-mannoside (Sigma) added. The amount of protein in the column fractions was determined by the Lowry method (Lowry et al., 1951). Peak fractions of the bound or unbound material were pooled for analysis.

Electrophoresis and Fluorography:

The Laemmli (1970) procedure for SDS-polyacrylamide gel electrophoresis was used to resolve proteins, and the gels were stained with Coomassie Brilliant Blue R (Sigma) for proteins in general, or Periodic Acid-Schiff reagent (ORTEC, 1970) for glycoproteins. Liquid samples to be analyzed by electrophoresis were mixed with an equal volume of two-fold

concentrated sample buffer (Laemmli, 1970) and then boiled. Cells and tissues were homogenized in boiling sample buffer. Gels containing radioactively labeled proteins were soaked in the fluor sodium salicylate, dried and exposed to X-Omat AR X-ray film (Kodak, Rochester, New York) at -80^{o}C (Chamberlain, 1979). The X-ray film was developed in D-19 developer (Kodak).

Experimental Numbers:

The number of animals used in experiments depended upon the echinoid species. For S. purpuratus, enough plasma, cells or tissue could be obtained from one or two animals per experiment. For D. excentricus, material from up to six animals was pooled for an individual experiment. The results presented in the various figures and tables were representative of experiments repeated at least three times, except for the results presented in Tables 2, 3 and 4, which contain complete summaries of those particular experiments.

RESULTS

The yolk glycoprotein precursor particle of the plasma in echinoids:

Not only is a 200K dalton yolk glycopeptide precursor found in the accessory cells of the previtellogenic ovary (Ozaki et al., 1986), but also in the plasma of the perivisceral coelomic fluid. For S. purpuratus, the plasma contains as its most prominent protein constituent, a 200K dalton glycopeptide (Harrington and Easton, 1982). For D. excentricus, the plasma contains a component which cross-reacts with the antiserum made against the major yolk glycoprotein of the egg (Ozaki and Harrington, 1986). A comparison between total plasma protein and total previtellogenic ovary protein from D. excentricus by SDS-polyacrylamide gel electrophoresis shows that the plasma, like the ovary, contains a prominent 200K dalton protein which is likely to be the cross-reacting material in the plasma (Figure 1). Since the glycopeptides of the ovarian accessory cells and the yolk glycopeptides of the egg are complexed into a larger particulate form, the plasma was examined to see if its 200K dalton glycopeptide was also complexed into a larger particle.

The plasma was separated from the perivisceral coelomic fluid by centrifugation. Since the protein content of the

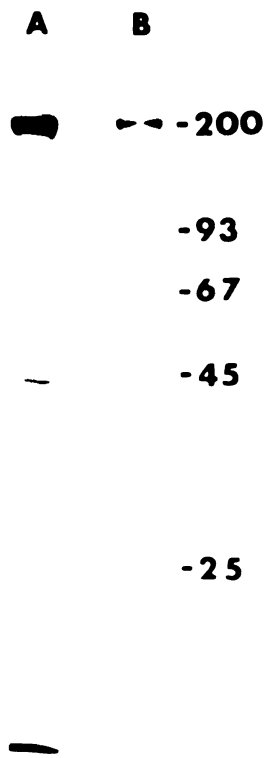


Figure 1. Plasma and previtellogenic ovary protein of D. excentricus analyzed by SDS-polyacrylamide gel electrophoresis.
Shown is a 10% polyacrylamide gel (Laemmli, 1970) stained with Coomassie Brilliant Blue: A) total previtellogenic ovary protein; B) total plasma protein. The molecular weight standards calibration is given in kilodaltons.

plasma is low, it was concentrated with a Micro-ProDiCon apparatus. Then, the concentrated plasma proteins were subjected to sucrose density centrifugation. In D. excentricus, the plasma contained a fast-sedimenting protein particle with the sedimentation coefficient of 22S (Figure 2A). Similarly, in S. purpuratus, the plasma contained a protein particle with the sedimentation coefficient of 24S (Figure 2B).

SDS-polyacrylamide gel electrophoretic analysis of the fast-sedimenting particles from the plasma revealed that their constituents were high molecular weight polypeptides, of which a 200K dalton protein predominated (Figure 3B and 4B). Therefore for both species, the glycoprotein component of the plasma had the same properties as the ovarian component immediately prior to the transfer step from accessory cell to oocyte (summarized in table 1).

A comparison between the total plasma protein (Figure 3A and 4A) and the high molecular weight plasma glycoprotein particles (Figure 3B and 4B) on an electrophoretogram shows that the particles make up a significant portion of the total plasma protein. This is shown further in an elution profile of plasma of S. purpuratus passed through a Concanavalin A-column (Figure 5). The material specifically eluted with α-methyl-D-mannoside was a 200K dalton protein which is glycosylated (Figure 6B), while the unbound material contained smaller molecular weight, non-glycosylated plasma proteins (Figure 6C). The amount of

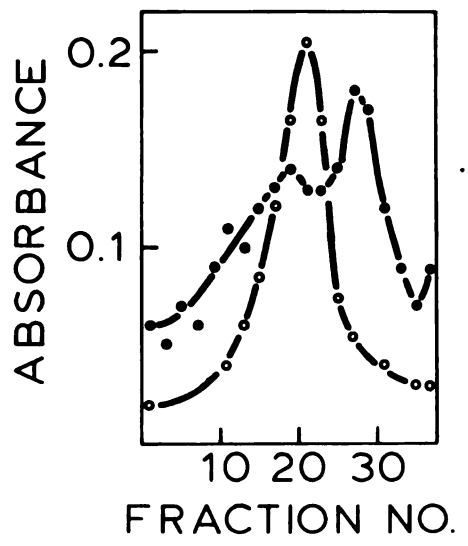


Figure 2A.

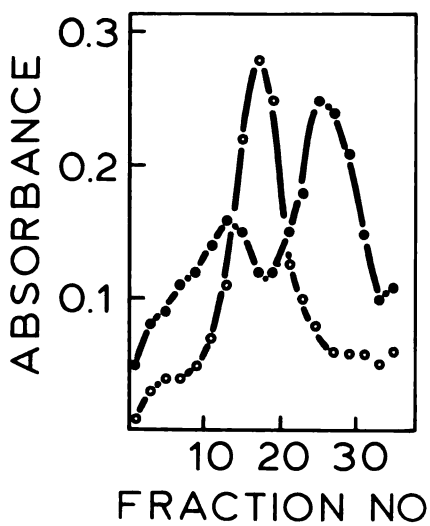


Figure 2B. Sucrose density gradient centrifugation of plasma proteins.
Shown are the fractionation profiles of 5-20% linear sucrose gradients loaded with either concentrated plasma proteins or porcine thyroglobulin (19.4S) as a standard, and centrifuged at 160 k x g for 5 h (Figure 2A) and 6 h (Figure 2B). The gradients were fractionated from the bottom of the tube (sedimentation direction right to left on figure), and the protein concentration of each fraction was determined by the Lowry method and is given as absorbance at 750 nm. Closed circles-plasma, and open circles-thyroglobulin. A) D. excentricus plasma protein profile with the fast-sedimenting material having a sedimentation coefficient of 22S; B) S. purpuratus plasma protein profile with the fast-sedimenting material having a sedimentation coefficient of 24S.

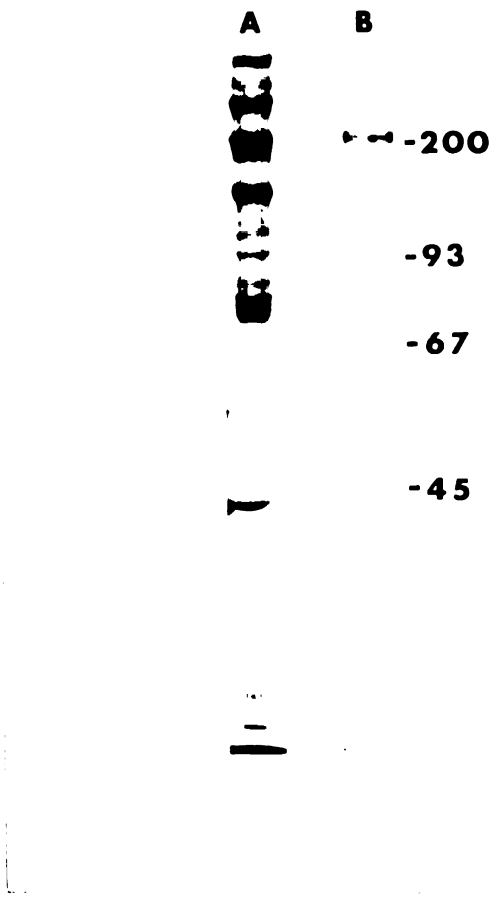


Figure 3. SDS-polyacrylamide gel electrophoresis of D. excentricus plasma proteins.
The electrophoretic conditions were the same as in Figure 1.
A) total plasma protein; B) 22S plasma particles.

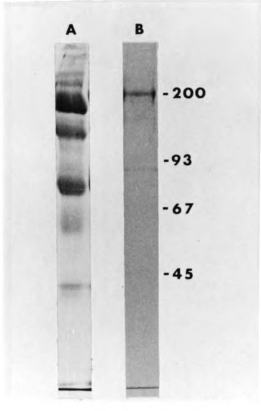


Figure 4. SDS-polyacrylamide gel electrophoresis of S. purpuratus plasma proteins.
The electrophoretic conditions were the same as in Figure 1.
A) total plasma protein; B) 24S plasma particles.

Table 1. Comparison of yolk glycoprotein and their precursor in various compartments for two echinoid species.

Dendraster excentricus

Compartment	Glycoprotein Particle Sedimentation Coefficient	Molecular Weight of Major Glycopeptide in Particle
coelomocyte[1]	ND	200K[+] daltons
plasma	22S	200K daltons
accessory cell[2]	22S	200K daltons
egg[2]	11S	100K daltons

Strongylocentrotus purpuratus

Compartment	Glycoprotein Particle Sedimentation Coefficient	Molecular Weight of Major Glycopeptide in Particle
coelomocyte	?	?
plasma	24S	200K daltons
accessory cell[2]	24S	200K daltons
egg3	24S	200K daltons

1- Ozaki and Harrington, 1986; 2- Ozaki et al., 1986; 3- unpublished result; ?- not determined; ND- not detectable.

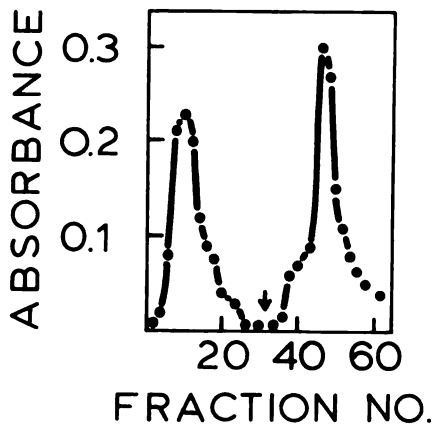


Figure 5. Concanavalin A-chromatography of S. purpuratus plasma.
The plasma proteins were passed through a Concanvalin A-Sepharose column. The non-bound material emerged immediately from the column and formed the first peak. An arrow marks the beginning of the elution of the bound proteins with α-methyl-D-mannoside. The amount of protein in each fraction was determined by the Lowry method and is given as absorbance at 750 nm.

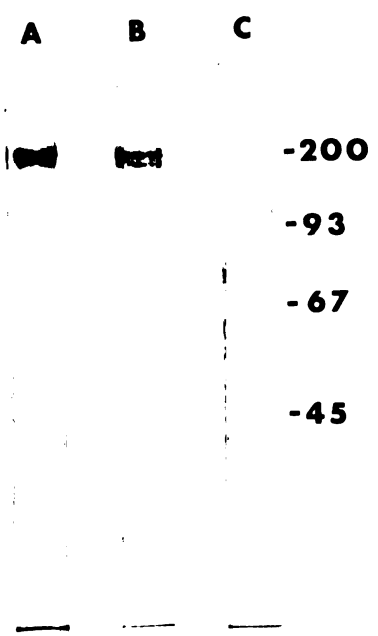


Figure 6. SDS-polyacrylamide gel electrophoresis of Concanavalin A-selected S. purpuratus plasma glycoproteins. The electrophoretic conditions were the same as in Figure 1. A) total plasma protein; B) pooled peak fractions of bound protein eluted from the Con A-chromatographic column (profiled in Figure 5); C) pooled peak fractions of unbound protein eluted from the column.

protein eluted with mannoside was approximately equal to the amount of unbound protein. Since the total plasma protein concentration is in the range of 0.1-0.3 mg/ml (table 2), and since the recovery of the protein from the chromatographic column was estimated to be nearly 100%, the concentration of the high molecular weight glycoprotein may be 0.05-0.15 mg per ml of plasma. Furthermore, since the plasma glycoprotein was always the most abundant protein in the coelomic fluid regardless of the reproductive season of the animal, the coelom could by the primary site of precursor production.

Secretion of the yolk glycoprotein precursor by echinoid coelomocytes:

The coelomic fluid is composed of plasma and a large number of coelomocytes. Both of these components of the coelomic fluid have been found to contain a possible yolk glycoprotein precursor with the coelomocytes being very active in the precursor synthesis (Ozaki and Harrington, 1986; this report). Table 2 shows the amount of total protein in the plasma and coelomocyte fractions from an equivalent volume of coelomic fluid. In D. excentricus, the amounts of total protein in the two fractions are nearly equal, but the relative amount of 200K dalton glycoprotein in the two fractions is quite different. Based on SDS-polyacrylamide gel electrophoresis, the 200K dalton glycoprotein is the most abundant plasma protein, but only a

Table 2. Concentration of total protein in echinoid coelomocyte and plasma fraction of the perivisceral coelomic fluid.

Coelomic fluid was collected and centrifuged to obtain the plasma and coelomocyte fractions. The plasma fractions were concentrated in a Micro-ProDiCon apparatus, and the protein amount was determined by the Lowry method. The coelomocytes were solubilized in Laemmli samble buffer and the protein determined by the Lowry method after TCA precipitation. The protein concentrations of the fractions correspond to the amount of protein in the original volumes of coelomic fluid collected.

A) Plasma

Dendraster excentricus		Strongylocentrotus purpuratus	
Animal Number	Plasma Protein Concentration	Animal Number	Plasma Protein Concentration
1	0.09 mg/ml	1	0.08 mg/ml
2	0.15 mg/ml	2	0.26 mg/ml
3	0.11 mg/m/	3	0.13 mg/ml
4	0.12 mg/ml	4	0.08 mg/ml

Table 2 (continued)

B) Coelomocytes

Dendraster excentricus		*Strongylocentrotus purpuratus*	
Animal Number	Coelomocyte Protein Concentration	Animal Number	Coelomocyte Protein Concentration
1	0.20 mg/ml	1	0.02 mg/ml
2	0.20 mg/ml	2	0.02 mg/ml
3	0.18 mg/ml	3	0.03 mg/ml
4	0.16 mg/ml	4	0.02 mg/ml
5	0.25 mg/ml		
6	0.28 mg/ml		
7	0.20 mg/ml		
8	0.25 mg/ml		

minor coelomocyte protein. In S. purpuratus, the difference between the coelomocyte and plasma fractions is even greater, with the plasma containing a magnitude greater total protein per equivalent volume of coelomic fluid. As stated above, the 200K dalton glycoprotein may be as much as 50% of the total protein, while in the coelomocytes it is not readily detectable. The glycoprotein present in the plasma could be a secretory product of the coelomocytes. In order to test this secretion hypothesis, the coelomocytes of S. purpuratus were cultured in spinner vessels (Figure 7), and the conditioned media were analyzed for the presence of secretory products. The coelomocytes could be cultured for up to 24 h with basically a sea water medium, or at least 48 h with proteose peptone added as a nutrient. These cells remained viable as judged by light microscopy and their ability to incorporate radioactively labeled amino acids into protein. Because of the tendancy of coelomocytes to form clots upon removal from the animal, some of the cells formed long strings of aggregated cells during culture while others remained as single cells. Even when the coelomocytes are allowed to form an extensive clot, a large amount of the glycoprotein remains in the plasma fraction (Harrington and Easton, 1982) suggesting that the plasma glycoprotein does not participate extensively in cell clotting.

The electrophoretic analysis of the conditioned medium (Figure 8B) showed the presence of a 200K dalton protein which was demonstrated to be glycosylated by Concanavalin

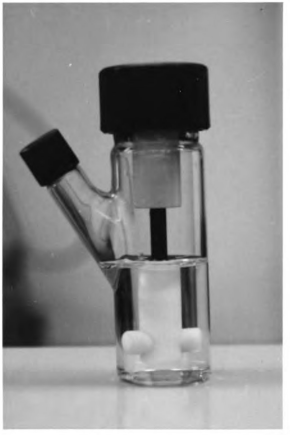


Figure 7A.

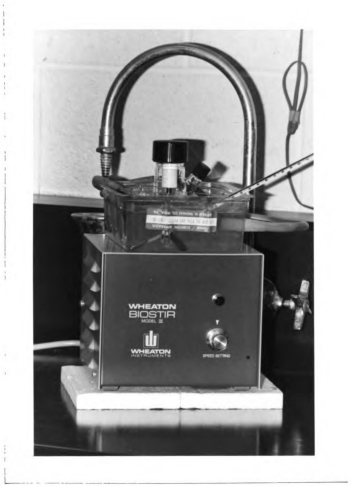


Figure 7B. Spinner culture.
A) Shown is a 25 ml spinner culture flask (Wheaton) used to culture coelomocytes; B) it was used by placing it in a constant temperature bath on a magnetic stirrir.

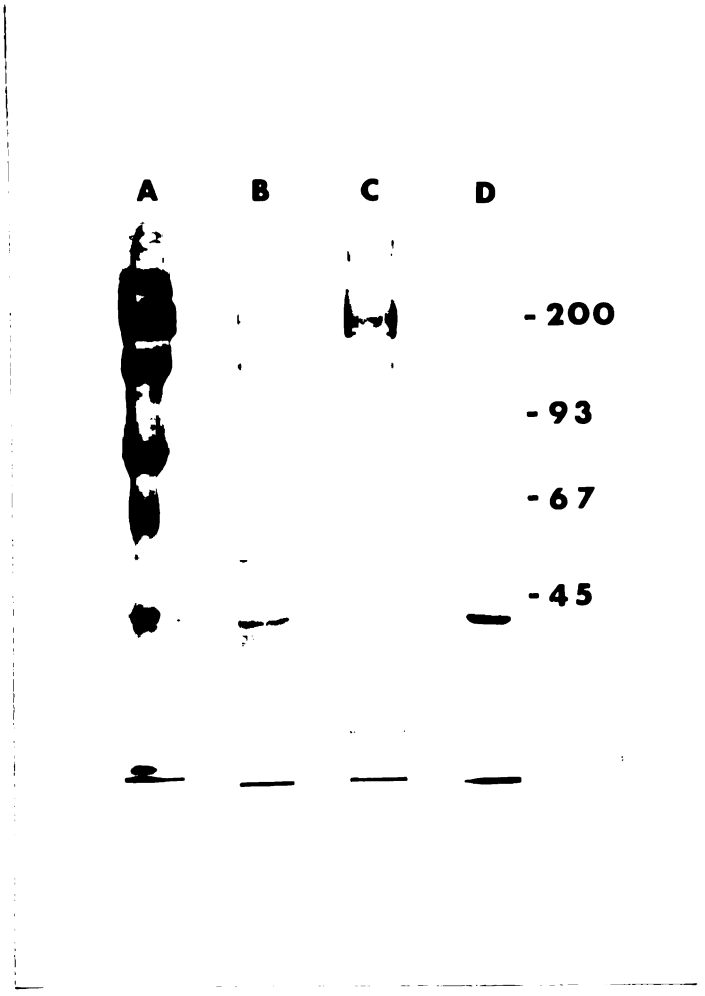


Figure 8. SDS-polyacrylamide gel electrophoresis of S. purpuratus coelomocyte cultures.
The electrophoretic conditions were the same as in Figure 1. A) total plasma protein; B) total conditioned medium protein from a 24 h culture; C) Concanavalin A-selected conditioned medium protein from a 48 h culture; D) total coelomocyte protein.

A-chromatography (Figure 8C). The electrophoretic pattern of the conditioned medium (Figure 8B) is quite different from the pattern of the coelomocyte proteins (Figure 8D). This difference and the absence of microscopic evidence for cytolysis during culture suggests that the 200K dalton-glycopeptide present in the conditioned medium was not an artifact of incidental cell lysis, but the consequence of selective secretion of coelomocyte products. The electrophoretic pattern of the conditioned medium (Figure 8B) is, moreover, similar to the pattern of plasma proteins (Figure 8A); thus it would appear that coelomocytes secrete the plasma glycoprotein, and that the circulation of the plasma could be a method of transfer of precursor into the ovary.

The perivisceral coelom as the major production site of the yolk glycoprotein precursor in echinoids:

In the foregoing section, it has been demonstrated that coelomocytes in the coelom do secrete the high molecular weight glycoprotein into the plasma. The coelomocytes in the coelom of D. excentricus produce the 200K dalton-yolk glycoprotein precursor throughout the year. The coelomocytes, however, are widely distributed within the echinoid body; therefore, the potential contribution by coelomocytes at sites other than the coelom to high molecular weight glycoprotein production was examined. The two main tissues of the echinoid, the ovary and the

digestive tract, were excised from D. excentricus, labeled in vitro with radioactive amino acids, and the labeled proteins were analyzed by electrophoresis and fluorography. Figure 9 shows the synthetic patterns of these two organs in comparison to the products of the coelomocytes labeled in vitro. The synthesis of the 200K dalton-yolk glycoprotein precursor was not apparent in the synthetic profile of the ovary or digestive tract, even though these tissues contain some coelomocytes.

It is important to show that the time the ovary was tested for the synthesis of the yolk glycoprotein coincided with the time the glycoprotein was accumulating in the ovary. During the months preceding vitellogenesis, the ovary undergoes a period of growth where yolk glycoprotein precursor accumulates in the accessory cells. Ovarian proteins from animals early in this previtellogenic stage were examined by SDS-polyacrylamide gel electrophoresis. In January, the ovaries of D. excentricus could be divided in to two categories (the end of oogenesis is in May and June when spawning can occur). Some ovaries already had the the 200K-dalton glycoprotein as a prominent constituent (Figure 10A), and other ovaries from animals collected from the same population at the same time had very low levels of the 200K-dalton glycoprotein (Figure 10B). This dichotomy in levels of ovarian yolk precursor in the month of January can be interpreted as the time when the accessory cells are just beginning to accumulate the precursor. Since labeling

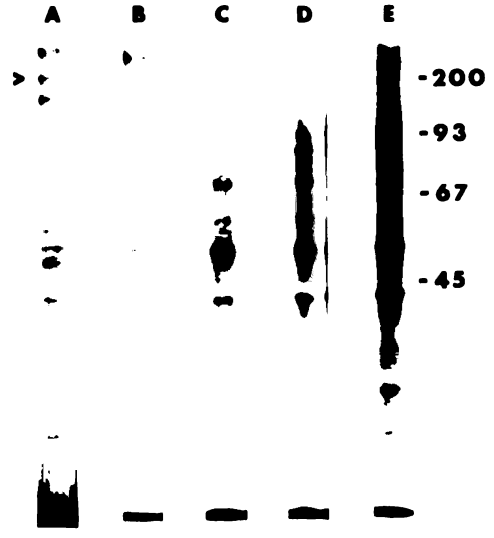


Figure 9. Fluorogram of labeled D. excentricus cells and tissues.
The cells and tissues were labeled in vitro for 1 h with radioactive amino acids. Labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The electrophoretic conditions were the same as in Figure 1. An arrow marks the position of the yolk glycoprotein precursor synthesized by the coelomocytes. A) coelomocytes; B) ovary; C) foregut; D) midgut; E) hindgut.

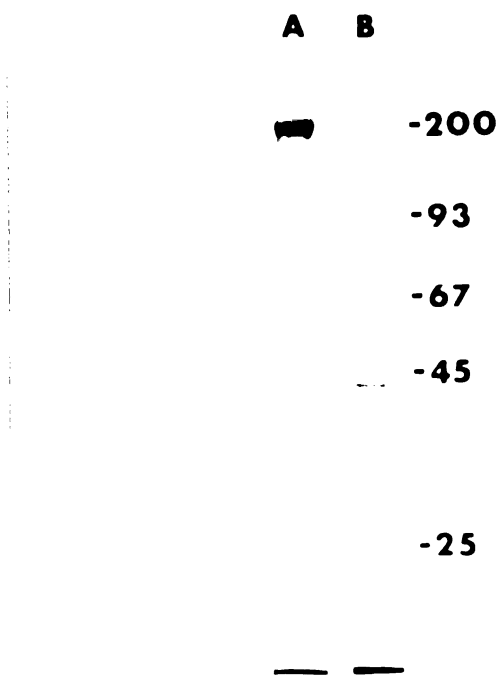


Figure 10. SDS-polyacrylamide gel electrophoresis of D. excentricus early previtellogenic ovaries.
The electrophoretic conditions were the same as in Figure 1. The ovaries were analyzed in January in animals from a population which would produce mature gametes for spawning in 5-6 months. A) ovary with a high concentration of 200K dalton glycoprotein (total ovary protein loaded was 1.2 µg); B) ovary with a low concentration of 200K dalton glycoprotein (total ovary protein loaded was 1.5 µg).

experiments were carried out from January to March (example in Figure 9), and the synthesis of the glycoprotein was not detectable in either the ovary or the intestines, it can be concluded that the predominant site of the major yolk glycoprotein precursor synthesis is the perivisceral coelom in D. excentricus.

Effect of estrogen on protein synthesis by echinoid coelomocytes:

In order to test the effect of estrogen on yolk protein synthesis in echinoids, coelomocytes of D. excentricus and S. purpuratus were maintained in vitro for 4 or 24 h, with or without estradiol in the culture media. A response was obtained in coelomocytes from nongravid females of D. excentricus after 4 h of hormone treatment. A comparison of the synthetic patterns of 4 h cultures shows that a protein of about 82K daltons is synthesized by treated cells (Figure 11A), but not the control cells (Figure 11B). The induction of the synthesis of this 82K dalton-protein appears to be transitory since its synthesis was seen in a 4 h hormone treated culture, but not in a 24 h culture (Table 3). Similarly, in coelomocytes from nongravid female S. purpuratus, the response was seen as the synthesis of a 78K dalton protein. However, the response required a longer culture period than in D. excentricus, since it was observed in the 24 h, but not 4 h treated cultures (Figure 11D and E; Table 4). The coelomocytes from gravid female of S.

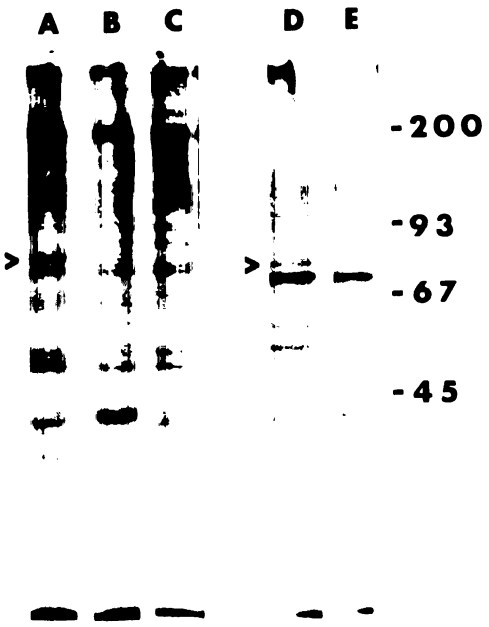


Figure 11. Induction by estradiol of the synthesis of a novel protein in coelomocytes from nongravid female echinoids.
Coelomocytes were labeled with L-[^{14}C]-valine for 1 h after culturing the cells for various periods of time with or without hormone in the media, and the labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography, with the electrophoretic conditions the same as in Figure 1: A) D. excentricus culture, 4 h with estradiol; B) D. excentricus culture, 4 h without hormone; C) D. excentricus culture, 4 h with progesterone; D) S. purpuratus culture, 24 h with estradiol; E) S. purpuratus culture, 24 h without hormone. The arrow in Lane A marks the position of the induced 82K-dalton protein in D. excentricus, and the arrow in Lane D marks the position of the induced 78K-dalton protein in S. purpuratus.

Table 3. The effect of steroid hormone on the protein synthesis in coelomocytes from nongravid female D. excentricus.
Cells were placed in spinner culture with and without hormone for the periods indicated, and then recovered and labeled with radioactive amino acids for 1 h. Hormone concentrations in the media were 10^{-6} M. Cell lysates were analyzed for radioactivity and protein as described in the material and methods, and the relative rate of amino acid incorporation into protein (protein incorporated counts/ total intracellular counts) is reported as a ratio of treated cells to control cells. A positive hormonal response (+) was the synthesis of a 82K dalton protein by the treated cells (see Figure 11A).

Experiment Number	Culture Length	Hormone Treatment	Incorporation Ratio	Hormonal Response
1	4 h	estradiol	0.9	+
2	24 h	estradiol	0.9	-
3	4 h	progesterone	1.0	-

Table 4. The effect of estradiol on protein synthesis in coelomocytes from female S. purpuratus.
The experimental conditions were the same as in Table 3, except that the hormonal response in this species was the synthesis of a 78K dalton protein by coelomocytes from nongravid females (see Figure 11D), but not from gravid females.

Experiment Number	Reproductive State	Culture Length	Incorporation Ratio	Hormonal Response
1	nongravid	4 h	0.7	-
2	nongravid	24 h	0.8	+
3	gravid	24 h	0.8	-
4	gravid	24 h	1.0	-

purpuratus, on the other hand, did not respond to the hormonal treatment; the novel protein was not induced in this case in the 24 h treated cultures (table 4). Thus, the hormone response may be different depending on the reproductive season of the animal.

The induction of the synthesis of the 82K-protein by estrogen treatment of D. excentricus coelomocytes was specific to estrogen as compared to progesterone. When the synthetic profiles of proteins of cultures with and without progesterone were compared, the profiles were the same for both control (Figure 11B) and hormone treated (Figure 11C) cultures. Cells treated with progesterone for 4 h had the same relative rate of amino acid incorporation into protein as the 4 h control (Table 3). Progesterone neither affected the rate of protein synthesis nor the kinds of proteins being synthesized by the coelomocytes, even though the cells from the same animals at the same time had responded to estrogen.

While the hormone treatment induced the synthesis of a novel protein, it did not affect the synthesis of the yolk glycoprotein precursor by D. excentricus coelomocytes; the density of the precurosr band on the fluorogram appears to be the same for both the treated and control cells (Figure 11A and B). A distinct band for the yolk glycoprotein precursor is not readily detectable in the synthetic pattern of S. purpuratus coelomocytes since, unlike D. excentricus coelomocytes, it is only a minor component of the total

protein synthetic profile.

Estrogen treatment as long as 24 h did not appreciably alter the relative rate of total protein synthesis by coelomocytes, even in the cases where a qualitative change in the protein synthetic pattern due to the hormone was observed. The rate of amino acid incorporation into protein by either the hormonally treated cells or the control cells was essentially the same as indicated by a ratio close to 1 between them (Table 3 and 4).

High molecular weight glycoproteins in echinoid and asteroid eggs:

The effect of estrogen on the echinoid cells which are involved in the synthesis of yolk precursor is of particular interest due to the regulation of asteroid vitellogenesis by this hormone. If the yolk proteins of asteroids and echinoids are similar, which would suggest that the yolk genes were conserved between the asteroids and echinoids, then the regulation of vitellogenesis might also have been conserved through phylogeny. The electrophoretic pattern of glycoproteins from an asteroid egg, Pisaster giganteus was compared with echinoid yolk glycoproteins. The result is shown in Figure 12 in which glycoproteins from three sources were analyzed: P. giganteus egg proteins (Figure 12A) and D. excentricus precursor proteins (Figure 12B) stained with Periodic Acid-Schiff reagent, and proteins from S. purpuratus eggs prepared by concanavalin A-chromatography

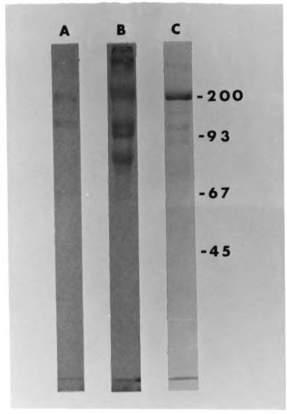


Figure 12. Comparison of asteroid and echinoid yolk glycoproteins by SDS-polyacrylamide gel electrophoresis. The electrophoretic conditions were the same as in Figure 1, except for the staining of A and B: A) Pisaster giganteus egg proteins stained with Periodic Acid-Schiff reagent; B) D. excentricus yolk glycoprotein precursor particles isolated from the previtellogenic ovary by sucrose density gradient centrifugation, stained as in (A); C) yolk glycoproteins purified by Concanavalin A-chromatography from S. purpuratus eggs, stained with Coomassie Brilliant Blue.

and stained with Coomassie Brilliant Blue (Figure 12C). The asteroid egg was found to contain a prominent 200K dalton glycopeptide, similar in molecular weight to the ones seen in the two echinoid sources. It is likely that the presence of similar glycopeptides in both asteroid and echinoid eggs reflects the close phylogenetic relationship as members of the phylum Echinodermata. Since the 200K-dalton yolk glycoprotein is highly conserved among several echinoids (Harrington and Easton, 1982), and possibly in both asteroids and echinoids, its pathway of accumulation during oogenesis and the regulation of the pathway could have been conserved within the these two classes of echinoderms as well.

DISCUSSION

Yolk Glycoprotein Precursor in the Echinoid Plasma:

For a number of different echinoid species, the plasma of the coelomic fluid has been shown to contain glycoproteins (Johnson, 1970; Harrington and Easton, 1982; Giga and Ikai, 1985a). Furthermore, plasma and eggs have been shown to have an immunologically similar component (Kondo and Koshihara, 1972; Giga and Ikai, 1985a and b; Ozaki and Harrington, 1986), and these egg-related plasma components appear to be high molecular weight yolk glycoprotein precursors (Harrington and Easton, 1982; Ozaki and Harrington, 1986). The present study has shown that the 200K dalton plasma glycopeptide is complexed into a larger, fast-sedimenting particle; the plasma glycoprotein particle has a sedimentation coefficient of 22S in D. excentricus and 24S in S. purpuratus, which is the same for the respective species as the glycoprotein particle found in the ovarian accessory cells (Table 1). This is in agreement with the results of Giga and Ikai (1985a and b) on a Japanese echinoid; they suggest that there are four 200K dalton glycopeptide subunits arranged in the particle to give a protein with the sedimentation coefficient of about 24S. Thus, in this dissertation, further evidence has been

obtained that the plasma glycoprotein is a likely candidate as a precursor to the yolk glycoprotein.

Site of Plasma Glycoprotein Synthesis:

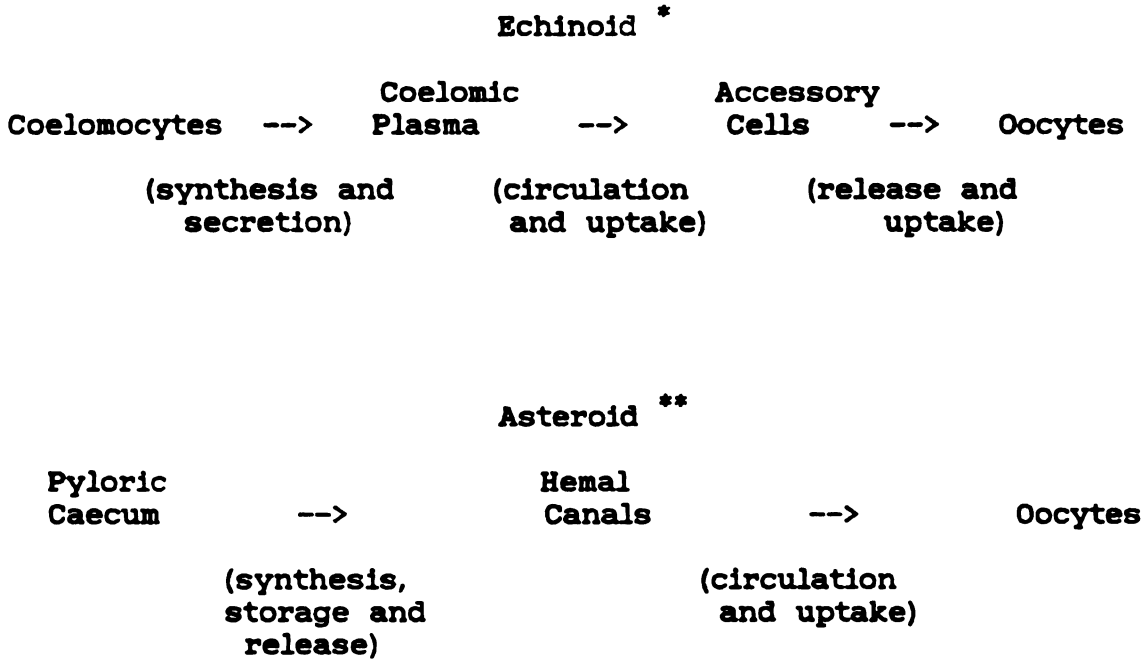
The present study has shown that other than coelomocytes in the coelom, neither of the other two major body compartments synthesize the high molecular weight glycopeptide at a detectable level in D. excentricus. Therefore, the principal contribution to yolk protein precursor synthesis seems to be by the coelomocytes of the coelom, and that the precursor would have to be translocated to the ovary. This translocation could involve either one or both of the coelomic components, the coelomocytes which contain the glycoprotein could carry it directly into the ovary, or the plasma could circulate into the ovary and carry the glycoprotein which had been secreted by the coelomocytes.

Plasma Glycoprotein Translocation:

Nutrient translocation is carried out extensively by the circulation of the perivisceral coelomic fluids, and to a much lesser extent by two associated parts of the circulation, the hemal and water vascular systems (Farmanfarmaian and Phillips, 1962; Ferguson, 1982). Liebman (1950) considered that the cellular components (the coelomocytes) of the circulatory fluid carry nutrients, invade the ovary and might themselves become the accessory

cells; but, this does not seem to be a significant part of the translocation process. Of the two coelomic fluid components, the plasma seems to contain more significant levels of the glycoprotein. In both species studied, it was the most prominent plasma protein, but only a minor coelomocyte protein. As far as total protein in the coelomic fluid fractions, for D. excentricus, there is almost as much total protein in the plasma fraction as in the coelomocyte fraction. Measurements taken over the course of a year have shown that the total plasma protein level ranged from 0.2-0.45 mg/ml for S. purpuratus (Holland et al., 1967). The measurements made in the present research for total plasma protein in the same species were 0.1-0.3 mg/ml, of which approximately one half was the 200K dalton-yolk glycoprotein precursor, while the total protein in the coelomocyte fraction was an order of magnitude less (0.02 mg/ml). Since the glycoprotein is produced and secreted by the coelomocytes, it seems that most of it is released into the plasma rather than being held intracellularly. Therefore, it has been concluded that the glycoprotein which is translocated into the ovary is the component found in the plasma, and that the coelomocytes do not play a direct role in carrying the glycoprotein. The pathway for yolk glycoprotein accumulation is inferred to be: coelomocyte > plasma > accessory cell > oocyte (Figure 13).

Figure 13. Comparison of models for asteroid and echinoid vitellogenic pathways.



* - based on Ozaki et al., 1986; Ozaki and Harrington, 1986; and this dissertation.

** - based on Voogt et al., 1985.

Comparison of Vitellogenesis in Echinoids and Asteroids:

Echinoids yolk proteins consist in part of high molecular weight glycoproteins (see introduction). Based upon partial proteolysis peptide mapping of several species, the yolk glycoprotein genes appear to be evolutionarily conserved within the echinoids (Harrington and Easton, 1982). By comparing asteroid egg glycoproteins to echinoid yolk glycoproteins, it was found that there are similar proteins within these two phylogenetically related groups. This is in agreement with the report by Broertjes et al. (1984b) that the yolk proteins of a European asteroid Asterias rubens have a glycoprotein component and two lipoglycoprotein components which might correspond to the yolk protein fractions published by Ii et al. (1978) for a Japanese species of echinoid.

Not only do the yolk protein genes appear to be conserved within the asteroids and echinoids, but there are several parts to the vitellogenic process which are analogous between these two classes of echinoderms. The outline of the vitellogenic pathway for the two type of echinoderms is shown in Figure 13. The echinoid lacks a pyloric caecum which is the site of yolk synthesis in the asteroid, but the coelomocytes, which the echinoid possesses, may take its place. Coelomocytes in asteroids may also synthesize the precursor; these cells are found in the pyloric caecum and they are intensely stained immunocytochemically with anti-yolk serum (Beijnink et al.,

1984). In asteroids, the yolk precursor is temporarily stored in the cells which line the lumen of the pyloric caecum, while in echinoids it is stored in the ovarian accessory cells which asteroids do not have. Due to these anatomical differences, in echinoids the precursor is translocated into the ovary prior to storage, while in asteroids the translocation takes place afterwards. Furthermore, the mechanism of translocation is different in the two groups of echinoderms; in asteroids, the hemal system plays a greater role in circulation than it does in echinoids (Ferguson, 1982), and is the route for precursor translocation into the ovary (Beijnink et al., 1984). In contrast, precursor translocation into the ovary in echinoids appears to be carried out by the circulation of the coelomic fluid rather than the hemal circulation. Again, in asteroids, the yolk precursor may be present in the coelomic fluid plasma, so the differences between asteroids and echinoids may not be as great as they seem.

Hormonal Regulation of Echinoderm Vitellogenesis:

The levels of estrogen in the asteroid fluctuate in relation to the reproductive cycle (Schoenmakers and Dieleman, 1981), and the treatment of an asteroid in vivo (Schoenmakers et al., 1981; Takahashi, 1982) or asteroid ovaries in vitro (Takahashi and Kanatani, 1981) with estrogen seems to stimulate vitellogenesis. The pyloric caecum which temporarily stores yolk precursor is a target

tissue of the hormone since it contains estrogen receptors (De Waal et al., 1982); and the hormone appears to regulate the mobilization of stored yolk precursor and its translocation into the oocyte (Voogt and Dieleman, 1984; Voogt et al., 1985).

In our testing of estrogen function in echinoids we have found that the cells which synthesize the yolk glycoprotein precursor are indeed target cells. In response to estrogen in the culture medium, coelomocytes of both echinoid species were found to synthesize a novel protein, 78K daltons in S. purpuratus and 82K daltons in D. excentricus. However, estrogen treatment for 24 h did not stimulate yolk protein synthesis in the coelomocytes, nor protein synthesis as a whole. Increased transcription of the yolk precursor mRNA is a consequence of estrogen stimulation observed in many vertebrates (Jost et al., 1978; Brock and Shapiro, 1983). However, echinoderms are metabolically slow relative to vertebrates (Lawrence and Lane, 1982); therefore to stimulate yolk precursor production in the coelomocytes may require much longer treatment periods than 24 h.

The novel protein which was induced by the hormone may ultimately result in stimulating yolk precursor synthesis in one of two ways. First, it could be part of an initial response analogous to what has been seen in chromosomal puffing in Drosophila after steroid hormone treatment (Walker and Ashburner, 1981). In Drosophila, ecdysterone

initially triggers the transcription of a few genes, which then in turn lead to a secondary burst of transcriptional activity. A second possibility is that the induced protein described here for echinoids could be the estrogen receptor being synthesized in response to hormonal stimulation. Receptor initially present at low levels may need to be augmented before increased transcription of the yolk protein precursor gene can occur. The estrogen receptor levels are regulated by the hormone itself in vertebrates (Cidlowski and Muldoon, 1978), and indeed, it has been observed that the pyloric caecum of asteroids increases synthesis of receptors after the organ is exposed to the hormone (De Waal et al., 1982). Therefore, the novel protein might be the receptor itself which would be required for the subsequent hormonal response.

In contrast to the mechanism of hormonal regulation of yolk precursor synthesis in vertebrates, estrogen's effect on echinoid vitellogenesis could be on processes taking place after yolk precursor synthesis. In this study, the coelomic fluid components from D. excentricus were analyzed throughout the yearly reproductive cycle, and the coelomocytes always actively synthesize the yolk precursor at a high rate, and the precursor is always the most predominant protein component of the plasma. This suggests that precursor synthesis is constitutive and does not undergoing any great seasonal modulations. However, the amount and location of the yolk precuror within the ovary

does undergo seasonal changes. In D. excentricus, yolk precursor was found in this study to start to be stored in the ovarian accessory cells in January. This is followed by several months of continued accumulation in the accessory cells and finally a short period of translocation into the oocytes which become fully mature in May or June (Ozaki et al., 1986). Similarly, for S. purpuratus, a histological study (Chatlynne, 1969) of oogenesis has shown a period of glycoprotein accumulation in the accessory cells, followed by a period of vitellogenic growth in the oocytes. Either one of these steps could be regulated by estrogen in the echinoid, which would be analogous to what has been shown for another echinoderm. In asteroids, it is the step of precursor transfer by the circulatory system, a step taking place after the original precursor synthesis, that appears to be regulated by estrogen. Therefore, precursor production in echinoderms may not be regulated as it is in vertebrates, but the estrogen-regulated step in echinoderms could be a point in the vitellogenic pathway subsequent to the step of yolk precursor synthesis.

While the complete function of estrogen in echinoids is still obscure, a target cell for the hormone has been identified in this study. This target cell, the coelomocyte, is involved in a complex reproductive pathway and is capable of undergoing a hormonally-induced change. Future studies on hormonal regulation of vitellogenesis in echinoderms will be required to further elucidate the

regulatory mechanisms, and to possibly understand the evolutionary changes which may have taken place following the divergence of the echinoderm and the chordate lineages.

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