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Abstract

The ovary was found to be the major synthesizing organ of vitellin (Vt) in penaeid shrimps, with a significantly lower contribution of the hepatopancreas and no contribution of the subepidermal adipose tissue. This conclusion is based on protein synthesis by in vitro incubations of these three organs, in presence of labeled amino acids.

In vivo synthesized Vg/Vt was found in both the hepatopancreas and the hemolymph of vitellogenic females, following injections of radioactively labeled amino acid; but the role of this Vg and the direction of its transportation is not yet known. The hemolymph of vitellogenic females contains Vg, up to 0.4 mg/ml.

In vitro incubated ovarian fragments synthesize reserve lipids but do not synthesize polyunsaturated fatty acids, which are present in the ovary and therefore have to be supplied from the diet.

The accumulation of vitellin in the ovary is confined to a limited stage during mid-oogenesis, in contrast to other proteins which are accumulated throughout the process.

A neuropeptide which inhibits protein and vitellin synthesis in the in vitro incubated ovarian fragments was isolated from sinus gland extracts. Its N-terminus (35 residues) was sequenced and it was found to belong to the recently established CHH- related peptide family.

In a related decapod, Procambarus clarkii it was established that serotonin (5-HT) stimulated ovarian development in vivo but not in vitro. In contrast, a gonad stimulating factor extracted from parts of the CNS, is effective also in vitro. Therefore it is postulated that 5-HT exerts its effect by stimulating the release of a gonad stimulating factor.

Objectives of the original research proposal

The aim of the present proposal was to develop specific bioassays that would permit the identification and quantitative evaluation of neurohormones or other neuropeptides involved in the control of vitellogenesis in species of shrimp belonging to the genus Penaeus which are of importance in aquaculture. In order to establish in vitro and in vivo specific bioassays, we had to identify the organs involved in the vitellogenic process, assuming that these are the target organs for hormones engaged in controlling the process. Unlike the in vitro bioassay system reported by Tsukimura and Kamemoto (1987) and the in vivo bioassay of Soyez et al. (1987), which were based on the measurement of oocyte diameter, we attempted to measure directly the levels of vitellogenin (Vg) or vitellin (Vt) already present or newly synthesized, using in vivo and in vitro assay systems that were developed in the course of the proposed research.

Preliminary work, carried out by the IOLR Haifa laboratory team before the commencement of the present project was aimed at: (a) identifying by immunohistological and immunochemical methods the organs suspected of being involved in vitellogenesis; (b) developing in vitro organ cultures of three tissues. Based on the literature, we anticipated that three tissues were involved in vitellogenesis: the subepidermal adipose tissue (SAT) and hepatopancreas (HP) as possible sites for extraovarian Vg synthesis, and the ovary as the site of Vt synthesis.

Hemolymph proteins separated by PAGE, revealed, by immunoblotting, the presence of one protein positively reacting with anti-Vt antibody (Ab-Vt) only in vitellogenic females. Using this antibody, we were able to detect, by immunohistological methods, immunoreactive intracellular sites only in the developing oocytes. Our work also included in vitro incubations of SAT, HP and ovaries. We were successful in obtaining incorporation of ^3H or ^{14}C -leucine into TCA precipitable proteins for up to 24 hours in in vitro cultures of ovarian and SAT sections removed from females at various stages of oocyte development. Using radioimmunoprecipitation (RIP), we were able to detect newly synthesized Vt in ovarian tissue. The vitellin level of synthesis was found to be related to the oocyte diameter. The relative amount of Vt to total TCA precipitable proteins synthesized in vitro was found to be similar in several replicate sections removed from one ovary. Thus, this system served later as a bioassay for evaluating the effect of hormones or other substances on Vt synthesis in the ovary.

Unlike what was found with the ovary, RIP revealed no Vt immunoreactive

synthesis in in vitro SAT incubations. The major protein component synthesized by SAT contains copper and is suspected to be hemocyanin. It is possible that the in vitro system of SAT lacked some essential humoral factors that were present in the hemolymph or tissues, or they became diluted or destroyed during the incubations, consequently preventing Vg synthesis.

Our attempts to culture HP were initially with little success, although Quackenbush and Keeley (1986) and Paulus and Laufer (1984) reported in their abstracts some successful results. We suspect our failure to be due to the presence of high amounts of proteolytic enzymes in the HP which must be specifically inhibited during the incubation.

In view of our preliminary results, we planned to proceed in two directions in developing the appropriate bioassays:

(1) Verify or reject the hypothesis of extraovarian sites for Vg synthesis, which is indicated by the presence of a Vt immunoreactive protein in the hemolymph of vitellogenic females.

In order to examine critically the possibility of extraovarian Vg synthesis, we proposed the following experimental plan:

(a) Isolation of Vg from the hemolymph of vitellogenic females and compare its molecular weight and polypeptide composition to that of the Vt.

(b) Analysis of the subunit labeling pattern of labelled Vt, synthesized in vitro by ovarian tissue and Vt/Vg synthesized in vivo by a vitellogenic female injected with a mixture of labeled amino acids. The labeling pattern in the latter case will be examined both in the ovary and in the hemolymph. A comparison of these subunit labeling patterns in both static and time course experiments may lead to a definitive conclusion with regard to the existence of an extraovarian Vg synthetic site. If we find proof or indication of extraovarian synthesis, we would proceed to develop and elaborate the in vitro cultures of the SAT and HP.

(c) Measurement of the Vg level in the hemolymph during oogenesis by rocket electrophoresis (Jurd, 1981) or by RIA, according to Asher et al. (1983).

The evidence that the ovary synthesizes at least part of the Vt molecule, along with the possibility that Vt transport from the ovary to the hemolymph might occur, as indicated by ovarian absorption (Browdy and Samocha, 1985a), combine to make it difficult to perform in vivo studies to follow Vt/Vg from its synthesis site to the ovary. Nevertheless, we think that our approach of determining the "fingerprints" of the various types of Vg and Vt is a reasonable approach for following Vt/Vg synthesis.

(2) Develop an in vivo bioassay that would permit quantitative evaluation of

the effect of various treatments, particularly gonad stimulatory factors, on vitellogenesis in intact females. The use of an in vivo assay would allow: (a) the evaluation of the amounts of hormones/peptides required for evoking a biological response; (b) the examination of the effect of the isolated factors on the full process of egg production; (c) the inspection of progeny produced by artificial induction of maturation. These studies will be carried out in the last phase of the project, and would be particularly significant because of their direct applicability to the mariculture industries.

In parallel with our attempts to establish appropriate bioassay systems, we planned to extract neuropeptides from the nervous system of Penaeus. These were to include the eyestalk, brain and thoracic ganglia. We followed at first, the method of Soyez et al. (1987) which recently isolated a GIH from a crustacean eyestalk. Furthermore, at that stage of our work only general outlines of the purification methods were available. The exact procedures of purification would have to depend on the nature of the peptides which would be active in our bioassays, with regard to their relative hydrophobicity, acidity and molecular weight. The general methods would have include various extraction methods, partial purification on gel chromatography columns, and final purification on RP-HPLC. We intended to use, in the beginning, our in vitro ovarian bioassay that was described above until we had additional ones. In addition to the isolation of neuropeptides, we intended to examine crustacean neuropeptides which we planned to obtain from other investigators. Furthermore, we hoped to examine the effects of ecdysone and methyl farnesoate in our bioassays.

IS THERE EXTRAOVARIAN SYNTHESIS OF VITELLOGENIN
IN PENAEID SHRIMP?

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Running head: Site of vitellogenin synthesis in penaeid shrimp

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Abstract

Extraovarian synthesis of vitellogenin (Vg), has been reported for a number of crustaceans, mainly in the subepidermal adipose tissue (SAT) or the hepatopancreas (HEP). The precise site(s) of Vg synthesis in penaeid shrimp is hitherto unknown and was investigated in a large local species - *Penaeus semisulcatus* de Haan. Protein synthesis was determined in SAT and HEP tissue pieces incubated *in vitro*.

Incubations were carried out at 25°C for 8 hours in an oxygen enriched atmosphere, under sterile conditions in a physiological medium, containing ¹⁴C-leucine. At the end of the incubation period, tissue homogenates and medium samples were analyzed for *de novo* protein synthesis. Total protein synthesis was determined by trichloroacetic acid precipitation. Specific vitellin (Vt) synthesis was determined by radioimmunoprecipitation with a polyclonal Vt-specific antiserum. Characterization of other *de novo* synthesized proteins was carried out by fluorography from polyacrylamide gels. SAT's removed from females at all stages of ovarian development did not synthesize Vt-specific proteins, in spite of the fact that total protein synthesis levels were high. The major protein synthesized *de novo* in the SAT of males and females is a protein with an identical electrophoretic mobility as hemocyanin in polyacrylamide gels. *In vitro* protein synthesis in HEP tissues was low compared to SAT or ovary systems. Vt-specific *de novo* synthesized protein was identified in HEP's from early vitellogenic females, but constituted less than 15% of total protein synthesis. We have previously shown that ovarian tissues from vitellogenic females incubated *in vitro* exhibit high levels of protein synthesis, an average of 38% of which is Vt-specific (Browdy *et al.*, 1990, J. Exp. Zool. 255: 205-215). The calculated Vt synthesis rates in ovaries were up to 23 times higher than in HEP. We conclude that the extraovarian contribution to vitellogenesis in *P. semisulcatus* is low.

Introduction

Vitellogenesis is associated with the formation of yolk globuli within the developing oocytes in the ovary. Proteins and lipids are the main constituents of yolk and vitellin (Vt) is the major protein that accumulates within the ovary during this process (Adiyodi and Subramoniam, 1983; Meusy and Payen, 1988). Vitellogenesis is an endocrine regulated process, and is therefore of interest as a central part of the female reproductive cycle (Quackenbush, 1986; Fingerman, 1987; Charniaux-Cotton and Payen, 1988). It has been studied intensively in the lower vertebrates (Tata, 1976; Lam *et al.*, 1978; Huang *et al.*, 1979) and in various groups of insects (Kanost *et al.*, 1990). In recent years there has been increasing interest in crustacean vitellogenesis, due to the growing commercial importance of crustaceans in aquaculture and the interest in new models for basic research (Laufer and Downer, 1988). A critical prerequisite for studying vitellogenesis at the endocrine and cellular levels is identification of the tissues that participate in the synthesis of yolk and its precursors.

The sites of synthesis of Vt or its precursor molecules have not yet been fully established for penaeid shrimp. A protein which reacts immunologically to antiserum prepared against purified Vt was found in the haemolymph of vitellogenic females. This protein, known as vitellogenin (Vg), has been reported in all species studied so far (Kerr, 1968; Ceccaldi, 1970; Wolin *et al.*, 1973; Fyffe and O'Connor, 1974; Caubere *et al.*, 1976; Meusy, 1980; Dehn *et al.*, 1983; Ferrero *et al.*, 1983; Marzari *et al.*, 1986; Tom *et al.*, 1987a; Suzuki, 1987; Nelson *et al.*, 1988; Quackenbush and Keeley, 1988; Quackenbush, 1989a). The presence of Vg in the haemolymph raises the question of possible extraovarian sites for synthesis of Vt or its precursor molecules.

Previous studies of crustacean vitellogenesis have focused on three tissues as possible sites for Vg synthesis - ovaries, adipose tissues and hepatopancreases (HEP). *De novo* synthesis of Vt in the ovaries has been shown for one isopod (Gohar *et al.*, 1985) and a number of decapods (Lui and O'Connor, 1976; Eastman-Reks and

Fingerman, 1985), including three species of penaeid shrimp (Yano and Chinzei, 1987; Quackenbush, 1989a, b; Browdy *et al.*, 1990). On the other hand there is convincing proof for extraovarian synthesis of Vg in the adipose tissues of isopods and amphipods (Picaud, 1980; Souty and Picaud, 1981; Junera and Meusy, 1982). Vt-positive immunoreactivity has been reported for the adipose tissue of *Parapenaeus longirostris* (Tom *et al.*, 1987a) and in the HEP of two brachyuran decapods (Paulus and Laufer, 1987). Different groups have reported conflicting results for *in vitro* Vg synthesis by the HEP in penaeids. Yano and Chinzei (1987) and Rankin *et al.* (1989) were unable to show *de novo* Vg synthesis in HEP of *Penaeus japonicus* and *Penaeus vannamei*, respectively. In contrast, Quackenbush (1989a, b) has reported *in vitro* synthesis of proteins immunoreactive with antibodies to yolk in cultures of the HEP of *Penaeus vannamei*. There have been no studies so far on *de novo* synthesis of Vg in penaeid adipose tissues.

Although there is sufficient evidence to support a role for the ovary in Vt synthesis in penaeids, the quantitative contribution of ovarian versus extraovarian synthesis is not clear. We have tried to clarify this question by studying protein synthesis *in vitro* in the three putative vitellogenic tissues of *Penaeus semisulcatus*. We have recently shown that the ovaries of this species synthesize Vt *in vitro*, and that the portion of Vt specific synthesis from total protein synthesis varies in accordance with ovarian developmental stages (Browdy *et al.*, 1990). In this report we summarize our studies on protein and Vg synthesis in the adipose and hepatopancreatic tissues of this marine shrimp species.

Materials and Methods

Chemicals

Amino acids, HEPES, Bis-Tris, vitamins and supplements for media were cell-culture tested grade and were obtained from Sigma. [$^{14}\text{C}(\text{U})$]leucine (308 mCi/mMol) was purchased from New England Nuclear (U.S.A.). All other reagents were of analytical grade.

Sea water

Mediterranean sea water (salinity 40 ‰) was pumped onshore into a central reservoir and used immediately. For *in vitro* cultures it was filtered through sieves and a 0.45 µm membrane filter, and finally sterilized by filtering through a heat sterilized 0.22 µm membrane filter. Dilutions were performed using deionized water (Ionex, Israel) prior to sterilization.

Animals

Adult *P. semisulcatus* were collected in Haifa Bay, Israel. They were held in 3 m³ running sea water tanks, at a density of 20 specimens per tank. Water was changed at a rate of 300% per day. Salinity was constant at 40 ‰, and temperature ranged from 18°C (winter) to 27°C (summer). The animals were fed once a day on a mixture of frozen fish, squid, shrimp and *Artemia*. Females were individually marked by clipping of the uropods, and ovarian development was monitored externally, according to the methods of Browdy and Samocha (1985).

Polyacrylamide gel electrophoresis (PAGE) and electroblotting

Electrophoresis in non-denaturing conditions was performed according to Davis (1964) in 5% polyacrylamide gels. Coomassie Blue stains for proteins and dithio-oxamide stains for copper-conjugated proteins were performed according to Hames and Rickwood (1981).

Western blots from PAGE were done using a Bio-Rad electroblotting apparatus at 400 mA for 4- 5 hours at 4°C. Proteins were transferred to nitrocellulose papers which were dried and stored at 4°C until staining. Immunostaining was carried out according to Browdy *et al.* (1990).

Purification of Vt and preparation of rabbit specific antiserum

Vt was identified as the main constituent of ripe ovaries by comparing the electrophoretic profiles in non-denaturing polyacrylamide gels (PAGE) of ripe and undeveloped ovarian homogenates. It was purified from homogenates of ripe ovaries by two steps of column chromatography and one of preparative PAGE as described by Browdy *et al.* (1990). Anti-Vt serum was prepared in rabbits after injection of 0.5 mg

Vt as described by Tom *et al.* (1987b). The specificity of the antiserum was verified by immunoblotting (Bio-Rad immunoblot assay system) against all the fractions obtained during the purification as shown in Browdy *et al.* (1990). The antiserum was found to react only with the Vt fraction.

Determination of oocyte diameter

Prior to *in vitro* incubations, a piece of tissue was removed from each tested ovary and fixed in 4% formalin in sea water. The average oocyte diameter was determined as described by Shlagman *et al.* (1986).

In vitro incubation experiments

Subepidermal adipose tissues (SAT's) were excised from females at different stages of vitellogenesis and from intermolt males. The SAT from each animal was divided into four pieces and each was incubated in Landureau's medium modified for penaeid tissues as described in Fainzilber *et al.* (1989). ^{14}C -leucine was added to a final concentration of 0.25 $\mu\text{Ci/ml}$. Cold leucine level in the media was 0.11 mM.

Incubations were carried out at 25°C with gentle shaking and under an oxygen enriched atmosphere. Following the incubation period, tissues were removed and homogenized in 0.1 M phosphate buffer. Homogenates were centrifuged at 14,000 rpm for 10 min in an Eppendorf Microfuge at 4°C. *De novo* synthesized proteins were determined by TCA precipitation. Samples of tissue homogenates, supernatant and the incubation media were pipetted onto Whatman 3 MM filter paper discs, dried, precipitated in 10% TCA and washed according to Mans and Novelli (1961). Discs were counted in scintillation vials with 5 ml Aquasol-2 (New England Nuclear) in a Kontron Betamatic liquid scintillation counter, and quenching was monitored by measuring sample channels ratio (SCR). The specific activities of the media (cpm/ μg leucine) were calculated from samples prepared on filter paper discs, and final results were calculated as μg leucine incorporated into TCA precipitable protein. Control incubations were carried out with media containing labelled leucine but lacking tissue and with tissue incubated without labelled leucine. Tissue protein was determined by the method of Bradford (1976).

Radioimmunoprecipitation (RIP) was carried out by taking 50 μ l samples from tissue homogenates, supernatants and media. These samples were mixed with precalibrated volumes of anti-Vt serum after the addition of 0.1 mg purified Vt, to ensure maximum precipitation. Reaction volumes were adjusted to 1 ml with 0.4 M NaCl. Incubation was carried out for 1 hr at room temperature, after which vials were further incubated at 4°C for 24 hrs. Normal rabbit serum (NRS) was added instead of anti-Vt serum in control vials. Additional controls included NRS with a calibrated amount of goat-anti-rabbit serum, to obtain a large non-specific pellet. Following the incubation at 4°C, samples were centrifuged and the pellet washed three times with 0.4 M NaCl. Finally the pellet was dissolved in 75 μ l of 0.5 M NaOH and incubated with vigorous shaking for 1 hr at 37°C. The dissolved pellet was neutralized with 2 M HCl and the complete sample was applied to Whatman 3 MM paper discs, dried and counted as described above.

HEP were excised from previtellogenic and vitellogenic females and intermolt males, weighed and each cut into 8-12 pieces. Each piece was weighed separately. They were prewashed for 1 hour with frequent changes of media and each piece was incubated separately. HEP pieces were homogenized in their incubation media, centrifuged, and supernatants taken for analysis. Protease inhibitors (0.2 mM PMSF, 1 mM Aprotinin, 1 mM Leupeptin) were added, and the samples were analyzed as described above. All controls were as described for SAT incubations, with the addition of a zero time incubation control. HEP pieces were immersed in incubation media containing 14 C-leucine and were immediately homogenized and processed for analysis. Zero-time controls and background counts were similar. There was no difference between incubations with or without protease inhibitors. A number of different media compositions were tried and the best composition was found to be the modified Landureau medium buffered with Bis-Tris to pH 6.5.

All incubations and RIP were performed in culture plates or tubes coated with Sigmacote (Sigma, U.S.A.).

For fluorography from PAGE, incubations were performed as described above, except that media contained 2.5 $\mu\text{Ci/ml}$ ^{14}C -leucine and no cold leucine. Samples from these incubations were separated on PAGE. Gels were fixed in 7.5% acetic acid for 1 hour, washed and immersed in Amplify (Amersham, U.K.) for 30 minutes. Afterwards, gels were dried at 60°C under vacuum and exposed to Kodak XAR5 films at -70°C .

Collection of haemolymph and identification of hemocyanin

Haemolymph was removed from shrimp by cutting the rostrum and bleeding into 1 ml of a 10% sodium citrate solution. The haemolymphs of several females or several males were pooled and placed separately into test tubes. Hemocyanin was identified as the main constituent of whole haemolymph, which stained positively for copper (Horn and Kerr, 1963, 1969).

Results

Protein synthesis in in vitro incubated SAT

De novo protein synthesis and secretion to the medium were measured at regular time intervals during the incubation of the adipose tissues, and were found to increase linearly after an initial lag period of about 4 hours, with a similar rate of protein synthesis in a male control (Fig. 1). Over 90% of the *de novo* synthesized TCA precipitable proteins were found in the medium fraction. A comparison of levels of total protein synthesis in SAT's of 13 females at different stages of vitellogenesis showed a large variability between females at the same vitellogenic stage, but failed to show any correlation between the vitellogenic stage and the synthesis of proteins in the adipose tissues (Table I). In excess antibody precipitations of all the samples, no Vt-specific proteins were detected. Ovaries from the same females all produced Vt *in vitro*, at levels averaging 38% of the total *de novo* synthesized protein (Browdy *et al.*, 1990; Table I).

SAT proteins were separated in non-denaturing polyacrylamide gels. Total proteins were visualized with Coomassie Blue (Fig. 2A), while incorporation of radiolabel into *de novo* synthesized SAT proteins was visualized by fluorography

(Fig. 2B). Vt-specific immunological staining was performed on Western blots (Fig. 2C) and copper conjugated proteins by dithio-oxamide staining (Fig. 2D). Samples from previtellogenic and vitellogenic females and control males were run on each gel. Figure 2C shows that samples from SAT's of vitellogenic females stained positive for Vt; however, Vt was not visualized in the fluorographic exposures (Fig. 2B). One major radioactive band is seen in the fluorography of all the samples, including the male control. Based on the position of this band versus the position of hemocyanin (Fig. 2A), and on a positive reaction with the copper stain (Fig. 2D), the main *de novo* synthesized protein in adipose tissues appears to be hemocyanin or a copper containing protein with a similar electrophoretic mobility in non-denaturing polyacrylamide gels.

Protein synthesis in in vitro incubated HEP

Protein synthesis was measured in HEP incubated *in vitro* in a number of media and culture conditions, including those of Quackenbush (1989a, b). Protein synthesis in these cultures was low, and typically only 4-7% of the available label was incorporated into TCA precipitable proteins. This compares to 14-23% for ovarian cultures and 50-61% for SAT. The best conditions were found to be incubation in Landureau's medium modified for penaeid tissue (Fainzilber *et al.*, 1989) at pH 6.5 with 20 mM Bis-Tris. Protein synthesis rates in HEP from 11 females under these conditions differed according to the stage of vitellogenesis (Fig. 3, Table I), and were highest for females at an early stage of vitellogenesis (OCD 130-200 μ m). Excess antibody precipitations revealed Vt specific label ranging from 3.7% of total proteins synthesized in late vitellogenic females to 14.4% in early vitellogenesis (Table I). Male HEP cultures were also found to contain Vt specific label, at near-background levels of 0.8%.

Comparison between different tissues

In order to evaluate the possible quantitative significance of the Vt synthesis in HEP cultures, leucine incorporation results were translated into Vt synthesis rates, calculated from the molar ratio of leucine in purified *Penaeus semisulcatus* Vt (Tom

et al., 1992). These calculated Vt synthesis values were corrected to allow for the 2 hour lag in protein synthesis observed in HEP incubations of early vitellogenic females (Fig. 3), and the final values obtained represent synthesis rates during the linear phase. Similar calculations were performed for ovaries incubated *in vitro*, from the data of Browdy *et al.* (1990). Ovary Vt synthesis rates ranged from 1.2 μg Vt/whole ovary/hour in previtellogenic females to 36.4 μg Vt/whole ovary/hour in vitellogenic females (Fig. 4). These values were 3-23 times higher than the corresponding Vt synthesis rates in HEP cultures (0.34-1.6 μg Vt/whole HEP/hour in previtellogenic and early vitellogenic females, Fig. 4).

Fluorography of TCA precipitates from female HEP samples run on polyacrylamide gels failed to show radioactivity in the region of the Vt band, which is clearly seen in the parallel lane of an ovary sample (Fig. 5). One of the main bands seen in fluorography of samples from HEP incubations has the same position as does the putative hemocyanin band from SAT samples (Fig. 5).

Discussion

The results shown above and those of Browdy *et al.* (1990) strongly suggest that the ovary is the main site of Vt synthesis in *Penaeus semisulcatus* and that the SAT has no role in this process. These conclusions are based on Vt specific immunoprecipitations (Table I) and fluorography of samples on PAGE (Fig. 2). The main protein synthesized in *in vitro* incubations of SAT appears to be hemocyanin or a related protein based on its positive copper stain and position in PAGE (Fig. 2). The fact that the SAT synthesized protein has the same position in PAGE as the main HEP product (Fig. 5) is noteworthy, since the HEP is a well characterized hemocyanin synthesizing tissue in decapod crustaceans (Senkbeil and Wriston, 1981; Bielefeld *et al.*, 1986; Preaux *et al.*, 1986). There are no previous reports of hemocyanin synthesis in adipose tissues of Crustacea.

Hemocyanin accounts for 90-95% of the haemolymph serum proteins in crustaceans (Horn and Kerr, 1969; Rochu and Fine, 1978; Ferrero *et al.*, 1983; Hagerman, 1983; de Fur *et al.*, 1985). Most of the copper found in the haemolymph

(about 95%) was found to be bound to hemocyanin (Horn and Kerr, 1963). Evidence in the literature strongly suggests the midgut gland (HEP) as a site of synthesis of hemocyanin in several crustacean species (see review of Mangum, 1983). However, Ghiretti-Magaldi *et al.* (1977) postulated a "lymphocytogluic" organ associated with the gizzard, and Senkbeil and Wriston (1981) observed synthesis on the "intestinal wall". The high levels of synthesis found in the present report suggest that the SAT may be a major hemocyanin synthesizing tissue in penaeid shrimp, and should motivate additional research into the role of adipose tissues in this process.

The quantitative significance of the HEP in Vg synthesis in penaeid shrimp is still unclear. The data shown above (Table I, Fig. 4), suggest that this tissue synthesizes a Vt immunoreactive protein in previtellogenic and early vitellogenic females. However, it seems that quantitatively the contribution of the HEP is low. Previous studies of protein synthesis *in vitro* in HEP of *Penaeus japonicus* and *Penaeus vannamei* have reported conflicting results. Yano and Chinzei (1987) did not detect synthesis of Vt specific proteins in *in vitro* incubated HEP of *P. japonicus*. However, their incubations were performed in salts ringer without buffer, vitamins and sugar supplements, that we have found to be essential for cultures of HEP, and the total protein synthesis they reported was very low. Rankin *et al.* (1989) reported that incorporation of radiolabelled leucine in *in vitro* incubated HEP of *P. vannamei* was relatively high early in the cycle of ovarian development. They did not observe changes in the SDS-PAGE peptide pattern during ovarian development, and did not use antibodies or other means of identifying Vg specific synthesis. The synthesis of yolk proteins reported by Quackenbush (1989a,b) for HEP of *Penaeus vannamei* is not clearly attributable to Vg synthesis. This is due to the use of an antigen for antibody production which does not represent the whole Vt. This antigen was a 97 kD protein resolved from SDS-PAGE (and therefore in denaturated form) and found in both ovaries and HEP. The antibody to this antigen reacted with three different ovarian proteins and one hepatopancreatic protein. The levels of immunoreactive proteins in ovaries of *P. vannamei*, when measured with this antibody, reached a

maximum level of 8% of total tissue protein (Quackenbush, 1989b), much lower than the expected levels for the main storage protein of a mature penaeid ovary (Rankin *et al.*, 1989).

Our comparison of the Vt synthesis rates per whole organ in HEP versus ovary (Fig. 4) clearly shows that there is a difference of one order of magnitude in Vt synthesis rates in these two organs incubated *in vitro*. We cannot rule out the possibility that this difference is less extreme *in vivo*. However, Vg haemolymph levels and turnover were found to be low in both previtellogenic and vitellogenic *P. semisulcatus* females (Shafir *et al.*, 1992 and unpublished results). These results lead us to the conclusion that although the HEP may be involved in the synthesis of some ovarian proteins, its quantitative significance is probably low. The possibility that the HEP may be involved in synthesis of an exclusively extraovarian Vt component has yet to be shown.

Vt specific immunoreactivity has been found in SAT samples of *Penaeus semisulcatus* (Fig. 2C), *P. japonicus* (Vazquez-Boucard, 1985) and *Parapenaeus longirostris* (Tom *et al.*, 1987a). Vt positive reactions have also been observed in the immunohistological preparations of HEP from two brachyuran decapods (Paulus and Laufer, 1987). These results show the presence of Vt immunoreactive compounds, but do not provide evidence for active synthesis of these compounds in the tissues examined. A high molecular weight Vt immunoreactive compound has been found in the haemolymph of a number of crustaceans. The Vt positive reactions in tissues may be due to absorption of haemolymph in the incubated tissues, or may suggest a role for these tissues in the catabolism of Vt following resorption of oocytes. Oocyte resorption provides one of the explanations for the presence of Vt immunoreactivity in the haemolymph. It is also possible that such immunoreactivity results from Vg synthesis in ovarian follicle cells, secretion of the Vg into the haemolymph and subsequent uptake into developing oocytes (Yano and Chinzei, 1987).

It should be noted that most of the data supporting the ovary as a single or main site of Vt synthesis in penaeids have been obtained from *in vitro* systems. Such

systems, although possessing many advantages for subsequent endocrinological studies, might not provide the necessary conditions for a specific tissue to function as it does *in vivo*. Conversely an *in vivo* experimental approach does not allow the clear isolation of a specific tissue's contribution. Identification of yolk protein mRNA from tissues of females at different stages of vitellogenesis, using *in vitro* translation systems could clarify this issue. These techniques have been employed with success for the study of hemocyanin synthesis in crayfish (Bielefeld *et al.*, 1986). The feasibility of this approach is currently under examination in our laboratory.

To summarize, our results and other recent publications suggest that the ovary is the most prominent site for Vt synthesis in penaeid shrimp. This is in contrast to the extraovarian synthesis of Vg in Isopoda and Amphipoda (Meusy and Payen, 1988). It seems that different groups of crustaceans utilize different strategies for the synthesis of their yolk proteins. In this respect, crustaceans are apparently similar to insects, in which at least three different strategies of yolk protein synthesis have been characterized (Kanost *et al.*, 1990). It is noteworthy that insects with different strategies of yolk synthesis also differ in various aspects of the endocrine regulation of this process, a fact with interesting implications for future studies of endocrine regulation of vitellogenesis in crustaceans.

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Table I. *In vitro* protein synthesis in tissues of female (F) and male (M)*Penaeus semisulcatus*

Tissue	Sex	Vitellogenic stage and oocyte diameter (μm)	No. of replicates ^a	Total protein synthesis ^b	Vt specific fraction ^c (%)
SAT	F	previtellogenic (≤ 100)	4	39.8 ± 15	0
	F	vitellogenic (100-300)	6	25.2 ± 9.2	0
	F	late vitellogenic (300-380)	3	28.2 ± 6.6	0
	M	-	4	26.6 ± 8.4	0
HEP	F	previtellogenic (≤ 100)	4	2.6 ± 1.3	5.2 ± 2.9
	F	vitellogenic (130-200)	4*	4.6 ± 1.6	14.4 ± 1.1
	F	vitellogenic (247-299)	5	0.4 ± 0.2	3.7 ± 2.0
	M	-	5	0.8 ± 0.3	0.8 ± 0.6
Ovary ^d	F	previtellogenic (≤ 100)	5	11.2 ± 3.1	5.0 ± 3.2
	F	vitellogenic (110-200)	4	55.2 ± 10.8	34.3 ± 4.7
	F	vitellogenic (220-281)	4	39.1 ± 4.5	42.3 ± 7.6

a Each replicate represents 2-3 pieces of tissue from a single animal, except where marked *. In this case, two pieces each from two animals were used.

b μg leucine incorporated into TCA precipitable protein per whole organ after 6 h of incubation (mean $\pm$ S.D.).

c Fraction of Vt specific immunoprecipitable protein from total *de novo* synthesized TCA precipitable protein (mean $\pm$ S.D.).

d Ovary data are based on Browdy et al. (1990; Figs. 5, 6) and are shown for comparison.

Legend to figures

- Fig. 1. The incorporation rate of leucine into protein secreted by *in vitro* incubated subepidermal adipose tissue (SAT). Tissues were removed from a male, and previtellogenic and vitellogenic females. Results were calculated for whole SAT. The mean $\pm$ S.D. for four replicates are given in each case. The average oocyte diameter (OCD) in ovaries is indicated (in μm) for each female.
- Fig. 2. Non-denaturing polyacrylamide gel electrophoresis (5%) of protein synthesized *in vitro* by subepidermal adipose tissue (SAT). Medium fractions only shown. (A) Coomassie blue stained gels. (B) Fluorography after 9 days of exposure. (C) Vt specific immunological stain after Western blotting. (D) Copper conjugated protein stain. Lanes: (1) male; (2) previtellogenic female (40 μm OCD); and (3) vitellogenic female (164 μm OCD). The positions of vitellin (V) and hemocyanin (H), which were run in separate lanes in the same gels, is indicated. Bovine serum albumin (BSA) was included in the culture media. Each lane in A, C and D was run with 25 μl aliquots. In gels visualized by fluorography each lane was loaded with a sample containing 15,000 - 30,000 cpm of TCA precipitable protein.
- Fig. 3. The incorporation rate of leucine into protein by *in vitro* incubated hepatopancreas pieces. Results (mean $\pm$ S.D.) were calculated per whole hepatopancreas and are presented for males and early and late vitellogenic females. Mean $\pm$ S.D. for four replicates at each time point are shown. The average oocyte diameters (OCD) in the ovaries of the females are indicated in μm .

- Fig. 4. Vitellin synthesis rates in hepatopancreases (HEP) versus ovaries incubated *in vitro*, in females at different stages of vitellogenesis. Ovary data are calculated from the results of Browdy *et al.* (1990). Synthesis rates are calculated for the whole organ, and represent the linear phase of protein synthesis, after the lag period (see Fig. 1).
- Fig. 5. Comparative fluorography of proteins synthesized *de novo* in the ovary (A), subepidermal adipose tissue (B) and hepatopancreas (C) during *in vitro* incubations of tissues from vitellogenic females. The positions of vitellin (V) and hemocyanin (H) are shown. 15,000 cpm of TCA precipitable proteins were loaded in each lane.

Fig 2

Fig 1

Fig 3

Fig 4

A B C

Fig 5

Running head:

Vitellogenin and vitellin during ovarian development in penaeids.

**Protein, vitellogenin and vitellin levels in the
hemolymph and ovaries during ovarian development
in *Penaeus semisulcatus* (de Haan)**

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Key words: vitellogenin and vitellin, concentrations, hemolymph volume, penaeid shrimp.

Abbreviations:

AOD - Average oocyte diameter.
SAT - Subepidermal adipose tissue.
Vg - Vitellogenin.
Vt - Vitellin.
HEP - Hepatopancreas.

Abstract

The concentration of vitellogenin (Vg) in the hemolymph of *Penaeus semisulcatus* was found to increase from an average of 50 $\mu\text{g ml}^{-1}$ to 439 $\mu\text{g ml}^{-1}$ in female shrimp during ovarian development. The most significant increase in Vg occurred concomitant with the increase in the vitellin (Vt) content of oocytes with an average diameter (AOD) ranging between 150-250 μm . The amount of Vt in the oocytes was found to increase linearly from a mean of 0.0126 mg to 4.55 mg per gm body weight. However, the percent of Vt in the total protein was found to decrease from 67% in ovaries with AOD of 150-250 μm to 39.7% in ovaries with AOD of 350 μm or larger. The volume of the hemolymph was found to be 0.4 ml per gm body weight and did not change significantly during ovarian development. Assuming that Vg in the hemolymph represents either an extraovarian origin of Vt or an active secretion from the ovary, a turnover rate of 2-3 times per day was calculated over one full cycle of oocyte development. However, during the most significant increase in Vt in the ovary (in ovaries with AOD of 150-250 μm), the turnover rate in the hemolymph could reach 7-8 times per day. The results lead to the conclusion that the contribution of Vg to the formation of Vt in the ovary is quantitatively insignificant.

Introduction

Vitellogenesis is associated with several complex processes which lead to the formation of mature oocytes within the ovary. In crustaceans, substantial quantities of yolk accumulate within the developing oocytes, and serve to meet the basic requirements of embryonic and larval development, independent of the maternal organism (Adiyodi and Subramoniam, 1983). One of the major components of yolk is the lipoglycoprotein, vitellin (Vt). In several crustacean species, Vt was found to be synthesized by the ovary (Lui et al., 1974; Lui and O'Connor, 1976, 1977; Dehn et al., 1983; Eastman-Reks and Fingerman, 1985; Yano and Chinzei, 1987; Quackenbush, 1989a,b; Browdy et al., 1990). Yano and Chinzei (1987) suggested that Vt was synthesized in the follicle cells of *Penaeus japonicus*. However, a protein which reacts immunologically to the antiserum prepared against purified Vt was detected in the hemolymph of vitellogenic females. This female-specific protein (FSP), known as vitellogenin (Vg), was reported in all species studied so far (Kerr, 1969; Ceccaldi, 1970; Fyffe and O'Connor, 1973; Wolin et al., 1973; Caubere et al., 1976; Meusy, 1980; Dehn et al., 1983; Ferrero et al., 1983; Marzari et al., 1986; Susuki, 1987; Tom et al., 1987; Nelson et al., 1988; Quackenbush and Keeley, 1988; Quackenbush, 1989a). Vg is one of the two lipoproteins known in crustacean hemolymph (Lee, 1990). A large increase in the concentration of Vg was reported in the hemolymph during vitellogenesis. While some reports show that the appearance of Vg in the hemolymph was correlated with morphological changes in the ovaries antecedent to egg release, others showed that Vg was at the highest concentrations prior to the maximum accumulation of yolk in the oocyte (Quackenbush, 1989b; Lee, 1990).

Several reports show evidence for extraovarian tissues engaged in Vg synthesis. Vg was reported to be produced by the subepidermal adipose tissue (SAT) of anostracean (Van Beek et al., 1987), isopod (Picaud, 1980; Souty and Picaud, 1981; Picaud et al., 1989) and amphipod crustaceans (Croisille and Junera, 1980; Junera and Croisille, 1980). Vg was also found in the SAT of decapods (Meusy et al., 1983; Vazquez-Boucard, 1985; Tom et al., 1987). However, it was not identified as being

synthesized by *in vitro* incubated SAT of *P. semisulcatus* (Fainzilber, 1988; Fainzilber et al., 1992). Conflicting results were reported for Vg synthesis by the hepatopancreas (HEP) in decapods. Quackenbush and Keeley (1988) and Quackenbush (1989a,b) reported *in vitro* Vg synthesis in *Uca pugilator* and *P. vannamei*, respectively. However, Yano and Chinzei (1987) and Rankin et al. (1989) were unable to detect *de novo* Vg synthesis in HEP of *P. japonicus* and *P. vannamei*, respectively. Low levels of a Vt-immunoreactive protein were detected in *in vitro* incubated HEP in females of *P. semisulcatus* (Fainzilber, 1988; Fainzilber et al., 1992). The possible contribution of Vg in the accumulation of Vt in the ovary is further suggested by work describing endocytosis in the ovary (Hinsch and Cone, 1969; Wolin et al., 1973; Duronslet et al., 1975; Zerbib, 1979; Beams and Kessel, 1980; Schade and Shivers, 1980; Zerbib and Mustel, 1984; Jugan and Soye, 1985). Furthermore, Vg receptors were isolated from the ovarian cytoplasmic membranes of two decapod species (Jugan, 1985; Lavrdure and Soye, 1988; Jugan and Van Herp, 1989). However, Lui and O'Connor (1976) suggested that Vg originated from resorbed oocytes.

The present investigation aims at elucidating the possible role of Vg and its contribution to oocyte development in *Penaeus semisulcatus*, which is a species of economic importance in Israel. We attempted to examine the hypothesis that Vg in the hemolymph was transported and taken up by the ovary, relying on the suggestion of Byard and Aiken (1984). They reported that the observed levels of FSP in the hemolymph were always higher prior to the maximum accumulation of yolk in the oocytes, and they dropped off markedly prior to oviposition. The conclusion drawn by Byard and Aiken was that this pattern is consistent with the idea that the FSP represents an externally synthesized protein and is found at the highest level in hemolymph during the period when oocytes are accumulating the maximum amount of yolk. In order to examine this possibility, the concentration of Vg in the hemolymph, Vt in the ovary and the volume of the hemolymph were determined during one cycle of ovarian maturation. The results enabled us to calculate the

hypothetical turnover rate assuming that the entire Vt within the ovary originated from Vg circulating in the hemolymph.

An approach to vitellogenesis as a flow system, in which Vg is produced by an extraovarian tissue, released into the hemolymph and subsequently taken up by the developing oocytes, was described in insects (Bakker-Grunwald and Applebaum, 1977). This line was attempted in the present paper, in order to reach conclusions on the possible dynamic flow of Vg in the hemolymph.

Materials and Methods

Chemicals

^3H Inuline (46/mCi/gr) was purchased from New England Nuclear (U.S.A.). All other reagents were of analytical grade and were purchased from Sigma (U.S.A.), Merck (Germany) or Bio-Rad (U.S.A.).

Animals

Adult *P. semisulcatus* were collected in Haifa Bay, Israel. They were held in 3 m³ running sea water tanks, at a density of 30 specimens per tank. Water was changed at a rate of 300% per day. Salinity was constant at 40 ‰, and temperature ranged from 18°C (winter) to 27°C (summer). The animals were fed once a day on a mixture of frozen fish, squid, shrimp and *Artemia*. Females were individually marked by clipping of the uropods, and ovarian development was monitored externally, according to the methods of Browdy and Samocha (1985). Moulting of marked females was also recorded by identifying the uropod coded cuts of the shedded exuviae.

Results of hemolymph volume, protein and Vg levels were related to the average oocyte diameter (AOD), measured according to Shlagman et al. (1986) in sacrificed animals.

Collection of hemolymph and the determination of its volume

Hemolymph was collected from animals bled after cutting off the anterior part of the cephalothorax near the base of the eyestalks. The hemolymph from each female was collected into a vial containing a known volume of 10% sodium citrate solution.

The hemolymph volume was determined by injecting 2 μ Ci of ^3H -inulin dissolved in 100 μ l of sterilized seawater diluted to 30 ppt. Preliminary experimental results showed that the hemolymph radioactivity was stable for 24 h after the injection of the inulin. The hemolymph volume was calculated by measuring the dilution in the radioactivity of samples withdrawn 2 h after the injection of the labeled inulin.

Determination of proteins, Vg and Vt concentrations

The total protein concentration in the hemolymph and ovaries was determined according to Bradford (1976), using bovine serum albumin as a standard.

The concentrations of Vg in the hemolymph samples and Vt in the ovarian homogenates were determined by rocket immunoelectrophoresis (Jurd, 1981). The area formed under the "rocket" precipitation lines was calculated using a Complot series digitizer 7000 and compared to known concentrations of purified Vt subjected to the same "rocket" preparation. The purified Vt and the rabbit polyclonal antibody used in these determinations were isolated and prepared as described in Browdy et al. (1990).

Experimental procedure

Two series of experiments were carried out. In Experiment 1, hemolymph samples were removed (as described previously) from 77 females that were at various stages of ovarian development, including females immediately after the moult. The hemolymph samples from each female were centrifuged (9000 g at 4°C for 10 min in a Sorvall RC-5C U.S.A.) and the supernatant was stored at -70°C. In parallel, a small piece was removed from the ovaries of each female and fixed in formaldehyde (4% in sea water) for the measurements of AOD (see above). In these females, the protein and Vg concentration in the hemolymph were determined (see above) and related to the AOD.

In Experiment 2, 21 females were chosen individually, after monitoring for several days the moult and ovarian developmental stages. Females following moulting and those showing arrested ovarian development, or with ovaries in

progress of oosorption, were rejected. Each female was injected with ^3H inulin (as described above). After two hours, the females were weighed and bled and the hemolymph volume was determined as described above. Ovaries were dissected out and weighed. A small piece was fixed in formaldehyde and used for measurements of AOD. Two other samples (each weighing 0.1-0.2 gm) were used for determinations of dry weight. They were placed in pre-weighed vials and dried at 60°C until reaching constant weight (about 48 hours). The remaining part of the ovary was homogenized in 10 ml of phosphate buffer (0.1 M, pH 7.4) and centrifuged at 4°C and 9000 g for 20 min (Sorvall RC-5C, U.S.A.). The supernatant of the ovarian homogenates and the hemolymph samples were stored at -70°C , until being used in the determinations of total protein, V_t and V_g concentrations.

Results

The concentration of total protein and V_t specific protein (V_g) in the hemolymph was found to increase with the progress of ovarian development, shown by the increase in AOD (Fig. 1a,b). The values obtained in Experiment 1 for total protein and V_g of 77 and 73 females, respectively, were pooled and divided into 4 size groups according to their AOD. It can be clearly seen that females with AOD of 150 μm or larger had similar levels of total protein and V_g (one way ANOVA, $p > 0.05$). However, females at the initial stage of oocyte development (50-150 μm in AOD) show lower protein levels ($p < 0.001$) and V_g levels ($p < 0.0001$). Four of the examined females which were taken immediately after spawning showed an average of $430.0 \pm 43.8 \mu\text{g ml}^{-1}$ of V_g in their hemolymph, and their results were not included in Fig. 1b. The highest average values of protein ($247 \pm 139 \text{ mg ml}^{-1}$) and V_g ($562 \pm 410 \mu\text{g ml}^{-1}$) were found in females with an AOD ranging between 250 and 300 μm . The lowest average values for protein ($116 \pm 32 \text{ mg ml}^{-1}$) and V_g ($60 \pm 95 \mu\text{g ml}^{-1}$) were found in females with AOD of less than 100 μm . The results from the 21 females (Experiment 2) were divided into 4 group sizes according to the AOD of the ovaries (Fig. 1a,b). Some of the females included in the class size of 50-150 μm AOD were after moult, and this may have contributed to the large standard deviations in this

group, relative to the others. Unlike the results shown earlier for Experiment 1, the total protein concentration in these females did not change with the AOD. However, Vg was virtually not present in the hemolymph samples removed from females with an AOD of 50-150 μm .

The mean concentrations of Vg in the hemolymph in the four oocyte size groups of Experiment 1 were not statistically different from those of Experiment 2 (p ranging from 0.2 to 0.9). However, the mean concentration of protein in the hemolymph in females from Experiment 1 with oocytes ranging in size from 50-150 μm was significantly lower ($p < 0.02$) than that of females from the same size group in Experiment 2. These differences may be due to the fact that in Experiment 1 this group included females after molt or following oviposition, which were eliminated in Experiment 2.

The hemolymph volume did not change in relation to the AOD of the examined females (results not shown) and its average was 0.403 ± 0.114 ml per gm body weight.

The total amount of protein in the ovary (in Experiment 2) increased linearly from an average of 1.14 ± 0.7 mg per gm body weight to 11.4 ± 0.4 mg per gm body weight (Fig. 2a). The amount of Vt in the ovaries increased significantly in ovaries with AOD of 150-250 μm and only slightly in ovaries with larger AOD (Fig. 2b). The Vt concentration in the ovaries was found to average 4.55 ± 0.66 mg per gm body weight in ovaries with an AOD of 300 μm or larger. A significant increase in the relative amount of Vt in the total ovarian proteins was found when the AOD increased from 50- 150 μm to 150-250 μm , similar to that found in the hemolymph. This ratio decreased significantly towards the end of ovarian development (Fig. 2b). The water content in the ovary was found to decrease linearly during oocyte maturation (Fig. 3) from 83% in immature females to 69.5% in mature ones. This could partially explain the increase in the total amount of protein in the ovaries mentioned earlier (Fig. 2a).

Discussion

The concentration of Vg in the hemolymph of *P. semisulcatus* was found to range from 50 $\mu\text{g ml}^{-1}$ in females with undeveloped ovaries to a high average concentration of 439 $\mu\text{g ml}^{-1}$ in females with oocytes larger than 150 μm AOD. These values constitute only $0.06 \pm 0.09\%$ to $0.27 \pm 0.15\%$ of the total proteins in the hemolymph of females with undeveloped or developed ovaries, respectively. The relatively large standard deviations probably stem from the attempt to correlate Vg level to the AOD in preset size classes. These values are much lower than those reported for *P. vannamei* (Quackenbush, 1989a), *P. japonicus* (20 $\mu\text{g }\mu\text{l}^{-1}$ at the maximum stage in vitellogenesis; Yano, 1987), *Homarus americanus* (Nelson et al., 1988) (1 mg ml^{-1}), *Cancer antennarius* (estimated as 175 mg dl^{-1} ; Fig. 1 in Spaziani, 1988), *Callinectes sapidus* (4.1 mg ml^{-1}) (Lee and Puppione, 1988), and *Macrobrachium rosenbergii* (12 mg ml^{-1}) (Derelle et al., 1986). Most of the protein in the hemolymph of several species of crustaceans was found to be associated with hemocyanin (Magnum, 1983; Depledge and Bjerregaard, 1989).

The concentration of total protein was found to increase (in Experiment 1) in the hemolymph with the increase in oocyte size, staying at a stable level at later stages of oocyte development. However, in Experiment 2, no such trend was found. It is possible that in Experiment 1, the lower concentrations could be attributed to females that were taken immediately after moulting, when their AOD was less than 100 μm . The hemolymph volume was reported to increase prior to moulting, and an inverse linear relationship between hemolymph volume and protein concentration was reported by Smith and Dall (1982).

As mentioned, the concentration of Vg in the hemolymph was found to increase dramatically during the initial stages of oocyte development, when the AOD was 150-250 μm . This increase occurred with a concomitant increase in the absolute and relative amount of Vt in the ovaries. A similar relationship between the increase of Vg in the hemolymph and oocyte development was reported by Lee and Puppione (1988) for *Callinectes sapidus*. However, in *Homarus americanus*, the increased Vg

levels in the hemolymph preceded ovarian development (Byard and Aiken, 1984). In ovaries with AOD of 150-250 μm , the Vt constituted $67.7 \pm 6.4\%$ of the total proteins. This was reduced to an average of $39.7 \pm 4.5\%$, suggesting that, in addition to Vt, other proteins (e.g. the cortical crypts protein, see below) were synthesized in the ovary taking lead towards the end of ovarian maturation. In *P. vannamei*, Vt was found to constitute 5.3-20.9% of the total protein in spawns of fertile eggs (Quackenbush, 1989b).

The presence and synthesis of a cortical crypts protein was reported by Bradfield et al. (1989) to take place mainly at the period immediately before spawning in *P. vannamei*. Ovaries that were incubated *in vitro* were also found to synthesize 40-60% of the total proteins. Furthermore, the proportion of Vt was reduced in ovaries with AOD of 350 μm and larger (Browdy et al., 1990). The increase in the total amount of protein deposited in the ovaries was inversely related to their water content, which was reduced from about 83% to 69.5% in the fully mature ovaries.

A flow system demonstrating the Vg flow in hemolymph towards the ovary in vitellogenic females was demonstrated in insects (Bakker-Grunwald and Applebaum, 1977). This model was based on the following data: (a) concentration of Vg and Vt in the hemolymph and ovary, respectively; (b) the rate of incorporation of injected labeled amino acid into Vg; and (c) the rate of incorporation of Vg by the ovary. In the present paper, we present results in *P. semisulcatus* comparable to (a) above.

In crustaceans, the origin of Vg in the hemolymph is still problematic. In the case that Vg originates from extraovarian tissue, it could be assumed to be directed towards the ovary, where it is taken up and constitutes at least part of the Vt. This is the case in some crustacean species and in most insects (Kanost et al., 1990), which are also arthropods. However, several reports show that Vt is synthesized in the ovary (see references in the Introduction). In this case, Vg originates either by controlled secretion from the ovary (Yano and Chinzei, 1987) or by passive leakage from oocytes in the process of resorption. If Vg is actively secreted from the ovary, it could have a role in the transport of nutrients (e.g. lipids) into the ovary from

extraovarian tissues (e.g. HEP). The data presented in the present paper allow us to consider the flow dynamics of such systems. From the total hemolymph volume, the Vg and Vt concentrations in the hemolymph and ovaries, respectively, it is possible to calculate the hypothetical turnover rate of Vg in the hemolymph. This is presented in Table 1. If it is assumed that the entire amount of Vt present in the ovaries is transported via the hemolymph as Vg, the calculated turnover rate is 2.5 times per day over one full cycle. In carrying out this calculation, it is assumed that one full cycle of oocyte development is linear and takes 10 days (Browdy, 1988). However, if the full cycle is divided into four stages, the turnover rate must reach 7.78 per day, in order to account for the Vt accumulated in oocytes of 150-250 μ m. The accumulation rate is reduced at later stages (Table 1), in order to account for the increment in the amount of Vt accumulated in the ovaries.

The results presented here on the increase in Vg concentration concomitant with the increase in the amount of Vt in the ovary, and those of Fainzilber et al. (1992) show that if there is an extraovarian source for Vt in *P. semisulcatus*, its contribution to Vt is quantitatively very low.

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Legends to Figures

- Fig. 1. The concentration of protein (a; in mg ml⁻¹) and vitellogenin (b; in µg ml⁻¹) in the hemolymph (Experiments 1 and 2) related to the average oocyte diameter (AOD). The mean $\pm$ S.D. are shown for samples pooled into four size groups of AOD.
- Fig. 2. The total amount of protein (a) and vitellin (b) in mg per gm fresh weight and the percent of vitellin out of the total amount of protein in ovaries of females at various stages of ovarian development. Results (mean $\pm$ S.D.) were pooled into four size groups according to the average oocyte diameter.
- Fig. 3. The percent of water in ovaries related to the average oocyte diameter ($R^2 = 0.814$).

Fig 2a

Fig 2b

Fig. 3

Running head: Synthesis of yolk in shrimps.

In vivo incorporation of labeled methionine into proteins, vitellogenin and vitellin in females of the penaeid shrimp *Penaeus semisulcatus* de Haan

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Abbreviations: AOD - Average oocyte diameter.

Mw - Molecular weight.

SAT - Subepidermal adipose tissue.

Vg - Vitellogenin.

Vt - Vitellin.

Key words: Crustacea, Penaeidae, Vitellogenin/vitellin.

Abstract

[³⁵S] - Methionine was injected into 11 intact vitellogenic females of the penaeid shrimp Penaeus semisulcatus de Haan. Levels of radiolabeled methionine, total protein and vitellogenin/vitellin (Vg/Vt) were measured in the hemolymph during 24 hours following methionine injections. The same parameters were measured 24 hours after injection, in the hepatopancreas and in the ovaries of the sacrificed females. Proteins were precipitated by trichloroacetic acid and Vg/Vt was immunoprecipitated by anti-Vt serum.

Hemolymphatic protein and Vg/Vt levels remained constant throughout the 24 hours of the experiment starting from the first sampling of hemolymph, two hours after injection. Similiar amounts of Vg/Vt were found in the hepatopancreas compared to the ovary twenty four hours after the injection, and 10% of the labeled protein in the ovary and 6.4% in the hepatopancreas were Vg/Vt. Free labeled methionine was still present in all examined tissues after 24 hours. The labeled protein and Vg/Vt in the ovary and the hepatopancreas could not be explained by the hemolymphatic content of the two organs. The hemolymphatic Vg is 24-fold labeled over the ovarian Vt, 24 hours after injection. The results indicate more intense involvement of the hepatopancreas in the vitellogenic process that can be deduced from earlier in vitro studies. It confirms the earlier definition of the oogenic stage of rapid Vt accumulation in the ovary (AOD range of 150-250 μ m) and also indicate a role for the hemolymph in transporting the Vg between its processing sites.

Introduction

Crustacean vitellin (Vt) is a lipoglycoprotein accumulated in the oocyte cytoplasm and is utilized later for the nutrition of the non-feeding embryo and larval stages. Vitellogenin (Vg), a vitellin-immunoidentical protein, is a female specific protein found in crustacean extraoocytic tissues and hemolymph (Meusy and Payen, 1988). Vg is produced by the subepidermal adipose tissue (SAT) of anostracan (Van Beek et al., 1987), isopod (Picaud, 1980; Souty and Picaud, 1981; Picaud et al., 1989) and amphipod crustaceans (Junera and Croisille, 1980; Croisille and Junera, 1981) before it is transported by the hemolymph into the developing oocyte.

Decapod Vg is present in the SAT (Meusy et al., 1983; Vazquez-Boucard, 1985; Tom et al., 1987), hepatopancreas (Paulus and Laufer, 1987; Quackenbush and Keeley, 1988; Quackenbush, 1989a,b; Shafir et al., in press; Fainzilber et al., submitted) and hemolymph (Kerr, 1969; Cecalldy, 1970; Wolin et al., 1973; Fyffe and O'Connor, 1974; Caubere et al., 1976; Dehn et al., 1983; Durliat, 1984; Marzari et al., 1986; Yano, 1987; Tom et al., 1987; Quackenbush, 1989a; Shafir et al., in press). A major source of decapod yolk is Vt synthesis by the ovary itself (Lui et al., 1974; Lui and O'Connor, 1976, 1977; Dehn et al., 1983; Eastman-Reks and Fingerman, 1985; Yano and Chinzei, 1987; Quackenbush, 1989a; Browdy et al., 1990). The synthetic site of the Vt in Penaeus japonicus was suggested by Yano and Chinzei (1987) to be the follicle cells. No Vg synthesis was found by the in vitro incubated SAT of Penaeus semisulcatus (Fainzilber et al., submitted), however, three studies have reported Vg synthesis in the decapod hepatopancreas: Quackenbush and Keeley (1988) in Uca pugilator; Quackenbush (1989a) in Penaeus vannamei and Fainzilber et al., (submitted) in Penaeus semisulcatus.

Vt endocytosis is described in the ovaries of several decapod species, including penaeids (Beams and Kessel, 1963; Hinsch and Cone, 1969; Wolin et al., 1973; Duronslet et al., 1975; Zerbib, 1979; Shade and Shivers, 1980; Zerbib and Mustel, 1984; Jugan and Soye, 1985). Vg receptors were found and isolated from the oocytic cytoplasm membrane of two decapod species (Jugan, 1985; Laverdure and Soye, 1988; Jugan and Van Herp, 1989).

The penaeid ovary is a well established major synthetic site of Vt compared to the minor quantitative contribution of the penaeid hepatopancreas to the Vg production, as was revealed by in vitro incubations of the penaeid hepatopancreas and ovaries in the presence of labeled amino acids. Yano and Chinzei (1987) found no Vg synthesis at all in P. japonicus hepatopancreas in

contrast to a high Vt synthesis in the ovary. Two additional studies, carried out in P. vannamei (Quackenbush, 1989a) and in P. semisulcatus (Fainzilber et al., submitted; Browdy et al., 1990) revealed smaller rates of yolk synthesis in the hepatopancreas compared to the respective ovarian rates. The rate of Vg production, normalized per mg protein, in the hepatopancreas of P. vannamei was around 10% of the ovarian one at early and mid vitellogenesis. The increase of this percentage to 50% at late vitellogenesis of P. vannamei, is an artifact caused by the normalization method, as the accumulation of yolk in the ovary during the vitellogenesis adds protein which does not contribute to the Vt synthesis but reduces the rate of synthesis per mg protein. A further reduction of the contribution of the whole hepatopancreas Vs. the whole ovary is due to its smaller protein content. The Vg rate of synthesis in the whole hepatopancreas of P. semisulcatus reached 4.35% of the Vt rate of synthesis in the whole ovary. The percentages of synthesized Vg from the total protein synthesis in the hepatopancreas were also low in both P. vannamei and P. semisulcatus (1% and 14.4%, respectively) compared to the similar percentages in the ovary (8% and 40%, respectively).

The small contribution of the in vitro incubated hepatopancreas to Vg synthesis should be further established by complementary in vivo methods, having better resemblance to the natural vitellogenic process. An appropriate method for the examination of the hepatopancreas share in yolk synthesis is the identification of Vg-mRNA in the penaeid hepatopancreas and ovary, a study waiting to be done. Another approach, the one taken by the present research includes determination of the presence of radioactively labeled Vg and Vt in the hemolymph, hepatopancreas and the ovary of vitellogenic penaeid females following injection of labeled amino acid. It is aimed at validation of the overall involvement of the hepatopancreas and the ovary, not distinguishing between production and processing of Vg/Vt and in addition at the examination of the dynamics of the circulating Vg.

Materials and Methods

Animals

Adult Peneaus semisulcatus were collected in Haifa Bay, Israel. Thirty shrimps were held in a 3,000 liter sea-water tank. Water temperature ranged between 18°C (winter) and 27°C (summer). Water was replaced at a rate of 300% per day. Animals were fed daily with defrosted Artemia and a mixture of

defrosted shrimp, fish and squids. Females were individually tagged by clipping of their uropods. Their oogenic stage was monitored periodically by visual examination of the ovaries according to the methods of Browdy and Samocha (1985). Ovarian developmental stages were determined more accurately after sacrificing the females, by measuring the average oocyte diameter (AOD) as described by Shlagman *et al.* (1986). Moulting was recorded daily by identification of the clipped uropods of the collected exuviae.

Chemicals

[³⁵S] - Methionine, 300 mCi/mMol was purchased from Amersham, U.K. All other reagents were of analytical grade.

[³⁵S]-methionine labeling and sampling of labeled tissues

Injections of labeled methionine were carried out with 1 ml disposable syringe through the thin cuticle connecting the thorax and the abdomen. Samples of hemolymph from live individuals were collected from the same site with 1 ml syringe containing premeasured volume of the anticoagulant 10% tri-sodium citrate. Hemolymph from sacrificed females was collected into a premeasured volume of 10% tri-sodium citrate through excision cut in the anterior part of their cephalothorax, at the eyestalk base level.

The ovary and the hepatopancreas were dissected out from each of the sacrificed females and weighed before a portion of the ovary was fixed in 4% formaldehyde in sea water for the measurement of oocyte diameter. The remaining part of the ovary was then weighed again. The hepatopancreas and the ovary from each of the females were homogenized separately in 0.1 M phosphate buffer, pH 7.4. Hemolymph samples and homogenized tissues were centrifuged and the supernatants were used for the following TCA precipitation and radioimmunoprecipitation (RIP).

TCA precipitation and measurement of total labeled methionine

Four 50 µl aliquots taken from each of the supernatants above were pipetted onto Whatman 3 MM filter paper discs and dried. The proteins were precipitated onto two of the discs in 10% TCA according to Mans and Novelli (1961) and all four discs were radioactively counted in scintillation vials with 4 ml Aquasol-2 (NEN) in a Kontron Betamatic liquid scintillation counter.

Radioimmunoprecipitation (RIP)

Radioimmunoprecipitation was carried out by mixing 25 μ l samples of each supernatant above with unlabeled purified vitellin (75 μ g in 25 μ l) and 200 μ l of anti-Vt serum. The anti-Vt serum was prepared and examined for Vt specificity according to Browdy et al. (1990) and Tom et al. (1992). Control samples contained normal rabbit serum (NRS) instead of anti-Vt serum. The total volume in the reaction tube was adjusted to 0.5 ml with 0.4 M NaCl and was mixed thoroughly before overnight incubation at 4°C. Following the incubation, samples were centrifuged and the pellets were washed three times with 0.4 M NaCl. Finally each pellet was dissolved in 75 μ l of 0.5 M NaOH by vigorous shaking for half an hour at room temperature and was neutralized with 25 μ l of 2 M HCl before it was counted.

Complete immunoprecipitation of the labeled antigen is achieved with adequate antigen-antibody ratio. The determination of suitable amounts of reactants was carried out by reacting fixed amounts of unlabeled Vt and labeled sample with increasing amounts of anti-Vt serum, followed by washing and counting of the pellets.

Results

Each of 11 vitellogenic females with AOD ranging between 180 - 350 μ m were injected with 127 μ Ci of [35 S] - methionine dissolved in 20 μ l of distilled water. Regular feeding, temperature and water circulation regimes were kept during the 24 hours experiment. Hemolymph samples were taken every two hours for the first 12 hours. Samples were taken at longer intervals during the last 12 hours of the incubation period to avoid stressing the sampled females. The females were sacrificed after 24 hours, their hemolymph bled and their ovaries and hepatopancreas homogenized separately in measured volume of 0.1 M phosphate buffer, pH 7.4. A piece of each ovary was taken for AOD measurement. The concentration of labeled methionine counted in the hemolymph two hours after injection served as normalization index, assuming that the injected methionine is evenly dissolved in the hemolymph following that period, as was previously found for [3 H] - inulin levels in the hemolymph (Shafir et al., in press). Normalizing of protein and Vg/Vt counts by this index compensates for differences in hemolymph volume and for the amounts of injected methionine which entered the hemolymph.

Three values of radioactivity were counted in each hemolymphatic sample: total labeled methionine, labeled TCA precipitable proteins and labeled

immunoprecipitated Vg (Fig. 1). There is no significant difference in the concentration of both labeled protein and Vg in the hemolymph during the entire experiment, starting from the first sampling two hours after injection. The standard deviations of the levels of labeled protein and Vg increase with time.

Incorporation of methionine into total protein and Vg/Vt in the hepatopancreas and in the ovary, 24 hours after injection, is presented in Table 1. The level of labeled Vg in the examined tissues could not be explained only by the labeled Vg found in its hemolymphatic component even when the whole tissue is considered to be composed of equal volume of hemolymph, assuming 1 gr wet weight of tissue is approximately equivalent to 1 ml of hemolymph (Table 2).

The percentage of the synthesized Vg/Vt from the total protein synthesized in the ovary and the hepatopancreas is presented in Figure 2, which shows a decrease of the relative methionine incorporation into ovarian Vt as the oogenesis proceeds, while the value in the hepatopancreas was constant.

Comparison of the levels of total labeled methionine and TCA precipitable labeled protein in the examined tissues (Fig 1a,b and Table 1) shows that free labeled methionine is present in the female even 24 hours after injection. Free labeled methionine was present in the above tissues also with reduced amounts of injected methionine (results not shown).

The molar ratio (P, in percents) of labeled methionine incorporated into Vg or Vt from the total methionine in the Vg or Vt was calculated using the parameters described in Table 3. Molar amounts of methionine are given per gram body weight.

$$P = \frac{\text{Mols of labeled methionine}}{\text{Mols of total methionine}} \times 100$$

For the hemolymphatic Vg the result was:

$$P = \frac{28210/2.2 \times 10^6/300}{177 \times 0.03/131.2} \times 100 = 0.1056\%$$

and for the ovarian Vt the result was:

$$P = \frac{30590/2.2 \times 10^6/300}{4550 \times 0.03/131.2} \times 100 = 0.0044\%$$

The results show that 24 hours after the injection of labeled methionine,

the ratio between labeled Vg in the hemolymph and the labeled Vt in the ovary is 24 (0.1056 / 0.0044).

Discussion

The possible roles of the ovary, hepatopancreas and hemolymph of the female penaeid shrimp in the vitellogenic process were investigated. This objective was accomplished in part by determining whether [35 S] - methionine had become incorporated into Vg/Vt present in these organs 24 hours after its injection to the hemolymph of vitellogenic females.

Free labeled methionine was found in the hemolymph, ovary and hepatopancreas 24 hours after injection of this amino acid when even an extremely small quantity of radioactive methionine was injected. This observation indicates high levels of endogenous methionine, competing with the incorporation of the labeled one into proteins preventing pulse labeling of proteins. The absence of a pulse of labeled proteins and Vg did not allow determination of Vg turnover in the hemolymph. Injection of labeled Vg into vitellogenic females might be a more suitable method to achieve its pulse labeling in the hemolymph.

A relatively high percentage of the in vivo labeled protein in the ovaries at early vitellogenesis (AOD = 180-250 μ m, figure 2) was Vt (15.5%), which is in agreement with the data of Shafir et al. (in press) who showed that oocytes of this size are rapidly accumulating Vt. The reduced percentage of labeled Vt from the labeled protein found in the present study in late vitellogenesis (4.4%, figure 2) when combined with the slow increase of accumulated Vt in ovaries in the same AOD range (Shafir et al., in press) indicates inhibition of Vt accumulation in the ovary of the intact female at AOD > 250 μ m. In contrast, in vitro incubated ovaries showed no inhibition of Vt synthesis at any stage of vitellogenesis although a decrease in the percentage of synthesized Vt was observed at AOD > 350 μ m, presumably due to the increased synthesis of other proteins (Browdy et al., 1990).

The amounts of labeled Vg and its percentage from the total labeled protein, in the hepatopancreas, were similar to the comparable values of labeled Vt in the ovary after the 24 hour experiment (Table 1 and Fig. 2). These labeled Vg/Vt amounts can not be explained only by the hemolymphatic Vg component of the two organs (Table 2) and they are probably the result of an equilibrium between influx of circulating Vg, contribution of in situ produced Vg and Vg secretion during the 24 hours of the experiment. These

findings strongly suggest that the hepatopancreas has more prominent role in the vitellogenic process than could be deduced from the results of earlier comparable in vitro incubations (Browdy et al., 1990; Fainzilber et al., submitted) (see also introduction). The in vitro studies favor the penaeid ovary as the major Vt synthetic site, a conclusion supported by other studies (Yano and Chinzei, 1987; Quackenbush, 1989a). The differences between the in vivo and the in vitro studies can be attributed to a more efficient Vg synthesis by the intact in vivo hepatopancreas, or to its functioning as a Vg processing site. This processing might involve addition of a polypeptide subunit which would explain the small amount of synthesis of a Vg-immunoidentical substance, or binding of a lipid component to the Vg molecule.

Absorption in penaeid ovaries of non-spawned ovulated oocytes immediately after spawning and in developing oocytes at the commencement of the premoult stage was reported by Browdy (1988). The absorption of ovulated oocytes is correlated with a relatively high Vg level in the hemolymph of recently-spawned females (Shafir et al., in press). Consequently, Vt secretion into the hemolymph is postulated for females with normally developing ovaries and is suggested as one explanation for the presence of Vg in the hemolymph. This Vg is assumed to be either newly synthesized Vt that will be processed further in extraovarian sites, prior to its reuptake by the developing oocytes or a degradation product of previously synthesized Vt. The 24-fold more intensive labeling of Vg compared with the Vt labeling found in the present study, argues against the possibility of unselected secretion of ovarian Vt, because if this were the case, similar Vg/Vt labeling should have been seen in both the ovary and the hemolymph.

In conclusion, the evidence from this and previous studies supports the hypothesis that the hepatopancreas has a more intense involvement in the vitellogenic process than could be deduced from the earlier in vitro studies, but not necessarily only as Vg synthetic site. The rapid Vt accumulation that occurs early in vitellogenesis (AOD range of 150-250 μ m) and the arrest of Vt accumulation later (AOD > 250 μ m), first found by Shafir et al., (in press), is confirmed by the present findings. Further more, the present study shows that the circulating Vg is not a degraded Vt which is secreted unselectively from the ovary.

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Parameters [*]	Ovary	Hepatopancreas
Total [^{35}S] - methionine	0.0573 $\pm$ 0.0450	0.2954 $\pm$ 0.1693
TCA precipitable protein	0.0278 $\pm$ 0.0260	0.0501 $\pm$ 0.0289
Immunoprecipitable Vt	0.0028 $\pm$ 0.0058	0.0032 $\pm$ 0.0021

Table 1: Incorporation of [^{35}S] - methionine into TCA precipitable proteins and Vg/Vt in the hepatopancreas and ovary 24 hours after injection.

[*] - dpm / total dpm in the hemolymph two hours after injection / gram dry weight of the organ (n=11).

Ratios [dpm/dpm]	TCA	RIP
Hepatopaneas/hemolymph	5.07±2.13	4.08±3.34
Ovary/hemolymph	4.82±3.21	2.71±1.33

Table 2: Average ratio (n=11) of labeled proteins in the hepatopaneas or the ovary to the labeled proteins found in an identical hemolymph volume. Tissue volume was estimated by assuming 1 gr wet weight equals 1 ml of hemolymph. The hepatopaneas and the ovary were dissected out 24 hours after the in vivo [^{35}S] - methionine injection. Protein labeling was determined from radioactive counting of the TCA-precipitable proteins (TCA) and the immunoprecipitated Vg/Vt (RIP).

Parameter	value	Source
Percentage of methionine in the Vt	3% (w/w)	Tom <u>et al.</u> (1992)
Mw of methionine residue	131.2.	
Maximum level of hemolymph Vg	177 µg/gr body weight	Shafir <u>et al.</u> (submitted)
Maximum level of ovarian Vt	4550 µg/gr body weight	Shafir <u>et al.</u> (submitted)
Specific activity of labeled methionine	300 µCi/µMol	NEN, U.S.A.
Maximum level of hemolymph labeled Vg	28210 dpm/gr body weight	Present study
Maximum level of ovarian labeled Vt	30590 dpm/gr body weight	Present study

Table 3: The parameters utilized for calculating the molar ratio of labeled methionine incorporated into Vg or Vt, from the total methionine in the Vg or Vt.

Legends to figures

Figure 1: Twenty four hour time course presentation of hemolymph total labeled methionine - (A), labeled protein; - (B) and labeled Vg - (C) normalized by the counts of total labeled methionine in the hemolymph two hours after injection (TC2h). Vertical lines show the standard deviations.

Figure 2: Percentage of labeled Vg/Vt from the labeled proteins in the ovary (filled bars), and the hepatopancreas (white bars), 24 hours after [35 S]-methionine injection. Vertical lines show the standard deviations.

Fig 1 78

Fig 2

Partial characterization of three hemolymph proteins of *Penaeus semisulcatus* de Haan (Crustacea, Decapoda, Penaeidae) and their specific antibodies

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Running title: Hemolymph proteins of *Penaeus semisulcatus*

Key words: Crustacea, Penaeidae, hemolymph, hemocyanin, lipoproteins, vitellogenin.

Footnotes to the title page:

Abbreviations: HDL - high density lipoprotein, LP - Lipoprotein, Hcy - Hemocyanin, Mw - molecular weight, PAGE - polyacrylamide gel electrophoresis, SDS - sodium dodecyl sulphate, Vg/Vt - vitellogenin/vitellin.

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Abstract

1) LP1, a high density lipoprotein common to both sexes of the penaeid shrimp Penaeus semisulcatus was isolated as the upper yellow-brown layer resulting from ultracentrifugation of male hemolymph. the hemolymph solution was adjusted to an hydrated density of 1.2 gr/ml. A similiar HDL fraction was isolated from the hemolymph of vitellogenic females containing, in addition to the LP1, a female-specific lipoprotein, the vitellogenin (Vg).

2) Hemocyanin (Hcy), the oxygen carrier of crustaceans was isolated as pellet, resulting from the same ultracentrifugation. Hcy was identified by its blue color, its copper content and its characteristic absorbance spectrum. It is the most abundant hemolymph protein composed of two major subunits of 85 and 95 kD.

3) Polyclonal antibodies were raised in rabbits against Hcy and LP1. The antisera were found to be specific and no crosreaction was observed between them and an anti-Vg/vt serum prepared during earlier studies. All three antisera proved to be suitable research tools for the identification of these three physiologically important proteins (Vg, LP1, Hcy).

Introduction

Circulating lipoproteins (Lp's) were identified in several crustacean species by specific lipid staining of PAGE profiles (reviewed by Lee, 1990). Two protein bands were stained for lipids in the hemolymph of both sexes of the penaeid P. japonicus (Teshima and Kanazawa, 1980). The LP's were isolated from crustacean hemolymph by gradient ultracentrifugation according to their hydrated densities (Lee and Puppione, 1978; Teshima and Kanazawa, 1980; Puppione et al., 1986; Spaziani et al., 1986; Lee and Puppione, 1988; Lee, 1990). Most crustacean LP's are High Density Lipoproteins (HDL's) revealing various HDL hydrated densities, which might not necessarily reflect presence of different proteins but various levels of lipid load on LP particles composed of similiar apoproteins. Functionally, two types of lipoproteins were identified in the hemolymph, as was recently reviewed by Lee (1990). One type, designated LP1, is common to both males and females. It was isolated from the hemolymph of the crab Cancer antennarius as a three subunits protein with molecular weights of 185, 100 and 84 kD with two hydrated densities, 1.112 gr/ml and 1.187 gr/ml. This difference is a result of the type and amount of their lipid load (Spaziani et al., 1986). A second study carried out on the same species revealed LP1 with only one subunit of 108 kD and a sedimentation coefficient of 5.35 Svedberg (Puppione et al., 1986). A similiar sedimentation coefficient was detected for the LP1 of the crab Callinectes sapidus which is composed of a single subunit of 112 kD with hydrated density of 1.116 gr/ml (Lee and Puppione, 1988). The LP1 of the penaeid shrimp P. japonicus contains two components, an HDL ($d > 1.125$) and a VHDL ($d > 1.21$) (Teshima and Kanazawa, 1980).

A female-specific protein was detected in the hemolymph of vitellogenic females of several crustacean species. In certain species it was also found to be immunoidentical to ovarian vitellin (Vt) and termed vitellogenin (Vg) (Kerr, 1969; Ceccaldi, 1970; Wolin et al., 1973; Fyffe and O'Connor, 1974; Caubere et al., 1976; Dehn et al., 1983; Durliat, 1984; Marzari et al., 1986; Tom et al., 1987a; Quackenbush, 1989; Shafir et al., in press). The lipoprotein characteristics of Vg were determined in several studies. It was isolated from the hemolymph of the crab C. sapidus as a three subunits protein with molecular weights of 190, 107 and 78 kD, a sedimentation coefficient of 10.4 Svedberg ($d = 1.16$ gr/ml) and termed LP2 (Lee and Puppione, 1988). LP2 with similiar characteristics was identified in Cancer antennarius with subunits of 152, 100 and 82 kD and a sedimentation

coefficient of 10.74 Svedberg (Puppione et al., 1986). This LP2 has higher density ($d > 1.125$) than the LP2 isolated from the same species ($d = 1.108$) by Spaziani et al., (1986). The presence of a female-specific Vg in penaeid hemolymph was demonstrated in several studies (Ceccaldi, 1970; Caubere et al., 1976; Tom et al., 1987a; Quackenbush, 1989; Shafir et al., in press). Vg concentration in the female hemolymph in relation to oogenesis was studied by Quackenbush, (1989) in P. vannamei and by Shafir et al., (in press) in P. semisulcatus, revealing maximum concentrations of 1 and 0.5 mg/ml respectively. Vg has never been isolated in penaeids.

Hemocyanin (Hcy) is the circulating oxygen carrier protein of arthropods and molluscs. Crustacean Hcy, as reviewed by Magnum (1983) and Herskovits (1988) contains 0.17% copper, small amounts of carbohydrates (4%) and lipids (Zatta, 1981). It exists as an hexamer with one or two aggregation states of di- and tetrahexamers. Most of the penaeids' Hcy is present in the basic hexameric form (Brouwer, 1978; Ellerton and Anderson, 1981). The six monomers composing each hexamer show two to six electrophoretically distinct variants of the basic subunit and are distinguished by their different Mw's, ranging between 60-90 kD. Subunits heterogeneity plays an important role in the formation of the native polymer. The absorption spectrum of a typical Hcy includes two peaks of light absorbance at 345 and 580 nm, in addition to the usual 280 nm protein band, (Nickerson and Van Holde, 1971).

The present study is aimed at the isolation of LP1 and Hcy from P. semisulcatus hemolymph and examination of the specificity of antisera raised against these two proteins. The two antisera, in addition to the anti-vg/vt serum (Tom et al., 1992) are valuable tools for identification of LP's and Hcy of P. semisulcatus.

Materials and Methods

Animals

Adult Peneaus semisulcatus were collected in Haifa bay, Israel. Up to thirty specimens were held in a 3,000 liter sea-water tank. Water temperature ranged between 18°C (winter) and 27°C (summer). Water was replaced at a rate of 300% per day. Animals were fed daily with defrosted Artemia and a mixture of defrosted shrimp, fish and squids.

Ovarian developmental stages were determined after sacrificing the females, by measuring the average oocyte diameter (AOD) as described by Shlagman et al., (1986).

Electrophoresis

Samples were subjected to 5% and 7% PAGE slab gels according to Hames and Rickwood (1981) and 5% SDS-PAGE slab gels according to Laemmli (1970). A solution of 15 mM Tris base, 5% 2-mercaptoethanol, 2% SDS and 10% glycerol (Sheiffer and Wensink, 1981) was used as the dissociation buffer. The sample was mixed with an equal volume of dissociation buffer (at twofold concentration) and was boiled for 5 min at 100°C before loading onto SDS-PAGE. Coomassie Blue staining for proteins and dithiooxamide staining method for copper-conjugated proteins were carried out according to Hames and Rickwood (1981).

The M_w of reduced SDS dissociated polypeptides was determined by running marker proteins of known M_w . The set of markers utilized for M_w determination (Fig. 4, lane M) contains myosin (205 kD), β -galactosidase (116 kD), phosphorylase B (97 kD), bovine serum albumin (66 kD) and egg albumin (45 kD). A second set of prestained M_w markers which is visualized on both the SDS-PAGE and on its immunoblots assisted the comparison between them. This set (Fig. 4, lane P) contains myosin (205 kD), β -galactosidase (116.5 kD), bovine serum albumin (77 kD) and egg albumin (46.5 kD).

Immunoblotting

The immunoreactivity of the ovarian proteins was examined according to Towbin *et al.*, (1979). Proteins were electroblotted from the gels onto nitrocellulose using a Bio-Rad electroblotting cell with 0.2 M glycine - 0.025 M Tris as running buffer. Methanol (20%) was added to this buffer during SDS-PAGE electroblotting. Electroblotting was performed for 4 hours at 100 mA at 4°C. Immunostaining of proteins bound to nitrocellulose was carried out by diluted primary antisera (1:20,000 or 1:40,000 dilution) and 1:1000 diluted goat anti rabbit IgG labeled with alkaline phosphatase as a secondary antibody. The color reaction was developed utilizing 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as alkaline phosphatase color reaction substrates (immunoblot assay kit, instruction manual, Bio-Rad).

Electroelution

Proteins were electroeluted from non-denatured PAGE using 0.2 M glycine - 0.025 M Tris as running buffer. The sliced bands were soaked in the buffer and subjected to electrical field for 24 hours. The proteins were eluted into dialysis tubes and frozen at -70°C.

Spectrophotometry

The hemocyanin spectrum of absorbance was measured by diode array spectrophotometer (Hewlett-Pacard, 8452A).

Ultracentrifugation

Hemolymph from sacrificed animals was collected into a premeasured volume of 10% tri-sodium citrate as anticoagulant through excision cut in the anterior part of their cephalothorax, at the eyestalk base level. The hemolymph solution was centrifuged at 10000 g for 15 min. and the resulted supernatant was brought to hydrated density of 1.2 gr/ml with solid KBr. The adjusted solution was subjected to ultracentrifugation on Sorvall OTD55B ultracentrifuge equipped with T-875 fixed angle rotor running at 125000 g for 40 hours at 12°C according to Lee and Puppione (1988).

Preparation of antisera

Polyclonal antisera against Vt, LP1 and Hcy were prepared in rabbits. LP1 and Hcy were isolated during the present study. The isolation of Vt and the preparation of anti-vitellin serum as well as the injection schedule of the three antigens were described by Tom et al., (1987b, 1992).

Results

Isolation and partial characterization of LP1 and Hcy

Male hemolymph of Penaeus semisulcatus was subjected to ultracentrifugation. The floating HDL, identified by its brownish color, and the blue pellet were collected. The pellet was dissolved in 0.325 M NaCl. The two fractions were dialysed against 0.325 M NaCl solution and applied on 7% (Hcy) and 5% (HDL) native PAGE (Figs 1,2). The main protein bands of the two respective fractions were cut and electroeluted. The HDL fraction contained only one band (Fig. 1) designated LP1. The dominant protein of the ultracentrifuge pellet was identified as Hcy by its blue color, its staining by specific copper stain (Fig. 2B) and its typical spectrum of absorbance revealing two absorbance bands, in 338 and 570 nm (Fig. 3) in addition to the usual protein absorbance band of 280 nm.

The electroeluted LP1 and Hcy were utilized as antigens. The anti-Hcy serum was immunoblotted against 7% non-denatured PAGE profiles of male and female hemolymph and isolated Hcy. In addition to the main band which served as antigen, two high molecular weight protein bands were immunostained (Fig. 2C).

The 5% SDS-PAGE profiles of ovarian homogenate, Hcy, Vt, male and female hemolymph and HDL were immunoblotted against the three produced antisera (anti-LP1, Vt and Hcy) (Fig. 4) and against nonimmunized rabbit serum as control (results not shown).

The SDS-PAGE subunits profile of the Hcy contains two main bands with Mw of 85 and 95 kD and several weaker bands with lower Mw (Fig. 4A, lane 5). The LP1 profile is composed of a blurred protein band which remained close to the gel origin and of two low molecular weight polypeptides (Fig. 4A, lane 6). The Vt profile (Fig. 4A, lane 2) is similar to the one already described (Tom et al., 1992).

The anti-LP1 serum immunostained the male and the female LP1 (Fig 4C, lanes 6,7). Only the low Mw proteins were faintly stained by this serum in the male and female hemolymph (Fig 4C, lanes 3,4).

the anti-Vt serum immunostained the Vt and the ovarian homogenate as already described by Tom et al., (1992). The female HDL also reacted with the anti-Vt serum but yielded a different profile compared to that of the Vt (Fig 4B, lanes 1,2,7). In contrast, Vt and hemolymph Vg have similar slow mobility in native PAGE as revealed by immunoblotting of ovarian homogenate and female hemolymph with anti-Vt serum (Fig. 5). The female hemolymph was not immunostained with this serum in SDS-PAGE probably due to

the small amounts of the hemolymph Vg. Male hemolymph and HDL did not react with anti-Vt serum nor in native PAGE neither in SDS-PAGE (respective unstained lanes were omitted from both Fig. 4B and Fig. 5).

The two main Hcy bands, immunostained with The anti-Hcy serum (Fig 4D, lane 5) are present in all the other profiles (Fig 4D, lanes 1,3,4,6,7) except the Vt lane (Fig 4D, lane 2). The two Hcy bands in the HDL lanes (Fig 4D, lanes 6,7) are contaminants of the purified LP's. Additional protein bands with lower Mw are also reacting with the anti-Hcy serum.

Discussion

Polyclonal antisera against three hemolymph proteins of the penaeid shrimp P. semisulcatus were prepared in our laboratory as identification tools for studying aspects of the metabolism of these proteins. The preparation and the specificity of the anti-Vt serum were described by Browdy et al., (1990) and Tom et al., (1992). The present study shows that the Vt-immunoidentical female-specific protein present in the hemolymph of P. semisulcatus (Shafir et al., in press) is part of the HDL fraction of the female hemolymph. On SDS-PAGE it reveals a different subunits profile than Vt, of much lower Mw subunits (Fig 4B, lanes 2,7). The SDS-PAGE profile of the LP1 is also composed of low Mw subunits of similar range as the Vg ones (Fig 4B,C, lanes 6,7), indicating the susceptibility of the circulating LP's to the dissociating treatment, in contrast to the ovarian Vt which is more resistant probably due to alteration of its structure while packed in the yolk globules.

Only the male HDL fraction of $d < 1.2$ g/ml was used as an antigen for the production of anti-LP1 serum. Ultracentrifugation at higher densities prevented the precipitation of the Hcy, resulting in severe contamination of the isolated LP1. VHDL fraction of $d > 1.2$ was reported by Teshima and Kanazawa (1980) as part of the LP found in male hemolymph of P. japonicus but its protein characteristics and its similarities to the HDL fraction is not known. Its contribution as a lipid carrier is small, due to its low lipid content but it is responsible for the exceptionally high concentration of LP (11-19 mg/ml) in the hemolymph of P. japonicus (Teshima and Kanazawa, 1980). Hcy also contains a small amount of lipids (Zatta, 1981) and it seems possible that the VHDL fraction is an Hcy fraction relatively highly loaded with lipids. Hcy, in the present study, contaminates even the less dense HDL fraction of $d < 1.2$ g/ml (Fig. 4D, lanes 6,7). A VHDL ($d = 1.3$) which contains Hcy fraction was found also in molluscs (Heras and Pollero, 1992).

The anti-Hcy serum, immunostained additional high Mw proteins (Fig. 2C, lanes 1,2,3) although it was prepared by using only the main Hcy PAGE band (Fig. 2A, lane 4) as an antigen indicating apparent nonspecificity. This apparent non-specificity is probably due to the well known phenomenon of aggregate formation of the basic hexamer unit of Hcy, resulting in high molecular weight proteins (Magnum, 1983, Durliat, 1984). These proteins dissociate into two subunits, representing variants of the basic Hcy monomer when subjected to denaturing PAGE (Fig. 4A,D). Several additional low Mw bands are immunostained by the anti-Hcy serum in the SDS-PAGE profile, a phenomenon which is particularly prominent in the female hemolymph profile presented in this study (Fig. 4D, lane 4). These low Mw polypeptides are probably degradation products of Hcy which could be detected at various intensities by immunostaining of SDS-PAGE profiles of hemolymph samples from different specimens (results not shown). They might also indicate contamination of the anti-Hcy serum by an unidentified antibody. It is therefore recommended to show that the purified Hcy is composed mainly of the two major monomers when using this anti-Hcy serum for identification or isolation of Hcy.

There is no crossreaction among the three antisera as was revealed by the immunostaining of their SDS-PAGE profiles (Fig 4). As expected from a hemolymph lipoprotein common to both sexes, the anti-LP1 serum reacted with both the male and female hemolymph in addition to its reaction with the isolated male and female HDL. The anti-Vt serum immunostained the protein bands in the ovary and the purified Vt described by Tom et al. (1992) (Fig 4B, lanes 1,2) as well as the female hemolymph and HDL (Fig 4B, Lane 7 and Fig. 5), as expected from a female-specific protein, but did not react with male HDL. The immunoreaction of both the anti-LP1 and the anti-Vt sera was much stronger with purified HDL than with hemolymph revealing additional stained bands in the purified HDL's probably due to the relatively low HDL concentrations in the hemolymph. The anti-Hcy serum immunostained two bands of Hcy and several lower Mw bands; none of them immunoreacted with the other two antisera. The two protein bands immunostained by both anti-Vt and anti-Hcy serum in the ovarian homogenate profile (Fig 4B,D lane 1) are due to an overlap between the Hcy and Vt bands in PAGE-SDS as was shown by an earlier publication (Tom et al., 1992). In that study, SDS-PAGE immunostained profiles of ovarian homogenates at various stages of oogenesis revealed gradual increasing immunostaining of the above protein bands with anti-Vt serum, in contrast to slightly decreasing staining with anti-Hcy serum. It is therefore not surprising that the present study

demonstrates that the Vt lane (Fig. 4B,D lane 2) which lacks the Hcy, does not react with the anti-Hcy serum. The Hcy was not identified as such by Tom et al., (1992) and was designated an unidentified peak A protein.

The concentration of LP1 in the ovary is below the detectable level (Fig. 4C lane 1) and it can not be considered as an oocytic storage protein, although the functionally-related insect lipophorine is known to enter and accumulate in the ovary during the vitellogenesis (Kanost et al., 1990).

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Legends to figures

Fig 1: Native 5% PAGE of male P. semisulcatus HDL, Coomassie Blue stained.

Fig 2: Native 7% PAGE of P. semisulcatus, (1) - male and (2) - female hemolymph, (3) - dissolved pellet resulted from the ultracentrifugation and (4) - Hcy electroeluted from PAGE. (A) -Coomassie Blue stained gel, (B) - dithiooxamide stained gel for detection of copper, (C) - nitrocellulose blot immunostained with anti-Hcy serum.

Fig 3: Absorbance spectrum of purified Hcy.

Fig 4:(A) - 5% SDS-PAGE of (1) - P. semisulcatus ovarian homogenat, (2) - Vt, (3) - male hemolymph, (4) - Female hemolymph, (5) - Hcy, (6) - male HDL and (7) - female HDL. (B,C,D) - Immunoblots of the above gel. (A) - Coomassie Blue stained gel, (B) - immunostaining using anti-Vt serum, (C) - immunostaining using anti-LP1 serum. (D) - immunostaining using anti-Hcy serum. M - Mw markers, 45, 66, 97, 116, 205 kD. P - prestained Mw markers, 46.5, 77, 116.5, 205 kD. Mw's of polypeptide subunits were calculated using the mobilities of the Mw markers of lane M. The Mw markers of lane P serves only for comparison between the SDS-PAGE and its immunoblotting profiles. Immunoblotting lanes which did not react with a particular serum were omitted from the figure.

Fig. 5: Immunoblotting of native 7% PAGE of P. semisulcatus (1) - ovarian homogenate, and (2) - hemolymph of vitellogenic female, with anti-Vt serum. Male hemolymph did not react with the anti-Vt serum and its lane was omitted from the figure.

LP1

Fig 1

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Fig 2

Fig 3

Fig 4

Fig 5

Running page head: Lipid synthesis by shrimp ovaries.

Lipid synthesis from acetate by the in vitro incubated ovaries of the
penaeid shrimp *Penaeus semisulcatus* de Haan

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Abbreviations

AOD-average oocyte diameter, DG-diacylglycerol, GAR-goat anti rabbit, MUFA-
monounsaturated fatty acids, PAGE-poly acrylamide electrophoresis, NL-neutral
lipids, NRS-nonimmunized rabbit serum, PC-phosphatidyl choline, PE-phosphatidyl
ethanol amine, PL-polar lipids, PUFA-polyunsaturated fatty acids, RIP-Radio-
immunoprecipitation, SDS-sodium dodecyl sulfate, SFA-saturated fatty acids,
TG-triacylglycerols, TCA-trichloro acetic acid, TLC-thin layer chromatography,
Vt-vitellin.

Abstract

The present research is aimed at (1) The study of lipid synthesis from ^{14}C -acetate, in vitro, by ovarian tissue. (2) The examination of the binding of newly synthesized lipids to ovarian proteins.

Ovarian pieces from Penaeus semisulcatus de Haan at various oogenic stages were incubated in vitro with ^{14}C -acetate for 8 hours. The pieces were homogenized and the homogenates were divided into two. One half was used for lipid analysis, the other half was centrifuged and vitellin was immunoprecipitated from the clear supernatant.

Lipid analysis - lipids were extracted with methanol-chloroform and neutral lipids and phospholipids were separated by TLC. Fatty acids were isolated after saponification, methylated with diazomethane and separated by TLC on AgNO_3 impregnated plates. Lipid synthesis gradually increased during oogenesis. Fifty percents of the newly synthesized lipids was phosphatidyl choline; triacylglycerols reached 30-35%; diacylglycerol was a relatively important component of the synthesized neutral lipids in immature ovaries. Cholesterol was not synthesized. Only saturated (73%) and monounsaturated (26%) fatty acids were labeled. Polyunsaturated fatty acids were not synthesized.

Lipid-protein interaction - vitellin was immunoprecipitated from the homogenate and the precipitate washed and counted. Binding of lipids to vitellin was observed at the commencement of vitellogenesis. Up to third of the newly synthesized lipids were bound to vitellin, with triacylglycerols and phosphatidyl choline as main bound lipids. Binding of lipids to ovarian proteins was also examined by subjecting the homogenates to native PAGE followed by autoradiography. Bound lipids in developing ovaries were conjugated mainly to vitellin. Lipids were bound to two other proteins in immature ovaries.

Introduction

Lipids are part of the reserve materials which accumulate in the developing oocytes of crustaceans during oogenesis. They are bound to vitellin which is packed in yolk globuli and are also present as free lipid droplets (Beams and Kessel, 1963; Zerbib, 1976, 1979, 1980; Schade and Shivers, 1980). The lipid droplets of the amphipod Orchestia gammarellus contains only triacylglycerols (TG) in contrast to the yolk globuli which contain mainly phosphatidyl choline (PC) bound to protein (Zerbib, 1976). No lipid droplets were observed by electron microscopy study in penaeid oocytes (Duronslet et al., 1975), but their existence can not be ruled out.

Ovarian lipid composition in relation to ovarian development was studied in the crab Pachygrapsus marmoratus (Lautier and Lagarrigue, 1988) and the freshwater palaemonid Macrobrachium borellii (Gonzalez-Baro and Pollero, 1988). Ovarian lipid composition in penaeid shrimps during oogenesis was studied by Gehring (1974), Teshima and Kanazawa (1983), Galois (1984), Teshima et al. (1989), Jeckel et al. (1989), Castille and Lawrence (1989) and Mourente and Rodriguez (1991). Lipid percentage ranged between 6.6%-10% of the ovarian wet weight in fully matured ovaries. A linear increase of lipid percentage was observed during oogenesis in most studies, except a decline of lipid percentage found by Gehring (1974) in Penaeus duorarum concurrent with the formation of the cortical crypts, a final stage of penaeid ovarian development.

Phospholipids were found to be the dominating ovarian lipids in most studies except in Teshima et al. (1989) who found triacylglycerols as main ovarian lipid in Penaeus japonicus. Faster accumulation of neutral lipids than of polar lipids was observed by Jeckel et al. (1989) and Mourente and Rodriguez (1991).

The fatty acids which are found in penaeid ovaries are mainly 16:0, 16:1, 18:1, 20:5 and 22:6. Saturated (SFA) and monounsaturated (MUFA) fatty acids prevailed in triacylglycerols, but polyunsaturated fatty acids (PUFA) were an additional important component of phospholipids.

Decapod crustaceans tissues which were examined so far are not capable of synthesizing PUFA (Chang and O'Connor, 1983) and have to obtain them from their diet. The ability of ovaries to synthesize PUFA was never examined in crustaceans.

Lipid synthesis by a decapod ovary was studied by Lui et al. (1974) who incubated the isolated ovary of the crayfish Procambarus sp. in the presence

of radioactively labeled glycerol. Glycerol was incorporated into neutral lipids (NL), Phosphatidyl ethanolamine (PE) and PC. Only labeled PC was bound to vitellin.

The present research is aimed at elucidating the ability of the developing ovary of penaeid shrimp to synthesize lipids in vitro when incubated in the presence of labeled acetate. The binding of the newly synthesized lipids to ovarian proteins is examined.

Materials and Methods

Animals

Adult Peneaus semisulcatus were collected in Haifa bay, Israel. Up to thirty shrimps were held in a 3,000 liter sea-water tank. Water temperature ranged between 18°C (winter) and 27°C (summer). Water was replaced at a rate of 300% per day. Animals were fed daily with defrosted Artemia and a mixture of defrosted shrimp, fish and squids. Females were individually tagged by clipping of their uropods. Their oogenic stage was monitored periodically by visual examination of the ovaries, according to the methods of Browdy and Samocha (1985). Ovarian developmental stages were determined more accurately after sacrificing the females, by measuring the average oocyte diameter (AOD) as described by Shlagman et al. (1986). Moulting was recorded daily by identification of the clipped uropods of the collected exuviae.

Chemicals

[1-¹⁴C]-sodium acetate 56 mCi/mmol was purchased from ARC, U.S.A., [L-¹⁴C]-leucine 300 mCi/mmol was purchased from Amersham U.K. All other reagents were of analytical grade.

Incubation procedures

Pieces of ovarian tissue were incubated in 1 ml MEM deficient medium (Sigma) in accordance to Fainzilber et al. (1989), in the presence of [1-¹⁴C]-sodium acetate. Similar incubations were carried out in the presence of [L-¹⁴C]-leucine to determine the vitality of the incubated ovary by monitoring its rate of protein synthesis and to detect bacterial contamination by incubating tissue-free medium with [L-¹⁴C]-leucine.

Following the incubation, the pieces were homogenized and kept at -70°C for subsequent processing.

Protein determination

Protein was determined according to Bradford (1976) using Bovine serum albumin as standard protein.

TCA precipitation of proteins

50 µl aliquots which were taken from homogenized ovaries and medium were pipetted onto Whatman 3MM filter paper discs and dried. The proteins were precipitated onto the discs in 10% TCA according to Mans and Novelli (1961) and the discs were radioactively counted in scintillation vials with 4 ml Safefluor (Lumac, Netherland) in a Kontron Betamatic liquid scintillation counter.

Radio-immunoprecipitation (RIP)

Radio-immunoprecipitation was carried out according to Browdy et al. (1990). Anti Vt serum was prepared and examined for its Vt specificity according to Browdy et al. (1990) and Tom et al. (1992). The suitable antigen- antiserum ratio which insured complete antigen precipitation was determined by reacting increasing amounts of antiserum with fixed amounts of labeled sample and in the case of immature ovaries also unlabeled purified vitellin. Control RIP tubes contained nonimmunized rabbit serum (NRS) and goat anti rabbit (GAR) serum. The total volume in the RIP tubes was adjusted to 0.5 ml with 0.4 M NaCl and mixed thoroughly before its incubation for one hour at room temperature followed by 24 hours at 4°C. Following the incubation, samples were centrifuged and the pellets were washed three times with 0.4 M NaCl. Finally each pellet was dissolved in 75 µl of 0.5 M NaOH by vigorous shaking for half an hour at room temperature and was neutralized with 25 µl of 2M HCl before it was counted.

Electrophoresis and fluorography

Ovarian homogenates were subjected to 7% native PAGE slab gels. The gels were stained by Commacie blue or Sudan Black B in accordance to Hames and Rickwood (1986).

Radioactive PAGE gels were soaked in EN³HANCE (NEN, U.S.A.) for an hour followed by one hour washing in water and then dried at 60°C in a gel drier. The dried gels were exposed to Kodak XAR5 film at -70°C for a couple of weeks.

Lipid extraction and separation of lipid classes

Lipid extraction was carried out according to Bligh and Dayer (1959).

Separation of the lipid classes was accomplished by thin layer chromatography (TLC) on Kieselgel 60 plates (Merck). The TLC developing solvent for the separation of neutral lipids was composed of n-hexane : diethyl ether : methanol : acetic acid in a ratio of 90 : 20 : 3 : 2 (v/v) and the composition of the solvent for polar lipids separation was chloroform : methanol : acetic acid : water in a ratio of 100 : 20 : 12 : 5 (v/v).

Fatty acids were isolated after saponification, methylated with diazomethane and separated by TLC on AgNO₃ impregnated plates into three classes, saturated, monounsaturated and polyunsaturated fatty acids according to Kates (1986).

Results

Time course incubations

Ovaries were dissected from three vitellogenic females and cut into approximately similar-size pieces. Duplicate pieces from each of the ovaries were incubated in the presence of [1-¹⁴C]-sodium acetate for 2, 4, 6, 8 and 16 hours. At the end of the incubation the tissue pieces were homogenized in NaCl 0.3 N and the lipids were extracted from both the homogenate and the medium and aliquots were radioactively counted. The protein content of the homogenate was determined from its concentration in the supernatant of an aliquot of centrifuged homogenate.

The rate of lipid synthesis per mg protein of ovarian homogenates is presented in Fig. 1. Eight hours were set as standard incubation duration because only small amounts (6.04±3.15%) of the synthesized lipids were secreted to the medium after 8 hours in comparison to a three fold higher percentage after 16 hours (18.07±2.71%). Constant rate of lipid synthesis is maintained during the first 8 hours of incubation.

The extracted lipids from the pieces which were incubated for 2, 8 and 16 hours were separated into their lipid classes. The results are presented in Table 1.

Lipid synthesis by the ovary

Four replicates of two lateral ovarian lobes were dissected from 16 females

in various stages of oogenesis. The tissue pieces were incubated in the presence of [1- 14 C]-sodium acetate for 8 hours. Following the incubation, the tissues were homogenized in the incubation medium, the lipids were extracted from an aliquot of the homogenate and separated into lipid classes by TLC. The radioactivity of the separated lipids was counted and the results are presented in Fig. 2 (total extracted lipids) and Fig. 3 (their separation to lipid classes). The rate of lipid synthesis is increasing during oogenesis (Fig. 2) and the main synthesized lipids are PC and TG. Synthesized DG is found in relatively higher amounts in immature ovaries (Fig. 3).

73.3 $\pm$ 8% of the synthesized fatty acid in the ovary were SFA and 26.3 $\pm$ 7.3% were MUFA. No PUFA were detected. Six females with AOD ranged between 50-313 μ m were examined.

Lipid binding into proteins

A lipid cap is formed during centrifugation of ovarian homogenate.

Preliminary examination of the possible trapping of protein-bound lipids in that lipid cap was tested by the following experiment: two vitellogenic ovaries were incubated in vitro in the presence of 14 C-leucine. Following 8 hours of incubation, the ovarian homogenates were subjected to the above centrifugation. The polypropylen centrifugation tube was immediately frozen in liquid nitrogen, the cap was cut out and suspended in 0.3 M NaCl by sonication. Residues of the infranatant were washed from the suspension by repeating twice the centrifugation, separation and suspension of the lipids. The proteins were precipitated from both the final lipid suspension and the infranatant by TCA. Only small amounts 2.53% (AOD of female=223 μ m) and 6.29% (AOD of female=317 μ m) of the radiolabeled proteins were trapped in the lipid cap. This allowed the estimation of the amount of lipid bound to vitellin by immunoprecipitation of the vitellin from the clear supernatant.

An aliquot from each ovarian homogenate was centrifuged for 15 minutes at 10000g, 4°C. the lipid cap, assumed to contain almost only free lipids, and the pellet were discarded. The vitellin was immunoprecipitated from the infranatant and counted. only the pellet which was precipitated from homogenate of developing ovaries revealed significant amounts of labeled lipids (Fig. 4). Lipids were extracted from the immunoprecipitate and separated into lipid classes (Fig. 5). PC contributed less to the Vt bound lipids than to the total synthesized lipids with the opposite for the TG, which contributed more to the Vt bound lipids.

The homogenates of incubated immature and developing ovaries were separated on 7% PAGE. The gels were subjected to fluorography. The ovarian protein bands on the gel were identified according to Tom et al. (1992). The results are presented in Fig. 6 and revealed that in developing females, almost all the bound labeled lipid was conjugated to vitellin. Two additional proteins were found to bind lipids in immature ovaries.

Discussion

Fatty-acid-containing lipids, which are accumulating in the crustacean ovary during oogenesis, could originate from the diet or could be synthesized by the ovary itself. The ovary of P. semisulcatus can synthesize from acetate only SFA and MUFA, which is in agreement with results obtained from other crustaceans tissues (Chang and O'Connor, 1983) but was never examined in the ovaries. PUFA, which are found also in penaeid ovaries has to be obtained from the diet and transported through the hemolymph. The synthesized lipids are mainly PC and TG and the in vitro rate of synthesis is much higher in developing ovaries than in immature ones. Two ovarian lobes which contain a constant portion of the ovarian weight were chosen as a normalizing unit for the comparison of the rate of lipid synthesis among ovaries. Using ovarian weight or its protein amount for this purpose is not adequate due to the ovarian gain of weight during oogenesis, caused by accumulation of reserve materials. These reserves, including proteins and lipids, do not participate in lipid synthesis but diminish the rate of synthesis per normalizing unit and lead to artificially lower rate of lipid synthesis towards maturity (see also Browdy et al., 1990).

The lipid class composition is not significantly changing with time during the 16 hours of the time course incubation except a significant decline in DG percentage (table 1) occurring probably because DG are intermediate products and converted into other lipid classes accumulated later in the incubation. Similarly during the longer time period of oogenesis, a decrease of the percentage of synthesized DG occurred in developing ovaries probably due to the accumulation of PC and TG, end products of the lipid biosynthesis. Most of the newly synthesized lipids were accumulated in the ovary and only minor secretion into the incubation medium was observed.

The most important result of this in vitro research is the finding that a significant percentage of the synthesized lipids in developing ovaries are bound to vitellin and most of it was TG and PC, in contrast to the findings of

Lui et al. (1974) that only PC is bound to vitellin of Procambarus sp. The Vt-bound lipids contained higher percentage of TG at the expense of PC compared to the lipid class profile of the total synthesized lipids. The lipid class profile of both bound and total lipids synthesized in the ovary, is different from that found in hemolymph which contains mostly PC (Allen, 1972; Lee and puppione, 1978; Teshima and Kanazawa, 1980; Spaziani et al., 1986; Lee and puppione, 1988; Vazquez-Boucard et al., 1989; Lee, 1990). Binding of lipids to vitellin and the alteration of the lipid class profile while entering the oocyte could be an efficient tool distinguishing genuine lipid uptake from lipoprotein binding to the external surface of the plasma membrane. Future experiments dealing with lipid uptake by the developing oocyte will be published separately.

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Time [h]	FFA [%]	DG [%]	TG [%]	PL [%]
2	2.38±2.38	13.36±5.86	32.55±3.73	51.71±10.37
8	1.29±1.67	5.04±1.23	39.54±14.67	54.13±15.45
16	4.76	2.18	45.24±14.31	52.45±18.31

Table 1: Separation of lipids synthesized in the ovary into lipid classes during time course incubation. Duplicates of ovarian tissue were incubated for each female at each incubation duration.

Legends to figures

Fig. 1: Time-course incorporation of ^{14}C -acetate into lipids. Replicates of ovarian pieces (n=4 per female per time point) from three vitellogenic females were incubated in the presence of ^{14}C -acetate for 2, 4, 6, 8 and 16 hours. Tissue was separated from medium, washed, homogenized and the lipids were extracted from the homogenate and the medium. Lipid synthesis is presented as ^{14}C -acetate incorporated into lipids per mg protein of homogenate.

Fig. 2: Rate of ^{14}C -acetate incorporation into lipids at various stages of oogenesis. Lipids were extracted from 16 ovarian homogenates after in vitro incubation in the presence of ^{14}C -acetate and counted.

Incubation unit is two ovarian lobes per 8 hours.

Fig. 3: Lipid class profil of synthesized lipids in ovaries at various

stages of oogenesis. Lipids were extracted from 16 ovarian homogenates after in vitro incubation in the presence of ^{14}C -acetate and neutral lipids and phospholipids were separated by TLC. Results are presented as the percentage of ^{14}C -acetate incorporated into lipid class compared to its incorporation to total synthesized lipid. AOD - average oocyte diameter, FFA - free fatty acids, DG - diacylglycerols, TG - triacylglycerol, PC - phosphatidyl choline, PE - phosphatidyl ethanol amine, PL's - other polar lipids: Black bar - AOD=43-50 μm , n=3, white bar - AOD=116-158 μm , n=4, dotted bar - AOD=222-367 μm , n=9.

Fig. 4: Vitellin was immunoprecipitated from homogenates of in vitro incubated ovaries and lipids were extracted from the precipitate. The percentage of their radioactive counts compared to the counts of total synthesized lipids is presented.

Fig. 5: Lipids were extracted from immunoprecipitated vitellin and separated to lipid classes. Results are presented as the percentage of ^{14}C -acetate incorporated into lipid class compared to its incorporation to total immunoprecipitated lipids. AOD - average oocyte diameter, FFA - free fatty acids, DG - diacylglycerols, TG - triacylglycerol, PC - phosphatidyl choline, PE - phosphatidyl ethanol amine, PL's - other polar lipids. white bar - AOD=88-158 μm , n=5, dotted bar - AOD=222-290 μm , n=7.

Fig. 6: Ovarian homogenates were centrifuged following incubation in the presence of ^{14}C -acetate. After removal of the upper fat layer, the infranatant was subjected to native PAGE followed by autoradiography and immunoblotting with anti-vitellin serum. (A)-Autoradiogram. (B)-Commassie Blue stained PAGE. (C)-Sudan Black B stained PAGE. (D)-Immunostaining with anti-Vt serum. (1)-Immature ovary. (2)-Vitellogenic ovary. The vitellin band is indicated by an arrow.

Fig 1 Fig 2

Fig 3

Fig 4

Fig 6

Fig 5

Isolation of peptide affecting protein and vitellin synthesis in the ovary.

Introduction

Previous results in our laboratory led us to the conclusion that the ovary is a major site engaged in the synthesis of vitellin (Fainzilber et al., 1992; Browdy et al., 1990). Consequently, we developed an in vitro system for testing the effect of hormones and other pharmacological agents on vitellin and protein synthesis in the ovary (Browdy et al., 1990).

The in vitro assay unit consisted of ovaries removed from a single female. The ovaries were dissected out and cut into 20- 30 pieces. One of these pieces of tissue was fixed in 4% formalin prepared in sea water and was used to measure the average oocyte diameter (AOD) and the others were used in the bioassay. Each piece was incubated in a well of a culture plate for 8 hours in the presence of labeled leucine. Each group consisted of 4 pieces and was subjected to different hormonal treatments. Each treatment, untreated control and sham treated groups were included in each experiment. The sham treated tissues were incubated with the hormone solvent.

After the incubation, each ovarian piece was homogenized in 1 ml of phosphate buffer, pH 7.4, and its protein concentration was determined (Bradford, 1976). The amount of leucine incorporated into newly synthesized proteins and specifically into vitellin (Vt) was determined by TCA precipitation and Vt-immunoprecipitation, respectively (for details refer to Browdy et al., 1990). The amount of newly synthesized proteins and Vt was related to the protein content (in mg) for each tested piece of ovary.

Results

1. Gonad inhibiting hormone (GIH) from *P. semisulcatus* sinus glands

X organ-sinus gland neurohormones affect many physiological processes in crustaceans, including ovarian development (Fingerman, 1987). Therefore, sinus glands (SG's) were dissected from eyestalks of *P. semisulcatus* males and from females with undeveloped ovaries. The SG's were homogenized in 10% acetic acid, centrifuged and the supernatant was used in the experiment. The crude extract and later its purified fractions were assayed for their effect on the synthesis of protein and vitellin in in vitro incubated ovaries.

Both male and female SG crude extract inhibited protein synthesis in the ovary (Table 1).

The activity of the SG extract was not affected by boiling or lyophilization

(Table 2). The effect of aliquots of SG extract of 0.1 Eye Stalk Equivalent (ESE) that were boiled or lyophilized and reconstituted in 10% acetic acid was similar to that of untreated SG extract. All three inhibited protein and Vt synthesis.

The crude SG extract was applied on RP-HPLC column and eluted with 0.1 TFA-70% acetonitrile gradient. The eluent was collected in five fractions (Fig. 1). The eluted fractions were freeze dried, reconstituted to the original injection volume by 10% acetic acid and assayed (Table 3).

Fraction 4 (F4) was the most potent inhibitor of protein synthesis. Fractions F3 and F5 also inhibited protein and Vt synthesis in one assay (female 81, Table 3). Male SG extract, subjected to HPLC gradients similar to those of Fig. 1 revealed similar chromatograms. Fraction 4 was further separated to 6 components on RP-HPLC (F4(1-6), Fig. 1) and peak 6 was found to be the active GIH (Table 4). The N-terminus of Peak F4(6) was sequenced (35 AA). It resembles the recently established CHH-related peptide family which is unique to crustaceans. We continue the sequencing in collaboration with Dr. Tim Hayes from the Texas A&M university.

Female #	AOD [μ m]	Treatment	TCA [cpm/mg pr]	RIP [cpm/mg pr]	TCA [%]*	RIP [%]*
104	242 $\pm$ 29	Control	19636 $\pm$ 2385	8436 $\pm$ 1750	100	127
		Sham	19692 $\pm$ 1034	6630 $\pm$ 309	100	100
		F SG ext.	12830 $\pm$ 1021	4905 $\pm$ 839	65	74
105	231 $\pm$ 48	Control	40633 $\pm$ 4690	7897 $\pm$ 2091	100	120
		Sham	40481 $\pm$ 4226	6580 $\pm$ 257	100	100
		F SG ext.	16989 $\pm$ 3012	4851 $\pm$ 863	42	73
106	225 $\pm$ 29	Control	32727 $\pm$ 1778	15402 $\pm$ 2249	94	93
		Sham	34904 $\pm$ 2299	16572 $\pm$ 1370	100	100
		F SG ext.	19939 $\pm$ 2573	10150 $\pm$ 2061	57	61
		M SG ext.	16412 $\pm$ 0998		47	
107	212 $\pm$ 29	Control	39583 $\pm$ 7910	15230 $\pm$ 2241	97	104
		Sham	40586 $\pm$ 5055	14648 $\pm$ 1453	100	100
		F SG ext.	19565 $\pm$ 6732	8315 $\pm$ 3798	48	57
		M SG ext.	18293 $\pm$ 4958	8807 $\pm$ 3159	45	60

Table 1: The effect of 0.1 ESE of sinus gland extracts from P. semisulcatus males and females on the amount of synthesized protein and vitellin in in vitro incubated ovaries. Each treatment was carried out with 4 replicates and results are presented as a mean $\pm$ S.D.

F-females, M-males, SG-sinus gland extract.

TCA - TCA precipitated proteins.

RIP - Immunoprecipitated Vt.

* - The percentage (in %) of labeled protein or vitellin of the treated and control groups was compared to the sham treated controls.

Female #	AOD [μ m]	Treatment	TCA [cpm/mg pr]	RIP [cpm/mg pr]	TCA [%]*	RIP [%]*
78	267 $\pm$ 34	Control	43654 $\pm$ 3982	11557 $\pm$ 869	104	93
		Sham	41637 $\pm$ 4892	12460 $\pm$ 1770	100	100
		Untreat.	26542 $\pm$ 3216	6192 $\pm$ 1073	64	50
		Boiled	28100 $\pm$ 3656	8095 $\pm$ 1200	67	65
		Lyophil.	27838 $\pm$ 828	6380 $\pm$ 1367	67	51
79	215 $\pm$ 38	Control	61094 $\pm$ 6361	17335 $\pm$ 2496	102	107
		Sham	59790 $\pm$ 3646	16248 $\pm$ 4301	100	100
		Untreat.	37930 $\pm$ 2824	10365 $\pm$ 1096	63	64
		Boiled	39600 $\pm$ 3674	7860 $\pm$ 897	58	49
		Lyophil.	37152 $\pm$ 6796	8577 $\pm$ 1231	62	53
80	184 $\pm$ 27	Control	51184 $\pm$ 5027	8932 $\pm$ 1201	106	99
		Sham	48284 $\pm$ 3448	9005 $\pm$ 1288	100	100
		Untreat.	34747 $\pm$ 5122	4020 $\pm$ 1133	72	45
		Boiled	31614 $\pm$ 1176	5462 $\pm$ 564	66	61
		Lyophil.	33200 $\pm$ 5868	4103 $\pm$ 571	69	46

Table 2: The effect of 0.1 ESE of SG extracts from P. semisulcatus females, subjected to various treatments, on the amount of synthesized protein and vitellin in in vitro incubated ovaries. Each treatment was carried out in 4 replicates and results are presented as a mean $\pm$ S.D.

TCA - TCA precipitated proteins.

RIP - Immunoprecipitated Vt.

* - The percentage (in %) of labeled protein or vitellin of the treated and control groups was compared to the sham treated controls.

Female #	AOD [μ m]	Treatment	TCA [cpm/mg pr]	RIP [cpm/mg pr]	TCA [%]*	RIP [%]*
81	235 $\pm$ 42	Control	86147 $\pm$ 31860	29289 $\pm$ 2451	104	98
		Sham	82964 $\pm$ 30675	29867 $\pm$ 2678	100	100
		F1	92531 $\pm$ 19345	28684 $\pm$ 1591	111	96
		F2	79182 $\pm$ 30122	24546 $\pm$ 2311	95	82
		F3	48290 $\pm$ 8316	15453 $\pm$ 1605	58	51
		F4	37711 $\pm$ 17463	10936 $\pm$ 1153	45	37
		F5	57299 $\pm$ 5468	17763 $\pm$ 1215	69	59
82	188 $\pm$ 31	Control	69983 $\pm$ 9936	22965 $\pm$ 2424	100	106
		Sham	69900 $\pm$ 9129	21725 $\pm$ 1457	100	100
		F1	82165 $\pm$ 9550	22592 $\pm$ 1900	117	104
		F2	71833 $\pm$ 4340	20705 $\pm$ 1567	103	95
		F3	74147 $\pm$ 7111	20880 $\pm$ 1767	106	96
		F4	47211 $\pm$ 8195	15853 $\pm$ 1133	67	73
		F5	79934 $\pm$ 9803	21269 $\pm$ 1111	114	98
83	113 $\pm$ 22	Control	88847 $\pm$ 22877	16273 $\pm$ 2327	126	109
		Sham	70104 $\pm$ 16640	14828 $\pm$ 2120	100	100
		F1	75192 $\pm$ 14315	17070 $\pm$ 3943	107	115
		F2	71872 $\pm$ 15487	12565 $\pm$ 642	102	85
		F3	74535 $\pm$ 8432	13338 $\pm$ 624	106	90
		F4	42022 $\pm$ 4228	7870 $\pm$ 2141	60	53

Table 3: The effect of five fractions purified by HPLC from the sinus gland (0.1 ESE) of *P. semisulcatus* females, on the amount of synthesized protein and vitellin in in vitro incubated ovaries. Each treatment was carried out in 4 replicates and results are presented as a mean $\pm$ S.D.

TCA - TCA precipitated proteins.

RIP - Immunoprecipitated Vt.

* - The percentage (in %) of labeled protein or vitellin of the treated and control groups was compared to the sham treated controls.

F1-F5 - Fractions 1-5 (see Fig. 1).

Female #	AOD [μm]	Treatment	TCA [cpm/mg pr]	TCA [%]*
126	258±30	Control	3319±422	70
		Sham	4737±181	100
		F4(6)	1763±556	37
127	187±29	Control	8634±2101	104
		Sham	8337±1801	100
		F4(6)	4587±778	55

Table 3: The effect of fractions purified by HPLC from the sinus gland of P. semisulcatus females, on the amount of synthesized protein in in vitro incubated ovaries. Each treatment was carried out in 4 replicates and results are presented as a mean ± S.D.

TCA - TCA precipitated proteins.

* - The percentage (in %) of labeled protein of the treated and control groups was compared to the sham treated controls.

(F4(6) see Fig. 1).

Figure 1: RP- HPLC chromatogram of SG extract from P. semisulcatus females with undeveloped ovaries. The extract was prepared in 10% acetic acid.

Solvent A - 0.1% TFA. Solvent B - 0.1% TFA in 100% acetonitrile. F1-5 and F4(1-6) are the collected fractions. (---) - Gradient curve [% B].

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In vivo stimulation of ovarian development in the red swamp crayfish, Procambarus clarkii (Girard) by 5-hydroxytryptamine

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Summary

The possibility that biogenic amines affect ovarian development in the red swamp crayfish, Procambarus clarkii, was investigated. Females were administered 15 $\mu\text{g/g}$ body weight (b.w.) of norepinephrine, dopamine, 5-hydroxytryptamine (5-HT) or octopamine on days 1, 5 and 10 and were sacrificed on day 15. Crayfish given 5-HT showed significant increases in ovarian index (30.5%) and oocyte size (34.0%) over the concurrent controls, while norepinephrine, dopamine and octopamine did not significantly affect either the ovarian index or oocyte size. Significantly more labeling by ^{14}C -leucine of ovarian proteins was found in ovaries of crayfish that were injected with 5-HT in vivo, but when ovarian lobes from crayfish that had not been injected with 5-HT were incubated in vitro with 5-HT added to the incubation medium no significant change in the level of incorporation of ^{14}C -leucine into ovarian proteins occurred.

The 5-HT receptor blocker LY53857 (25 $\mu\text{g/g}$ b.w.) retarded ovarian development. The 5-HT releaser fenfluramine and the 5-HT potentiator fluoxetine (both 15 $\mu\text{g/g}$ b.w.) were also used. Crayfish given fenfluramine, fluoxetine, fenfluramine plus 5-HT or fluoxetine plus 5-HT showed significant increases of ovarian index (24.0-102.8%), oocyte size (20.0-87.4%) and in vitro ^{14}C -leucine labeling of ovarian proteins (30.6-123.6%) over the concurrent controls. The ovaries of crayfish that received the 5-hydroxytryptaminergic neurotoxin 5,6-dihydroxytryptamine (10 $\mu\text{g/g}$ b.w.) did not show any significant change as compared with the initial control. These findings are consistent with the hypothesis that 5-HT, which is present in the central nervous system of Procambarus clarkii, exerts its stimulatory effect on the ovary of this crayfish indirectly, by triggering release of the ovary-stimulating hormone.

Introduction

Several neurotransmitters have been identified as affecting the release of neurohormones in crustaceans. Dopamine was found to stimulate release of chromatophorotropic red and black pigment-concentrating hormones (Fingerman and Fingerman, 1977; Fingerman and Rosenberg, 1988) and the distal retinal pigment dark-adapting hormone (Kulkarni and Fingerman, 1986). Octopamine inhibits the release of black pigment-dispersing hormone (Quackenbush and Fingerman, 1984), whereas norepinephrine stimulates the release of this hormone (Fingerman et al., 1981a) and the distal retinal pigment light-adapting hormone (Kulkarni and Fingerman, 1986). 5-Hydroxytryptamine (5-HT) was found to stimulate the release of several hormones, including the hyperglycemic hormone (Keller and Beyer, 1968), the red pigment-dispersing hormone (Rao and Fingerman, 1975), the neurodepressing hormone (Aréchiga et al., 1985) and the molt-inhibiting hormone (Mattson and Spaziani, 1985).

The biogenic amines, norepinephrine, dopamine, octopamine and 5-HT used in the study described herein occur in the central nervous system of crayfishes (Elofsson et al., 1982; Breen and Atwood, 1983). Recently, Aréchiga et al. (1990) demonstrated 5-HT-like immunoreactivity in axons of the lamina ganglionaris of the eyestalk of Procambarus clarkii, the crayfish used in this study. 5-HT- containing neurons have also been demonstrated by immunocytochemistry in the brain of the crayfishes, Pacifastacus leniusculus (Elofsson, 1983), Orconectes virilis (Sandeman and Sandeman, 1987) and Cherax destructor (Sandeman et al., 1988). Rao and Fingerman (1975) found that 5-HT functions as a neurotransmitter that stimulates release of the red pigment-dispersing hormone in the dwarf crayfish, Cambarellus shufeldti.

Development of the ovary in crustaceans is regulated by two antagonistically acting

neurohormones, the ovary-inhibiting hormone (OIH) reported for the first time by Panouse (1943) from the X-organ-sinus gland complex in the eyestalk of the prawn, Palaemon serratus, while Ōtsu (1960, 1963) using the crab, Potamon dehaani, provided the first evidence for an ovary-stimulating hormone (OSH) that he found in the brain and thoracic ganglion. Recently, Kulkarni et al. (1991), studying ovarian development in Procambarus clarkii, showed that the eyestalk neuroendocrine complex, presumably containing OIH, inhibited *in vitro* ^{14}C -leucine incorporation into ovarian proteins, whereas the brain, subesophageal ganglion and thoracic ganglia of this crayfish, presumably containing OSH, stimulated such *in vitro* ^{14}C -leucine incorporation into ovarian proteins. With the fiddler crab, Uca pugilator, Richardson et al. (1991) reported that 5-HT has a stimulatory effect on ovarian development, and Kulkarni and Fingerman (1992a), also using this fiddler crab, supported the findings of Richardson et al. by performing experiments that involved use of the 5-HT agonists fenfluramine and fluoxetine. 5-HT appears to exert its effect on the fiddler crab ovary indirectly, most likely by stimulating OSH release. The aim of this investigation was to extend the studies of the roles of neurotransmitters in regulating ovarian development to the ovary of the red swamp crayfish. The experiments described herein were designed to explore whether norepinephrine, dopamine, 5-HT or octopamine might influence ovarian development in Procambarus clarkii.

Materials and Methods

Animals

Female red swamp crayfish, Procambarus clarkii, in intermolt (stage C₄, Reddy et al., 1990), 30-35 mm carapace length, were purchased from a local seafood dealer in the month of

January, 1991. This period corresponds to the beginning of the annual period of ovarian growth and maturation. The crayfish were held in a recirculating freshwater facility (200 l/h) at 24°C and acclimatized for 3 days before use. The crayfish were fed commercial crayfish food (P.M.G. Inc. Palmetto, LA) ad libitum every third day.

Experimental protocol

In each experiment groups of 15 similar sized crayfish were used. The number of groups depended upon the experimental protocol. Always, in each experiment one group served as the initial control. This group of crayfish did not receive any treatment and was sacrificed on the first day of the experiment. These 15 crayfish were weighed; then their ovaries were removed and weighed. Five ovaries were fixed in aqueous Bouin's fluid for ultimate microscopic examination whereas the lobes of the rest of the ten ovaries were used to study ¹⁴C-leucine labeling of ovarian proteins. A second group in each experiment served as an additional control, which received vehicle alone, i.e. physiological saline (Van Harreveld, 1936) and ran concurrently with the experimental groups. In the initial set of experiments, the crayfish in four additional groups received norepinephrine hydrochloride, dopamine hydrochloride, 5-HT creatinine sulfate or octopamine hydrochloride (15 µg/g body weight, b.w.) in a dose of 100 µl per injection. As will be described below, of these four compounds, only the 5-HT stimulated ovarian development. Consequently, additional experiments were performed to provide further support that 5-HT produces ovarian development in this crayfish. These experiments involved the use of 5-HT agonists and antagonists. LY53857 (5-HT receptor blocker; Fuller and Snoddy, 1979), fenfluramine (5-HT releaser, Consolo et al., 1979), fluoxetine (5-HT potentiator, Wong et al., 1975) and 5,6-dihydroxytryptamine (5,6-DHT; 5-HT neurotoxin, Baumgarten et

al., 1982) were chosen to be used. In addition to the initial and concurrent controls, other groups received a 100 μ l dose of agonist alone or 15 μ g/g b.w. of fenfluramine or fluoxetine in 50 μ l dose followed by 15 μ g/g b.w. 5-HT in a 50 μ l dose 30 min after the first injection. 10 μ g/g b.w. of the 5-HT antagonist 5,6-DHT was injected alone in a dose of 100 μ l per injection. Each of these compounds was dissolved in isosmotic crayfish physiological saline. In all of the experiments, injections were given on the first, fifth and tenth days. All of these crayfish were sacrificed on day 15. As was done with the crayfish of the first set of experiments, these crayfish in the second set of experiments also were weighed and their ovaries were removed, weighed and a few were fixed in aqueous Bouin's fluid whereas the rest were used to study in vitro 14 C-leucine incorporation into ovarian proteins. Each experiment was performed three times, and the averaged results are presented below. Standard Error of the Mean (SEM) was calculated for each mean. The ovarian index (OI) for every crayfish used was determined using the conventional formula:

$$OI = \frac{\text{Wet weight of the ovary}}{\text{Wet weight of the crayfish}} \times 100$$

After 24 h of fixation, the ovaries were dehydrated in an alcohol series, and embedded in paraffin (m.p. 56-58 °C). Seven μ m sections were prepared and stained with Delafield's hematoxylin followed by counterstaining in alcoholic eosin (Bancroft and Stevens, 1982). One hundred oocytes from each group were measured to determine their diameters by using an ocular micrometer with a light microscope.

In vitro study

In vitro labeling of ovarian proteins by 14 C-leucine was studied using the same procedure

described earlier (Kulkarni et al., 1991). Briefly, the radiolabeled amino acid, ^{14}C -leucine (318.5 $\mu\text{Ci}/\text{mmole}$), was purchased from New England Nuclear Corp. (Westwood, MA) and equilibrated for 24 h before determining the counts per minute (CPM) with a Beckman LS6000IC liquid scintillation counter ($< 10\%$ error). Ovaries of initial and concurrent controls and of crayfish having been treated with either LY53857, 5-HT, fenfluramine, fluoxetine or 5,6-DHT individually, or fenfluramine or fluoxetine in combination with 5-HT were used. For the study of the incorporation of labeled leucine into total acid precipitable proteins, individual ovaries of untreated individuals (initial control) and the experimental ones treated for 15 days were rinsed in Van Harreveld's physiological saline and separated into lobes. One lobe, 6-7 mm long, was then transferred to a vial containing 2 ml of saline pH 7.2, and 2.5 μCi of ^{14}C -leucine. In addition, 5-HT (15 $\mu\text{g}/\text{g}$ of the lobe) was added to an additional vial containing an ovarian lobe (6-7 mm long) from a crayfish that had been treated in the same manner as were the concurrent control individuals to determine whether 5-HT would directly stimulate ^{14}C -leucine incorporation into ovarian proteins. These *in vitro* incorporation studies follow closely the methods of Fainzilber et al. (1989) and Browdy et al. (1990) who used 0.5-4.0 μCi of leucine in each 2 ml of incubation medium for their studies of protein synthesis in the ovary of the shrimp, Penaeus semisulcatus. The medium was gently aerated during incubation at 24°C . After 24 h of incubation, the tissue was removed, weighed and homogenized in 2 ml of cold 10% trichloroacetic acid (TCA) with a ground glass homogenizer. The suspension was centrifuged at $5000 \times g$ for 10 min at 4°C . The pellet was then washed twice with cold TCA, resuspended, and filtered with a Millipore suction filter (Millipore Corp. Bedford, MA), and washed finally with 5 ml of ethanol:ether (2:1). The filter paper discs were air dried and

submerged in Biofluor for radioactivity determination. Ovarian protein was determined with a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). The results are presented as Specific Radioactivity in CPM/ μ g protein.

Norepinephrine (arterenol hydrochloride), dopamine, (3-hydroxytyramine hydrochloride), 5-hydroxytryptamine creatinine sulfate, octopamine hydrochloride, fenfluramine hydrochloride and 5,6-dihydroxytryptamine creatinine sulfate were purchased from Sigma (St. Louis, MO). LY53857 [4-iso-propyl-7-methyl-9-(2-hydroxyl-1-ethylpropoxycarbonyl) 4,6,6A,7,8,9, 10, 10A-octahydroindolo (4,3-FG) quinoline maleate] and fluoxetine hydrochloride were gifts from Lilly Research Laboratories (Indianapolis, IN).

Results

Effects of biogenic amines on the ovary

Initially, the effects of the biogenic amines norepinephrine, dopamine, 5-HT and octopamine on oocyte growth of Procambarus clarkii were determined. The average OI and oocyte diameter data obtained from the three experiments are presented in Figure 1. These results reveal that only 5-HT (15 μ g/g b.w.) induced ovarian development as evidenced by the significantly larger OI's and oocyte diameters as compared with both the initial and concurrent control crayfishes. Comparison of the data for just the initial and concurrent controls shows that the ovaries in the concurrent control crayfishes were slowly developing during the course of these experiments, and 5-HT injection clearly accelerated this growth. Figures 2-4 show representative ovaries from initial control crayfish and those that received physiological saline (concurrent control) and 5-HT. The stimulatory effect of 5-HT can be seen.

Effects of 5-HT agonists and antagonists on the ovary

The objective of this portion of the study was to obtain further support that 5-HT stimulates ovarian development in Procambarus clarkii. The data presented in Figure 5 show that crayfish administered 25 $\mu\text{g/g}$ b.w. of the 5-HT receptor blocker LY53857 showed less ovarian development than did the saline injected concurrent controls. The ovaries of crayfish that were injected (15 $\mu\text{g/g}$ b.w.) with 5-HT, fenfluramine, fluoxetine, fenfluramine plus 5-HT or fluoxetine plus 5-HT showed significant increases in OI (24.0-102.8%) and oocyte size (20.0-87.4%), whereas those administered 10 $\mu\text{g/g}$ b.w. 5,6-DHT did not show any significant change in their structural profile when compared with the initial controls.

The ovaries of crayfish that received (15 $\mu\text{g/g}$ b.w.) 5-HT, and fenfluramine and fluoxetine alone, or in combination with 5-HT when incubated in vitro with ^{14}C -leucine at the end of each 15-day experiment, showed significant increases in labeling by ^{14}C -leucine of ovarian proteins (30.6-123.6%) as compared to the initial and concurrent controls. In contrast, the ovaries of crayfish that were given 25 $\mu\text{g/g}$ b.w. LY53857 or 10 $\mu\text{g/g}$ b.w. 5,6-DHT showed less ^{14}C -leucine incorporation into ovarian proteins than did the concurrent controls (Fig. 6). No significant difference in the labeling of ^{14}C -leucine of ovarian proteins was found in ovarian lobes of normal crayfish that were incubated in vitro with 5-HT (15 $\mu\text{g/g}$ of the lobe, 36.4 ± 5.1 CPM/ μg protein) when compared with the values for the concurrent (32.7 ± 3.2 CPM/ μg protein) controls.

Discussion

The annual breeding cycle of the red swamp crayfish, Procambarus clarkii, in Louisiana has been described as having three phases (Penn, 1943; Culley and Duobinis-Gray, 1990). I: Growth and maturation terminating in ovulation and extrusion of mature eggs (January-May);

II: Burrowing with fertilized eggs attached to the pleopods (June-September); and III: Hatching of eggs producing young-of-the-year (October-December). Kulkarni et al. (1991) divided oocyte development in this crayfish into seven stages according to structural features of the oocytes: (1) oogonial, (2) immature, (3) avitellogenic, (4) early vitellogenic, (5) midvitellogenic, (6) late vitellogenic and (7) postvitellogenic and resorptive. The present study was started and completed in the first phase of this breeding cycle with crayfish having oocytes in the third stage of development.

There is continuing, and increasing, interest in identifying the roles of neurotransmitters in inducing the release of neurohormones that regulate the reproductive activities in both vertebrates (Crim et al., 1984) and invertebrates (Fingerman, 1987). 5-HT, for example, has been reported to be involved in the functioning of the gonads of fishes (Emata et al., 1985; Somoza and Peter, 1991), insects (Raina, 1974; Gouddey-Pèriere et al., 1991) and mollusks (Matsutani and Nomoura, 1987; Kadam and Koide, 1990). From the present study norepinephrine, dopamine and octopamine do not appear to have any role in ovarian development in Procambarus clarkii, whereas 5-HT is an excellent candidate for a stimulatory role. The presence of 5-HT in the central nervous tissues of Procambarus clarkii has been demonstrated by immunocytochemistry (Fujii and Takeda, 1988; Aréchiga et al., 1990), and we in this laboratory have quantitatively measured its content in this crayfish using reverse phase high performance liquid chromatography (Kulkarni and Fingerman, 1992b). The quantity ranged between 54 and 168 pg/mg wet weight of tissue.

As mentioned above, ovarian development in crustaceans is regulated by two neurohormones, OIH from the X-organ-sinus gland neurosecretory complex in the eyestalks and

the OSH from the brain and ventral nerve cord (Panouse, 1943, 1944; Ōtsu, 1963, Eastman-Reks and Fingerman, 1984; Mohamed and Diwan, 1991; Amaldoss et al., 1991). Kulkarni et al. (1991), using ovaries of Procambarus clarkii *in vitro*, found that eyestalk neural tissue inhibits ^{14}C -leucine incorporation into ovarian proteins, whereas, brain, subesophageal ganglion and thoracic ganglia stimulate such incorporation. In the present study when ovarian lobes were incubated *in vitro* with 5-HT added to the medium, there was no significant change in the amount of labeling of ovarian proteins by ^{14}C -leucine as compared to the initial and concurrent controls. Therefore, it appears that the stimulatory action of injected 5-HT on the ovary of Procambarus clarkii is not due to a direct action on the ovary. Consequently, we favor the hypothesis that 5-HT exerted its observed *in vivo* effect on the ovary indirectly, by triggering release of OSH.

The crayfish administered the 5-HT receptor blocker LY53857 showed less ovarian development than did the saline injected concurrent controls (Fig. 5). This effect of LY53857 is consistent with the hypothesis that 5-HT triggers release of the OSH in Procambarus clarkii. LY53857 antagonizes the action of 5-HT by blocking 5-HT receptor sites (Fuller and Snoddy, 1979). Previously, Fingerman et al. (1981b) reported that injection of LY53857 blocks the 5-HT-stimulated release of the red pigment-dispersing hormone in the fiddler crab, Uca pugilator, while Richardson et al. (1991) found that LY53857 slows development of the ovary of the fiddler crab, Uca pugilator.

Fenfluramine, a 5-HT releaser, when injected into Procambarus clarkii presumably produced release of more 5-HT than occurred in the concurrent controls injected with saline, resulting in increased release of OSH. Therefore, the oocytes of the crayfish that received

fenfluramine would be expected, as occurred, to be larger than those in the concurrent controls (Figure 5). Likewise, fluoxetine, a 5-HT potentiator, when injected into Procambarus clarkii presumably produced its stimulating action on the ovaries through its effect as a 5-HT reuptake blocker. This action would potentiate the effect of 5-HT. As stated above, these experiments were performed at a time when the ovaries of even the control crayfish were developing on their own, presumably under the influence of endogenous 5-HT. Furthermore, when fenfluramine and fluoxetine were injected in combination with 5-HT, ovarian development was significantly greater than in those crayfish administered 5-HT, fenfluramine or fluoxetine alone (Fig. 5). When the ovarian lobes from crayfish that were injected with 5-HT, and fenfluramine or fluoxetine alone or in combination with 5-HT were incubated in vitro with ^{14}C -leucine at the conclusion of each 15-day experiment, significant increases in labeling in ovarian proteins were observed (Fig. 6). These results indicated in Figure 6 are not only consistent with the data in Figure 5 that showed that these ovaries were growing in size under the influence of 5-HT and its agonists but also that these stimulated ovaries were synthesizing proteins at a faster rate than were the control ovaries. Much of this newly formed protein was presumably being incorporated into vitellin.

It is now well known that 5,6-DHT produces selective degeneration of 5-hydroxytryptaminergic neurons, producing 5-HT depletion, in both vertebrates and invertebrates (Fingerman et al., 1974; Baumgarten et al., 1982). Fingerman et al. (1974) reported that administration of 5,6-DHT decreased the 5-HT concentration in the eyestalks and brain of the fiddler crab, Uca pugilator. During the 15-day period of each of the present experiments, the ovaries of the saline injected concurrent control Procambarus clarkii showed, a slow, but

normal, rate of development, exhibiting increased OI and oocyte size. In contrast, inhibition of ovarian development was observed in the crayfish injected with 10 $\mu\text{g/g}$ b.w. of 5,6-DHT. The observations that 5,6-DHT decreases 5-HT levels in the central nervous system tissues of Procambarus clarkii (Kulkarni and Fingerman, 1992b) and that 5-HT stimulates ovarian development, presumably by triggering release of OSH, can explain the effects of 5,6-DHT on the ovaries. Due to the depletion of 5-HT from central nervous tissues under the influence of injected 5,6-DHT little or no 5-HT was available to trigger release of OSH, and consequently the ovaries of the 5,6-DHT treated crayfish failed to develop at the same rate as those of the concurrent controls.

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LEGENDS

Fig. 1. Mean ovarian indices (OI) and oocyte diameters (μm) of Procambarus clarkii administered 15 $\mu\text{g/g}$ body weight on the first, fifth and tenth days of the experiment norepinephrine (NE), dopamine (DA), 5-hydroxytryptamine (5-HT) or octopamine (OA), and sacrificed on the fifteenth day. Numbers in parentheses indicate the percent change from the saline injected concurrent control values (CC). Error bars are SEM. IC, initial control.

Fig. 2. Procambarus clarkii. Transverse section of the ovary of initial control crayfish having mostly avitellogenic oocytes. X 430. Calibration bar = 24 μm . FC, Follicle cells (arrow); N, Nucleus; OT, ovarian tissue.

Fig. 3. Procambarus clarkii. Transverse section of the ovary of saline injected concurrent control crayfish after 15 days. Oocytes show increase in size as compared with initial control in Fig. 2, and a few avitellogenic oocytes advanced to early vitellogenic stage. X 210. Calibration bar = 45 μm . FC, Follicle cells (arrow); N, Nucleus.

Fig. 4. Procambarus clarkii. Transverse section of the ovary of 5-hydroxytryptamine injected crayfish after 15 days. Oocytes show increase in size as compared with concurrent control in Fig. 3, and most of the oocytes look matured with distinct yolk (y) granules. X 190. Calibration bar = 48 μm . FC, Follicle cells (arrows); N, Nucleus.

Fig. 5. Mean ovarian indices (OI) and oocyte diameters (μm) of saline (concurrent control, CC) or 5-HT, 5-HT agonists and 5-HT antagonists (alone or in combination with 5-HT; see Materials and Methods for details) injected Procambarus clarkii after 15 days. Numbers in parentheses indicate the percent change from the concurrent control values, whereas numbers in parentheses with asterisk show the percent change from the initial control values (IC). Error bars are SEM. 5,6-DHT, 5,6-dihydroxytryptamine; 5-HT, 5-hydroxytryptamine; FEN, fenfluramine; FLU, fluoxetine; LY, LY53857.

Fig. 6. Incorporation of ^{14}C -leucine into ovarian proteins of Procambarus clarkii after 15 days of injecting saline (concurrent control, CC) or 5-HT, 5-HT agonists and 5-HT antagonists (alone or in combination with 5-HT; see Materials and Methods for detail). Numbers in parentheses represent the percent change from the concurrent control values, whereas numbers in parentheses with asterisk denote the percent change from the initial control values (IC). Error bars are SEM. 5,6-DHT, 5,6-dihydroxytryptamine; 5-HT, 5-hydroxytryptamine; FEN, fenfluramine; FLU, fluoxetine; LY, LY53857.

MEAN OOCYTE DIAM. (μm)

Fig 1

O.I

TREATMENTS

Fig 2

Fig 3

Fig 4

Fig. 6

Quantitative Analysis by Reverse Phase High Performance
Liquid Chromatography of 5-Hydroxytryptamine in the Central
Nervous System of the Red Swamp Crayfish, Procambarus clarkii

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Abstract. The concentrations of 5-hydroxytryptamine (5-HT) in central nervous organs of the red swamp crayfish, Procambarus clarkii, were determined by reverse phase high performance liquid chromatography with electrochemical detection. The quantity ranged between 54 and 168 pg/mg wet weight of tissue. The amount is highest in the brain, followed in decreasing order by the thoracic ganglia, subesophageal ganglion, eyestalks and abdominal nerve cord. Significant increases in the levels of 5-HT in the eyestalks, brain, subesophageal ganglion and thoracic ganglia occurred in crayfish exposed for 3 days to continuous light on a white background, whereas the 5-HT levels in these tissues decreased in crayfish kept in darkness. Electrical stimulation of central nervous organs in vitro produced significant decreases in the levels of 5-HT. Fenfluramine (5-HT releaser), 5,6-DHT (5-HT neurotoxin) and reserpine (5-HT depletor) induced significant decreases in the 5-HT levels in the portions of the central nervous system tested.

Introduction

The biogenic amines, norepinephrine, dopamine, histamine, octopamine, 5-hydroxytryptamine (5-HT) and gamma aminobutyric acid, are known to function as neurotransmitters in various animals (Werman, 1966; Gerschenfeld, 1973; Krnjevic, 1974; Fingerman, 1985); and have been found in crustacean central nervous organs (Beltz and Kravitz, 1983; Elofsson, 1983; Laxmyr, 1984; Fingerman, 1985; Sandeman *et al.* 1988). Aréchiga *et al.* (1990) showed that in the red swamp crayfish, Procambarus clarkii, the species used in this study, there is in the lamina ganglionaris of the eyestalk a set of axons with 5-HT-like immunoreactivity. These investigators also found that the responsiveness of the retinal photoreceptors of this crayfish to light is enhanced by exposure to 5-HT, both *in vivo* and *in vitro*. With respect to other crayfishes, 5-HT- containing neurons have been reported in the optic lobes and proto-, deuto- and tritocerebral regions of the brain of Pacifastacus leniusculus (Myhrberg *et al.*, 1979; Elofsson, 1983), Orconectes virilis (Sandeman and Sandeman, 1987) and Cherax destructor (Sandeman *et al.*, 1988).

Kulkarni *et al.* (1991) have shown that 5-HT stimulates oocyte maturation in Procambarus clarkii. In view of this observation and the earlier study of Aréchiga *et al.* (1990), we felt it would be useful to determine for the first time the quantity of 5-HT in the central nervous system of this crayfish. In addition, we determined the effects on the 5-HT concentration in components of the central nervous system of (1) continuous exposure to light or darkness, (2) *in vitro* electrical stimulation and (3) pharmacological agents known to affect 5-HT levels in vertebrates.

Materials and Methods

Animals

Red swamp crayfish, Procambarus clarkii, were purchased from a local seafood dealer and maintained in the laboratory at 24°C in a recirculating freshwater system. They were acclimatized to

the laboratory conditions (12:12 L:D) for at least 2 days before being used in an experiment.

Medium sized (carapace length 40-50 mm), intermolt (Stage C₄, Reddy *et al.*, 1990) crayfish of both sexes were used. The crayfish were fed commercial crayfish food. Fiddler crabs, Uca pugilator, were obtained from the Gulf Specimen Co., Panacea, Florida, and acclimatized for 3 days to the laboratory conditions under 12:12 L:D in a recirculating artificial seawater system.

Tissue preparation and homogenization for 5-HT determinations

To determine initially the 5-HT concentrations in the components of the central nervous system of crayfish maintained in the stock tanks, the eyestalks, brain, subesophageal ganglion, thoracic ganglia and abdominal nerve cord of 100 crayfish were dissected out as rapidly as possible in cold Van Harreveld's crayfish physiological saline (Van Harreveld, 1936). These components were then distributed, 10 per tube, in tubes containing 1 ml of 0.4 M perchloric acid and sonicated in 4 cycles of 30 sec each with a sonicator (Biosonik-II, Bronwill Scientific) equipped with narrow probe for small volume work. The homogenates were centrifuged (10,000 x g) for 15 min. at 4°C. The pH of the supernatant, after decanting, was adjusted to 6.0 with 2 M potassium carbonate and the mixture was again centrifuged for 10 min., and the supernatants were used for further purification. The wet weight of each tissue was recorded. The averages for the 10 determinations of the 5-HT contents of each nervous system component was then calculated.

Purification and analysis of the sample

To determine 5-HT the supernatant was filtered and purified on a weakly acid cation exchanger column, Amberlite IRP-64 (Hansson and Rosengren, 1978). Purification was done using a glass column, 30 mm long with 5 mm i.d. equipped with a Millipore HV 0.45 µm filter (Nihon Millipore Kogyokk) at the bottom and filled with 300 µl of Amberlite IRP-64. The supernatant of the extract was loaded at a rate of 50 µl/min. The 5-HT adsorbed onto the resin was eluted by 1 ml of 1.2 N HCl and 100 µl aliquots were collected. The recovery of 5-HT from the ion exchange resin

was determined after elution with 1.2 N HCl. By eluting with 2 x 1.0 ml 1.2 N HCl and combining these two fractions, for faster analysis of only one sample instead of two, we obtained high recovery, which was always in the range of 80-82%. The data presented were corrected to reflect the recovery percentage. The remaining samples were stored at -70°C (Hansson and Rosengren, 1978; Elofsson, et al., 1982).

A Waters RP-HPLC unit, Model 501, fitted with a U6K universal LC injector and a 3.9 X 150 mm 5 µm silica C-18 steel column with a small guard column, coupled to a Waters electrochemical detector (Model 460) was used for the quantitative analysis of 5-HT in the samples. Samples (25 µl) were run five times, and the results were averaged. The variation among samples was less than 10%. The elution reagent was methane sulphonic acid (40 mM) and phosphoric acid (30 mM) in 17 % methanol, pH 2.5, and was thoroughly degassed before use (Hansson and Rosengren, 1978; Elofsson *et al.*, 1982; Nässel and Laxmyr, 1983). The pressure applied was 1500 psi with a flow rate of 1 ml/min. The detector was a glassy carbon electrode, and the working potential was set at +0.75 V against the reference electrode. The 5-HT concentrations were determined by comparing the peak height in the elution profile of the sample with that of the standards, and are presented as pg/mg of wet tissue. One additional criterion, other than elution time, was used to identify the 5-HT peak in the samples. To the samples was added a small amount (10 µg/ml) of synthetic 5-HT before analysis. In no case did the sample peak show any inhomogeneity due to the addition of 5-HT to the biological material when compared with the peak of the standard. With this analytical system we could detect as little as 25 pg of 5-HT.

Calibration curve

To prepare the calibration curve, to nine clean glass tubes each containing 2 ml of 0.4 M perchloric acid were added a known amount of 5-HT creatinine sulfate monohydrate (0-51.2 ng/ml

free base) and 10^{-5} M 3,4-dihydroxybenzylamine hydrobromide (DHBA) (internal standard). The samples were loaded onto the ion exchange column (Amberlite IRP-64) and treated as above. Samples (100 μ l) were collected and 25 μ l of the eluate was injected onto the RP-HPLC column. The peak heights were recorded and fitted in a graph against the concentration of 5-HT free base. Retention time with 1 ml/min flow rate and at 1500 psi was found 8.8-8.9 min for 5-HT and 3.8 min for DHBA (Fig. 1).

Experimental protocols

The 5-HT content in the central nervous organs of the crayfish were initially determined using specimens that had been exposed for 2 days to 12:12 L:D. In addition, 20 equal-sized crayfish were held continuously for an additional 3 days either under a fluorescent light (450-500 lx) or in darkness. After these three days, all of the central nervous organs from 10 crayfish were dissected out for 5-HT determinations. In addition, central nervous organs were removed from the rest of these crayfish and homogenized in 2 ml of crab physiological saline (Cooke *et al.*, 1977), pH 7.4. These extracts were then centrifuged (10,000 x g) at 4°C and the supernates were used to bioassay the red pigment-dispersing activity in eyestalkless fiddler crabs, *Uca pugilator*, of 10-15 mm carapace width. The pigment in the erythrophores of these eyestalkless crabs that were used to assay the extracts of the crayfish tissues was initially maximally concentrated. The erythrophores were staged according to the method of Hogben and Slome (1931) wherein stage 1 indicates maximal pigment concentration, stage 5 maximal pigment dispersion and stages 2, 3 and 4 the intermediate conditions. The Hogben and Slome stages for the experimental and control animals were then used to calculate Standard Integrated Responses (SIR) of the erythrophores of *Uca pugilator* to the nervous tissue extracts of *Procambarus clarkii* according to the method of Fingerman *et al.* (1967). Briefly, when pigment dispersion occurs the sum of the Hogben and Slome stages recorded for the duration of the experiment for the control group is subtracted from the corresponding sum for the experimental group. The difference is the

SIR. The SIR integrates the amplitude, which is based on the observed Hogben and Slome stages, and duration of the response of the erythrophores. A dose of 50 μ l containing the tissue extract equivalent to either one eyestalk, brain, subesophageal or thoracic ganglion was injected into each crab.

For experiments on in vitro electrical stimulation, the entire optic tract including the major ganglia and sinus gland was removed from the eyestalk, and maintained in 50 μ l physiological saline. The remaining nervous tissue from the brain to the end of thoracic nerve cord was also dissected out intact and carefully placed in 100 μ l of physiological saline. Electrical stimulation of the isolated tissue was performed as described in detail by Quackenbush and Fingerman (1984). Briefly, the eyestalk tissue was held in place with a suction electrode attached to the stump of the optic nerve, whereas the central nerve tract, from the brain to the end of the thoracic nerve cord, was held in place by a suction electrode attached at the brain end. The stimulation given via the suction electrode was 5 pps, 4 msec delay, with 40 msec duration pulses of various voltages (10, 15, 20 and 25 V). Stimulation was delivered by a Grass Stimulator, Model S44, with a stimulus isolation unit, Model SIU 5A (Grass Instrument Co. Quincy, Mass). After a stimulation bout of 2 min, the nervous tissues were homogenized in 0.4 M perchloric acid and processed for 5-HT determination as described above. A total of 75 crayfish were used, divided equally among unstimulated controls and four voltage stimulated groups.

Experiments on the effects of pharmacological agents, such as fenfluramine (5-HT releaser, Consolo et al., 1979), fluoxetine (5-HT potentiator, Wong et al., 1975), 5,6-dihydroxytryptamine (5,6-DHT) (5-HT neurotoxin, Baumgarten et al., 1982) and reserpine (5-HT depletor, Myhrberg et al., 1979; Elofsson et al., 1982), on the levels of 5-HT in the central nervous tissues of Procambarus clarkii were performed according to the procedure of Myhrberg et al. (1979). Crayfish were divided into five groups of 15 each. The first group received physiological saline alone, and served as the

control. The crayfish in the second, through fifth groups were administered various concentrations (10-25 $\mu\text{g/g}$ body weight) of either fenfluramine, fluoxetine, 5,6-DHT or reserpine respectively. All injections were given once in a dose of 50 μl and the crayfish were sacrificed after 2 h and their nervous tissues were removed and processed for 5-HT analysis as described earlier.

Fenfluramine hydrochloride, 5,6-DHT, reserpine and Amberlite IRP-64 were purchased from Sigma. Fluoxetine hydrochloride was a gift from Lilly Research Laboratories, whereas the 5-HT creatinine sulfate monohydrate and DHBA were purchased from Aldrich. All drugs were dissolved in isosmotic crayfish physiological saline.

The data obtained from these experiments were analyzed statistically by calculating Standard Error for each of the means (SEM).

Results

The results of the measurements quantifying 5-HT in the central nervous organs, eyestalks, brain, subesophageal ganglion, thoracic ganglia and abdominal nerve cord, of the crayfish maintained under laboratory conditions (12:12 L:D; 24°C) in recirculating freshwater for 2 days are summarized in Figure 2. The 5-HT concentration was highest in the brain (168 pg/mg) and lowest in the abdominal nerve cord (54 pg/mg), with the eyestalk, subesophageal ganglion and thoracic ganglia having intermediate concentrations. Because of the small concentration of 5-HT in the abdominal nerve cord relative to the rest of the central nervous organs, only eyestalks, brains, subesophageal ganglia and thoracic ganglia were used in the rest of the experiments.

The pigment in the erythrophores of the illuminated Procambarus clarkii on the white background was concentrated whereas the red pigment of the crayfish kept in darkness was dispersed. The results presented in Figure 3 show that there were significant increases in the 5-HT concentrations in the eyestalks, brain, subesophageal ganglion and thoracic ganglia of crayfish held under continuous light for three days on a white background. The corresponding red pigment-

dispersing SIR values evoked in the fiddler crabs by these extracts also increased. In contrast, the 5-HT levels in the eyestalks, brains, subesophageal ganglia and thoracic ganglia of crayfish held in darkness, and the SIR values showed significant decreases when compared to the controls, as the basis of the SEMs.

In the experiments where eyestalk neural ganglia and the central nerve tract, brain to the end of the thoracic nerve cord, were electrically stimulated with various voltages (10, 15, 20 and 25 V), 25 V were found to be most effective. The data in Figure 4 are for tissues stimulated with 25 V. The stimulation produced significant decreases in the concentration of 5-HT in all the tissues, with the maximum decrease occurring in the eyestalks (-37.4%) and the minimum in the thoracic ganglia (-22.8%). Furthermore, when extracts of the stimulated tissues were bioassayed for red pigment-dispersing activity in crabs, it was found that stimulation also caused a significant decrease in the SIR values evoked by the extracts of the electrically stimulated tissues as compared to the tissue extracts from the control crayfish. Interestingly, the percentage decrease in 5-HT content decreased progressively in the tissues along the central nervous chain from the brain to the thoracic ganglia.

Of the concentrations tested, the smallest concentrations that produced significant effects on the 5-HT level after 2 h were 15 $\mu\text{g/g}$ body weight of fenfluramine, 10 $\mu\text{g/g}$ body weight of 5,6-DHT and 15 $\mu\text{g/g}$ body weight of reserpine. Fenfluramine induced a significant decrease of 5-HT from all of the nervous tissues (Fig. 5). Release was maximum from the eyestalks and least from the thoracic ganglia. None of the concentrations (10-25 $\mu\text{g/g}$ body weight) of fluoxetine produced any significant effect on the 5-HT concentration in any of the tissues. 5,6-DHT and reserpine produced significant decreases in the 5-HT concentration of all the nervous tissues tested. The maximum decrease was produced in the eyestalks, whereas the minimum decrease occurred in the thoracic ganglia.

Discussion

Histochemical studies by means of fluorescence microscopy have revealed the presence of

yellow-fluorescing cells, indicative of 5-HT, in the eyestalks, brain and ventral nerve cord of the crayfishes, Astacus astacus (Elofsson *et al.*, 1966) and Pacifastacus leniusculus (Myhrberg *et al.*, 1979). The identification of biogenic amines and determination of their concentrations in arthropods had earlier depended on fluorometric methods. Those methods required relatively large tissue samples and yield readings only in the microgram or nanogram range. The advent of HPLC technology enabled investigators to analyze the 5-HT content of tissues from small arthropods with only moderate amounts of biogenic amines, and the sensitivity has been extended down to the picogram level.

A comparison of the amount of 5-HT present in various tissues of the central nervous system of the red swamp crayfish, Procambarus clarkii, revealed distinct differences among them (Fig. 2). These experiments provide for the first time data on the amount of 5-HT in different nervous tissues of this crayfish. The level of 5-HT found in the eyestalks (102 pg/mg) of Procambarus clarkii is comparable to the value reported by Elofsson *et al.* (1982) for the eyestalks (100 pg/mg) of another crayfish, Pacifastacus leniusculus, whereas the brain (168 pg/mg) of Procambarus clarkii contained slightly more 5-HT than the brain (150 pg/mg) of Pacifastacus leniusculus (Elofsson *et al.*, 1982), but like Pacifastacus the 5-HT level in the brain of Procambarus is higher than in the eyestalk.

The 5-HT levels in all nervous tissues of crayfish held for three days under constant illumination on a white background were higher than the corresponding values for the crayfish held for three days in complete darkness and of the control crayfish held under 12:12 L:D (Fig. 3). Furthermore, the red pigment-dispersing SIR values for the erythrophores of the eyestalkless fiddler crabs, Uca pugilator, that received the extracts of eyestalks, brain, subesophageal ganglion and thoracic ganglia of the light adapted crayfish were also significantly higher than the corresponding SIR values evoked by the nervous tissues of the dark adapted crayfish and the initial controls. Earlier studies with the fiddler crab, Uca pugilator, and dwarf crayfish, Cambarellus shufeldti, that revealed

5-HT functions as a neurotransmitter that stimulates release of red pigment-dispersing hormone (Rao and Fingerman, 1970, 1975). The changes that occurred in the 5-HT concentrations in the central nervous organs of the crayfish in darkness or illuminated while on a white background presumably reflect this role of 5-HT in color change hormone release. The red pigment of the crayfish kept in light on a white background was concentrated. The central nervous tissues of these crayfish on a white background not only contained higher 5-HT levels than the tissues of the control crayfish but also evoked higher red pigment-dispersing SIR values, observations that are consistent with a pigment-dispersing hormone releasing role of 5-HT. Presumably because the red pigment was concentrated, neither 5-HT nor pigment-dispersing hormone was being used, thereby accounting for the increased levels of both substances. Earlier, Fingerman *et al.* (1964) reported the presence of erythrophorotropic hormones in the eyestalks and brain of juvenile and adult Procambarus clarkii. They observed that the injection of an extract containing one-third of an organ complement per dose significantly evoked pigment migration in eyestalkless animals. More recently, McCallum *et al.* (1988, 1989) confirmed the previous findings of Fingerman *et al.* (1964) by isolating and amino acid sequencing of the pigment-dispersing hormone (PDH), which is an octadecapeptide, from the eyestalks of Procambarus clarkii.

Rao and Fingerman (1975) later reported that 5-HT, when injected into the dwarf crayfish, Cambarellus shufeldti, caused dispersion of the red pigment in the erythrophores, as in the fiddler crab, but was ineffective when tested *in vitro* on isolated chromatophore-bearing pieces of the crayfish carapace. In these crayfish on a white background where their red pigment was concentrated 5-HT turnover would have presumably decreased because with the red pigment concentrated, 5-HT would not be used to stimulate release of red pigment-dispersing hormone, and consequently a rise in the intraneuronal concentration of this neurotransmitter would occur. On the other hand, because darkness fostered red pigment dispersion, the crayfish in darkness would be utilizing the intraneuronal

stores of 5-HT to effect red pigment dispersion and would according to the hypothesis be expected to have a lower intraneuronal concentration of 5-HT than the crayfish on white background under light, which the present data show is indeed the case.

Previously, Berlind and Cooke (1970) reported the release of a neurosecretory peptide hormone from the pericardial organs of the spider crabs, Libinia emarginata and Libinia dubia, following electrical stimulation. Later, Quackenbush and Fingerman (1984) found that electrical stimulation of the isolated eyestalks of the fiddler crab, Uca pugilator, produces release of chromatophorotropic peptides from the sinus gland. Recently, Kulkarni and Fingerman (1991), also using Uca pugilator, showed that the distal retinal pigment light-adapting hormone is released by electrical stimulation of isolated eyestalk neuroendocrine tissues. The data presented in Figure 4 clearly show that 25 V stimulation reduced the 5-HT levels in the central nervous tissues of Procambarus clarkii. Furthermore, the stimulation appears also to have reduced the amount of stored red pigment-dispersing hormone in the central nervous system because the extracts of the stimulated tissues produced lesser SIR values for red pigment dispersion than did the tissues of the unstimulated controls. In their studies, both Quackenbush and Fingerman (1984) and Kulkarni and Fingerman (1991) bioassayed only the bathing fluid and not the actual stimulated tissue. In the present study the stimulated tissues were extracted and bioassayed for red pigment-dispersing activity by injecting the extracts into eyestalkless crabs, Uca pugilator.

Fenfluramine (5-HT releaser), 5,6-DHT (5-HT neurotoxin) and reserpine (5-HT depletor) decreased the amount of 5-HT in the central nervous system, although their modes of action are different (Fig. 5). However, the 5-HT potentiator fluoxetine had no appreciable effect on the 5-HT concentration. These findings are consistent with earlier 5-HT depletion studies where reserpine was used with crustaceans. Myhrberg *et al.* (1979), who used the histochemical fluorescence method of Falck and Hillarp, and Elofsson *et al.* (1982), who used HPLC, found that reserpine decreases the

5-HT content of nervous tissues in the crayfish, Pacifastacus leniusculus. Likewise, the 5-HT content of the brain and eyestalks of Uca pugilator decreased after injection of reserpine and 5,6-DHT (Fingerman et al., 1974).

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Legends

FIGURE 1. Chromatograms of (A) brain supernatant of Procambarus clarkii and (B) aqueous standard containing 5-HT and DHBA. Column: 5 μ m silica C-18 steel. Mobile phase: Methane sulphonic acid and phosphoric acid in methanol. Detector: Glassy carbon electrode at +0.75 V potential. Retention time for 5-HT 8.8-8.9 min. and for DHBA 3.8 min.

FIGURE 2. 5-HT concentration in pg/mg of tissue, assayed by HPLC and electrochemical detection, in eyestalks (ES), brain (BR), subesophageal ganglion (SG), thoracic ganglia (ThG) and abdominal nerve cord (ANC) of Procambarus clarkii. Error bars are SEM for 10 separate extracts of tissue pooled from 10 animals.

FIGURE 3. 5-HT concentration in eyestalks (ES), brain (BR), subesophageal ganglion (SG) and thoracic ganglia (ThG) of Procambarus clarkii exposed for 3 days to continuous light (light adapted, LA) or held in total darkness (dark adapted, DA). Error bars are SEM of 10 separate extracts of tissues pooled from 10 animals. Figures in parentheses are percent change from the 12:12 L:D control (C) and those in the columns are the red pigment-dispersing Standard Integrated Responses (SIR) of the erythrophores of eyestalkless fiddler crabs, Uca pugilator, to extract of that tissue of Procambarus clarkii.

FIGURE 4. Effect of 25 V electrical stimulation (ST) for 2 min. on the concentration of 5-HT in eyestalks (ES), brain (BR), subesophageal ganglion (SG), and thoracic ganglia (ThG) of Procambarus clarkii. Error bars are SEM of 10 separate extracts of tissues pooled from 10 animals. Figures in

parentheses

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denote percent change from the control (C) and those in columns are the red pigment-dispersing Standard Integrated Responses (SIR) of the erythrophores of eyestalkless fiddler crabs, Uca pugilator, to extracts of that particular tissue of Procambarus clarkii.

FIGURE 5. Effects of pharmacological agents on the concentration of 5-HT in eyestalks (ES), brain (BR), subesophageal ganglion (SG), and thoracic ganglia (ThG) of Procambarus clarkii. Error bars are SEM of 10 separate extracts of tissues pooled from 10 animals. Values in parentheses show the percent change from the control (C). 5,6-DHT = 5,6-dihydroxytryptamine, 10 $\mu\text{g/g}$ body weight; FN = fenfluramine, 15 $\mu\text{g/g}$ body weight; FL = fluoxetine, 25 $\mu\text{g/g}$ body weight; RS = reserpine, 15 $\mu\text{g/g}$ body weight. The volume of drug solution or saline injected was 50 μl . The tissues were removed from the crayfish 2 h after the injections were given.

Fig 1

Fig 2

Fig 3

182
5-HT CONC.(Pg/mg)

20 50 80 110 140 170

Fig 5

ES

C FNFL5,6-RS
DHT

BR

C FNFL5,6-RS
DHT

SG

C FNFL5,6-RS
DHT

ThG

C FNFL5,6-RS
DHT

Description of cooperation

Except from the characterization of the ovarian proteins and their specific antibodies which was carried out by both the Israeli and American participants, most of the research was done separately and the cooperation was mainly through exchange of views and through coordination of the research subjects to reach the common objective; namely better understanding of the factors controlling the ovarian development of the decapod crustacean, and in particular the penaeid ovary. The Israeli laboratory dealt mainly with the physiology of the vitellogenic process and the identification and characterization of the gonad inhibiting hormone. The American laboratory dealt mainly with the effects of bioamines and a gonad stimulating factor on ovarian development. During the course of the research we established cooperation with a second American researcher, Dr. Tim Hayes from the Texas A&M university which contributed to the separation and sequencing of the GIH.

Evaluation of the research achievements in respect to the aims of the original research proposal

We carried out most of the experiments planned to verify or reject the existence of an extraovarian site of Vg synthesis, except the isolation of the Vg from the hemolymph. the conclusions are:

1. The subunits profile of the ovarian Vt was found by us to change during both the oogenesis and the purification procedure in P. vannamei but not in P. semisulcatus, making it a questionable tool for tracing the origin of the Vt. The native Vg from the hemolymph was found to have the same mobility on non-denatured PAGE as Vt but its subunits profile is different compared to the Vt one.

2. The hepatopancreas is involved in the vitellogenic process. Labeled Vg was found in it, subsequent to injection of labeled amino acid to vitellogenic females in proportion that was higher than that found in the hemolymph. In addition, a relatively low level of Vg synthesis was found in in vitro incubated hepatopancreas in the presence of labeled amino acid.

3. The origin of the Vg in the hemolymph is still not clearly established. It is probably not a result of passive leakage of degraded ovarian Vt. This is indicated by its much higher relative level of radioactive label compared to the ovarian Vt, after injection of labeled amino acid to vitellogenic females. Comparison of the hemolymph Vg level and ovarian Vt content during oogenesis reveals no indications of extraovarian source. Vg level increases concurrently with the Vt level, and there is no Vg decline in the hemolymph toward the end of

the oogenic process.

In view of these results, we took two additional approaches (one of them was supported by the present project) aimed at better understanding of the roles of extraovarian sites in the processing of Vg/Vt. They are currently being carried out.

1. (Not supported by the present project) Searching for the Vg/Vt encoded mRNA in all suspected tissues using the Vt antiserum as a probe, to detect Vg/Vt synthesis sites.

2. Examining the role of Vg/Vt in lipid transport to the ovary and lipid accumulation in it. The ovary was found to synthesize lipids, part of which is bound to Vt and part exists as free lipid droplets. There is no synthesis of poly unsaturated fatty acids in the ovary, which are present in the ovary and have to be transported from extraovarian sites.

We are studying the mechanism of lipid uptake by the ovary and the involvement of both Vg and LP1 in the process but have yet not reached any conclusive results.

Using the in vitro incubation assay, we found a direct effect of male and female sinus glands extract on the rate of protein and Vt synthesis in the ovary. The active peptide was isolated and partially sequenced. It belongs to the recently established CHH-related peptide family.

Serotonin had no direct effect on protein synthesis in the in vitro incubated ovary but serotonin has an indirect effect on ovarian development and protein synthesis in the ovary, when injected to the intact female of a crab and a crayfish species.

Most penaeid culture is conducted in Israel and elsewhere in ponds with varying degrees of control and management. An abundant, reliable and inexpensive source of seedstock is an absolute necessity for shrimp mariculture. A large number of enterprises today depend on seed obtained from gravid females caught in the sea. Control over the reproductive cycle is imperative for successful commercial cultivation. The commercial activity in shrimp production has increased dramatically in the past few years and is estimated annually at 2-3 billion dollars. Most of the shrimp cultured today are in extensive ponds. Techniques for high density, controlled cultures are urgently needed.

Wild seed is essentially unselected and uncontrolled with respect to genetic background. Urgently needed genetic programs aimed at shrimp stock improvement cannot begin until a basic understanding of the reproductive process leads to true control over reproduction. The use of specific reproductive hormones is

widespread in vertebrate animal husbandry, including finfish, but as yet has not been applied to crustacean cultures. The contribution of this project to the understanding of the reproductive system of shrimp will assist in achieving these goals.

List of Publications originating from the project

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3. Kulkarni, G. K., Ngabhushanam, R., Amaldoss, G., Jaiswal, R.G., Fingerman, M. 1991. 5-hydroxytryptamine stimulation of the ovary in the crayfish Procambarus clarkii. Am. Zool. 31: 115A.
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