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ASPECTS OF VITELLOGENESIS IN THE LOBSTER HOMARUS AMERICANUS

by

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ABSTRACT

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In crustaceans evidence exists for both an exogenous and endogenous origin of lipovitellin, the major yolk protein. In the lobster, Homarus americanus, a female-specific serum lipoprotein is electrophoretically and immunologically identical to lipovitellin. This lipoprotein, vitellogenin, is assumed to be the precursor to or to be lipovitellin, and is presumably sequestered by the ovary. Data obtained from Homarus americanus, from a variety of in vivo and in vitro labeling techniques, support current theories for a dual origin of yolk proteins in crustaceans. Extra-ovarian serum vitellogenin has a role in yolk formation during secondary vitellogenesis, while endogenous synthesis of yolk proteins seems to be more active in primary vitellogenesis.

Secondary vitellogenesis is characterized by high levels of vitellogenin in the serum, which are the result of protein in transit from the synthetic site to the ovary. Vitellogenin production declines and ceases prior to extrusion and/or resorption. Total levels of serum proteins and serum vitellogenin decrease prior to extrusion or resorption. With resorption, levels of total serum protein and serum vitellogenin increase markedly and are the result of protein salvage from the ovary. Eyestalk ablation had little effect on secondary vitellogenesis.

The ovary is capable of synthesizing proteins and lipovitellin. The greatest amount of synthetic activity is during primary vitellogenesis. The ovary is also capable of sequestering proteins during secondary vitellogenesis as purified lipovitellin was taken up in vitro. The exogenous source of serum vitellogenin is still unknown.

Key words: vitellogenesis, Homarus americanus

RÉSUMÉ

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Chez les crustacés, on a réussi à prouver l'origine exogène et endogène de la lipovitelline, la protéine principale du jaune d'oeuf. Chez le homard d'Amérique, une lipoprotéine du sérum propre aux femelles est au point de vue électrophorétique et immunologique, identique à la lipovitelline. Il est admis que cette lipoprotéine, la vitellogénine, est le précurseur de la lipovitelline ou constitue la lipovitelline elle-même; elle est probablement séquestrée par l'ovaire. Les données obtenues au sujet du homard d'Amérique à l'aide de diverses techniques de marquage in vivo et in vitro, appuient les théories actuelles sur la double origine des protéines du jaune d'oeuf chez les crustacés. La vitellogénine du sérum extra-ovarien participe à la formation du jaune d'oeuf au cours de la vitellogenèse secondaire, tandis que la synthèse endogène des protéines du jaune d'oeuf semble être plus active au cours de la vitellogenèse primaire.

La vitellogenèse secondaire se caractérise par une teneur élevée de vitellogénine dans le sérum, causée par la présence de protéines passant du lieu de synthèse à l'ovaire. La production de vitellogénine diminue et cesse complètement avant l'expulsion ou la résorption. La teneur totale en protéines et en vitellogénine du sérum diminue avant l'expulsion ou la résorption; au moment de la résorption, elle augmente sensiblement à cause de la présence de protéines échappées de l'ovaire. L'ablation des yeux pédonculés a eu peu d'effet sur la vitellogenèse secondaire.

L'ovaire peut faire la synthèse des protéines et de la lipovitelline. La plus grande partie de la synthèse s'effectue au cours de la vitellogenèse primaire. L'ovaire peut également séquestrer les protéines au cours de la vitellogenèse secondaire, tout comme la lipovitelline purifiée peut être absorbée in vitro. La source exogène de la vitellogénine du sérum demeure inconnue.

OVERVIEW

In lecithotropic marine organisms, embryos are dependent on yolk in the eggs for their nutritional requirements. The process of yolk deposition, or vitellogenesis, in oocytes is a seasonal or cyclical phenomenon. Vitellogenesis occurs in two stages in crustaceans (see Adiyodi and Adiyodi 1974 and Aiken and Waddy 1980 for reviews). Primary vitellogenesis may extend over several months and results in a slow increase in oocyte size, while secondary vitellogenesis results in a rapid increase in oocyte size and leads to oviposition (Aiken and Waddy 1980).

Synthesis of proteins and their incorporation into yolk during vitellogenesis have been studied in many different groups of animals. In addition to the chemical characterization and identification of the various yolk proteins and precursors, the site(s) of synthesis has been of major concern. In birds and amphibians, the yolk precursors are produced in the liver and transported to the ovary where pinocytosis by the developing oocytes occurs (Dehn and Wallace 1973; Wallace 1970; Wallace and Bergink 1974; Wallace and Dumont 1968). In insects, the yolk protein is produced extra-oocytically by the fat body (Hagedorn and Kunkel 1979). In crustaceans, evidence exists for both an endogenous (Beams and Kessel 1963; Ganion and Kessel 1972; Kessel 1968; Kessel and Beams 1963; Lui et al. 1974; Lui and O'Connor 1976, 1977) and a exogenous (Croissile et al. 1974; Ganion et al. 1972; Junera et al. 1977a, 1977b; Kerr 1968, 1969; Meusy et al. 1974; Varadarajan and Subramoniam 1980; Wolin et al. 1973) origin of vitellogenin. Accumulating evidence suggests that yolk protein synthesis may be both endogenous and exogenous (Beams and Kessel 1980; DeLeersynder et al. 1980; Dhainaut and DeLeersynder 1976; Eurenius 1973; Fyffe and O'Connor 1974; Hinsch and Cone 1969; Schade and Shivers 1980; Varadarajan and Subramonian 1980; Zerbib 1977, 1979). Endogenous synthesis of yolk proteins (primarily glyoproteins in nature) occurs during primary vitellogenesis, while exogenous synthesis occurs during secondary vitellogenesis (DeLeersynder et al. 1980; Varadarajan and Subramoniam 1980; Zerbib 1977). During the exogenous phase, the yolk (primarily a lipoglycocartenoprotein complex) is produced from a lipoprotein complex present in the hemolymph, which is then sequestered by the oocyte via micropinocytosis (DeLeersynder et al. 1980; Varadarajan and Subramoniam 1980; Zerbib 1977).

A female-specific protein (FSP), vitellogenin, exists in the hemolymph of crustaceans and is electrophoretically and immunologically identical to the major yolk protein, lipovitellin (see Meusy 1980 for a review). Vitellogenin and its role in vitellogenesis is not clearly understood in crustaceans. A positive correlation exists between vitellogenin levels and oocyte maturation (Aiken and Waddy 1980 for review). Whether vitellogenin and lipovitellin are one and the same is not known, although immunologically and electrophoretically they appear to be (Meusy 1980).

In crustaceans, the synthetic site of serum vitellogenin has not been clearly demonstrated, although Junera et al. (1977b) have shown it to be extra-ovarian. The hepatopancreas (Besse et al. 1970; Ceccaldi and Martin 1969; Ceccaldi 1970; Kessel 1968; Wolin et al. 1973) and hemocytes (Kerr 1968) have been suspected as the synthetic sites of vitellogenin. Recently Junera and Croisille (1980), Picaud (1980), Picaud and Souty (1980), and Souty and Picaud (1981) have presented evidence that the subepidermal adipose tissue produces vitellogenin.

The controls of vitellogenesis are just beginning to be investigated in crustaceans (Meusy 1980). It is assumed that the neurosecretory tissue produces three hormones: gonad inhibitory hormone (GIH), gonad stimulating hormone (GSH), and molt inhibitory hormone (MIH) which suppress molting during the reproductive cycle (Adiyodi and Adiyodi 1970; Aiken and Waddy 1980; Bomirski et al. 1981). Eyestalk ablation accelerates vitellogenesis in a variety of decapods (Adiyodi and Adiyodi 1970; DeLeersynder et al. 1980; Meusy 1980). Ablation of this structure removes the X-organ-sinus gland complex which acts as the neurosecretory center and presumably removes the effects of GIH. It is assumed that the neurosecretory center stimulates the vitellogenin synthesizing tissue, ovaries, and possibly the y-organs, which produce the molting hormone (MH) although neurosecretory controls have not been demonstrated (Meusy 1980). Eyestalk hormones may control the maintenance, resorption, or uptake of vitellogenin directly or indirectly through an intermediate factor.

In the lobster, little is known of vitellogenesis or of the possible endogenous controls of the vitellogenic process (Aiken and Waddy 1980). A lipoprotein, lipovitellin, has been isolated from the ovary (Wallace et al. 1967). A female-limited lipoprotein, vitellogenin, has been found in the hemolymph and is immunologically and electrophoretically identical to lipovitellin (Byard 1975). The origin of vitellogenin is unknown as is its role in vitellogenesis. The controls of vitellogenesis are also unknown, although eyestalk ablation results in increased levels of serum lipoprotein which may be the result of protein mobilization leading to uptake by, or resorption from, maturing oocytes (Aiken and Waddy 1980).

The purpose of this study was to examine: the role of serum vitellogenin during secondary vitellogenesis; the role of the ovary in the synthesis of lipovitellin; the effects of neurosecretory center removal on vitellogenic activity; and possible extra-ovarian synthetic sites in the lobster, Homarus americanus.

GENERAL METHODS

ANIMALS

Female lobsters were obtained from the southern Gulf of St. Lawrence in the North Rustico and Miminegash areas of Prince Edward Island. All were in intermolt (C_4), had less than 6 wk to extrusion as indicated by cement gland development (Stage C) (Aiken and Waddy 1982), had carapace lengths of 72-80 mm, and had wet weights of 300-420 g. Animals were maintained in a flow-through water system at collection temperatures (6°C). Two weeks prior to experimentation, females were moved into 12°C water and maintained at this temperature throughout the experimental period. All animals were fed five times weekly.

CHOICE OF RADIOLABEL AND MARKER

Leucine was chosen as the marker as it is a major component of both the hemolymph (Florkin 1960; Kessel 1968; Stewart et al. 1966) and the oocytes (Kessel 1968) during the reproductive cycle. Most labeling was done with ^{3}H-leucine as Kerr (1968) reported that she obtained inconclusive results with C^{14} when attempting to label serum vitellogenin.

GENERAL TREATMENT OF RADIOACTIVE SAMPLES

Serum was processed according to Byard (1975). Crude ovarian homogenates were prepared in 0.5 M NaCl-5mM EDTA. Prior to centrifugation 25 µM PMSF (phenylmethyl sulfonyl fluoride) was added to the crude homogenate to inhibit enzymatic activity. Purified ovarian lipoproteins were prepared from crude homogenates by ammonium sulfate precipitation (Wallace et al. 1967). The purification proposed by Wallace et al. (1967) does not isolate just one protein. However, lipovitellin is the major protein isolated, as all other proteins can only be detected with the use of a silver stain (Fig. 1). All preparative work was done in the cold (4°C). Protein levels were measured by either the biuret (Gornall et al. 1949) or the Lowry (Lowry et al. 1951) method, using BSA as the standard. Radiolabel incorporation/accumulation into total TCA precipitable proteins was determined by the filter paper disc method (Mans and Novelli 1961). As this method could not measure the incorporation of labeled amino acid into a specific serum or ovarian protein directly, horizontal thin-layer gel electrophoresis, with a 5% polyacrylamide gel (PAGE) and Tris-glycine buffer system (pH 8.9), was used to separate proteins for examination of label incorporation into specific hemolymph and ovarian proteins. Electrophoretic samples were run in duplicate so that half of the gel could be stained with Coomassie brilliant blue (R-250; Eastman Kodak) to provide a record of protein migration distances, while the duplicate half could be prepared for liquid scintillation counting. The unstained gel was used for scintillation counting as the dye was found to severely quench the counts present (p. 5). The gel was first separated into sample strips, then each strip was cut by hand into 2-mm sections. Each 2-mm section was placed into a numbered scintillation vial and treated with 0.5 mL NCS tissue solubilizer (9:1 NCS:HOH) (Amersham) at 50°C for 2 h to release the proteins from the gel. Following solubilization, the vials were cooled and 17 mL OCS (Amersham), a premixed scintillant recommended for use with PAGE and NCS, was added. To prevent spurious counts, samples were left in the dark for 24 h, then counted in a Beckman L-100C. To determine the total counts (free and bound leucine) in the serum and in the material loaded on the gel for electrophoresis, duplicate 50-µL samples were placed in scintillation vials to which NCS (0.5 mL) was added to absorb the water. After thorough mixing, 17 mL of OCS was added, and the samples were then kept in the dark for 24 h prior to counting. All counts were corrected for background and are expressed as CPM (counts per minute)/mL and/or as CPM/mg protein/mL.

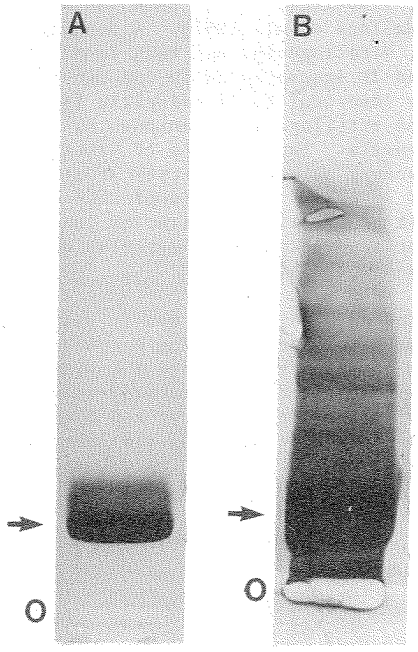

Fig. 1. Representative polyacrylamide strip of a purified lipovitellin fraction prepared by the method of Wallace et al. (1967) from a vitellogenic H. americanus ovary. A. Strip stained with Coomassie brillant blue R-250 (levels of detectable protein as low as 0.5 µg/cm^2). B. Strip stained with silver stain (Bio-Rad, levels of detectable protein 0.05-0.01 µg/cm^3). Lipovitellin is the major protein present (arrows). The sample origin is indicated (O).

I

ROLE OF SERUM VITELLOGENIN DURING SECONDARY VITELLOGENESIS

INTRODUCTION

Secondary vitellogenesis in Homarus americanus is characterized by the presence of a female specific protein (FSP), vitellogenin, which is immunologically and electrophoretically identical to the major yolk protein, lipovitellin, which can be extracted from mature oocytes (Byard 1975; Wallace et al. 1967). A positive correlation exists between vitellogenin levels in the hemolymph and successful ovarian maturation that results in oviposition, levels being maximal during times of final oocyte maturation (Byard 1975). The role of serum vitellogenin is still unknown, as actual uptake of this serum component by the oocytes has not been demonstrated. It is presumed that serum vitellogenin is sequestered by the oocyte via micropinocytosis during the final stages of ovarian maturation (DeLeersynder et al. 1980; Varadarajan and Subramonium 1980; Zerbib 1977). The purpose of this phase of the work was to examine the role of vitellogenin during secondary vitellogenesis (final stages of ovarian maturation) by radioactively labeling this serum component and then following it throughout the final period of the reproductive cycle.

MATERIALS AND METHODS

1. DETERMINATION OF THE HEMOLYMPH RADIOLABEL CHARACTERISTICS AND IN VIVO UPTAKE INTO OVARIAN COMPONENTS

^{3}H-leucine

On May 28, 1981, L-(4, 5-^{3}H)-leucine (Amersham), 1.5 uCi (sp. act. 65 Ci/mmol)/g wet body weight was injected into the ventral abdominal sinus of a pre-ovigerous female. A 1-mL serum sample was removed from the sinus at 0, 2, 4, 8, 12, 24, 48, 72, 96, 124, 168, and 212 h. After processing, duplicate 50-µL samples were withdrawn to examine the incorporation of label into total TCA precipitable proteins. In addition, the 12-, 24-, 96-, 168-, and 212-h samples were used to electrophoretically examine incorporation into serum vitellogenin. Levels of serum protein of samples used for electrophoresis were measured by the biuret method. Protein levels loaded onto the gels were between 7 and 9 mg/mL (0.07-0.09 mg/10 µL). Both dyed and undyed gel strips were sectioned and counted to observe what effect the dye might have on counting efficiencies. Duplicate gel strips were sectioned and counted to observe what variation might exist as a result of hand sectioning. After the 10th day, the female was sacrificed and the ovary was homogenized and purified as previously described (p. 2). The amount of label associated with lipovitellin was examined electrophoretically. The purified fraction was thoroughly dialyzed against sample buffer prior to electrophoresis to remove salts which interfere with protein mobility.

^{14}C-leucine

On May 29, 1982, L-(^{14}C(U))-leucine (NEN), 1.5 µCi(sp. act. 353 mCi/mmol)/g wet body weight was injected into the ventral abdominal sinus of three pre-ovigerous females to determine whether or not this isotope could be used to label vitellogenin (p. 2). A 1-mL serum sample was taken from the sinus at 0, 1, 2, 4, and 7 d. After processing, duplicate 50-µL samples were withdrawn to examine incorporation of label into total TCA precipitable proteins. Serum was examined electrophoretically to measure incorporation into serum vitellogenin. Levels of serum protein were measured for all samples by the biuret method. Protein levels loaded onto the gels were between 7 and 8 mg/mL (0.07-0.08 mg/10 µL). After the 7th day, females were sacrificed, and ovaries homogenized and purified as previously described (p. 2). The amount of label associated with both total TCA precipitable proteins and lipovitellin was examined in crude and purified fractions. Both fractions were thoroughly dialyzed against sample buffer prior to electrophoresis.

2. FATE OF THE LABELED VITELLOGENIN IN THE HEMOLYMPH DURING SECONDARY VITELLOGENESIS

On June 27, 1981, L-(3,4,5-^{3}H)-leucine (NEN), 1.5 uCi (sp. act. 115.2 Ci/mmol)/g wet body weight was injected into the ventral abdominal sinus of five pre-ovigerous females. A 1-mL serum sample was taken from the sinus at approximately 3-4 d intervals for the first 33 d following the initial injection, then once weekly for the first month following either extrusion or resorption; intervals of 3-4 d were chosen for sampling as Barlow and Ridgeway (1969) reported that Cole (1941) found a significant decrease in hemolymph protein content with more frequent bleedings. It should be noted that their bleedings consisted of 2-5 mL and smaller animals were used. Nevertheless, this guide was used to ensure that protein levels would be unaffected by sampling regimen and would accurately represent real differences in serum protein level (see section III. Endocrine Control, for further discussion). Incorporation of label into serum vitellogenin was examined electrophoretically. Levels of serum protein were measured for all samples by the biuret method. Protein levels loaded onto the gels were between 5 and 7 mg/mL (0.05-0.07 mg/10 µL). After the 7th day, one female was sacrificed, and the ovary was homogenized and purified (p. 2). The amount of label associated with lipovitellin was examined electrophoretically. The purified fraction was thoroughly dialyzed against sample buffer prior to electrophoresis.

RESULTS AND DISCUSSION

1. DETERMINATION OF THE HEMOLYMPH RADIOLABEL CHARACTERISTICS AND IN VIVO UPTAKE INTO OVARIAN COMPONENTS

^{3}H-Leucine

Incorporation of label into total TCA precipitable serum proteins was evident at 2-h post-injection, reached highest measured levels on day 3 and then slowly declined through day 10 (Fig. 2). Label was associated with vitellogenin at 12 h, reached highest measured levels on day 4, and declined through day 10 (Fig. 2). Figure 3 shows the day 4 gel as well as both the counts associated

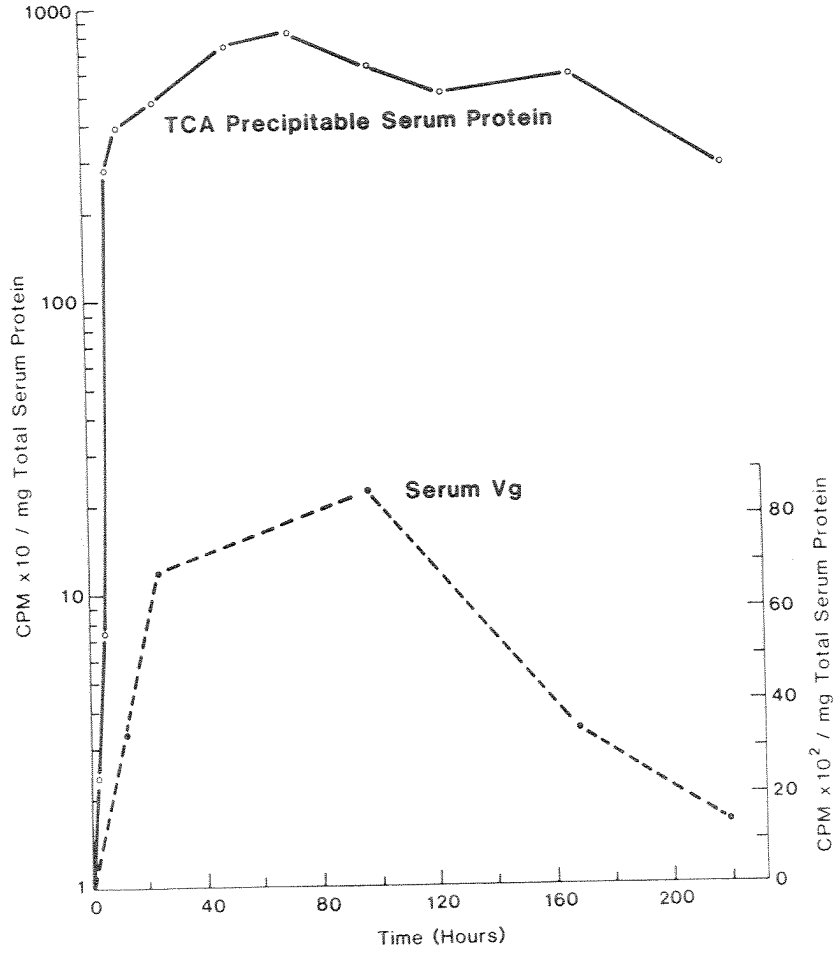

Fig. 2. Time course of incorporation of label into total TCA precipitable serum proteins and serum vitellogenin for a vitellogenic H. americanus injected with ^{3}H-leucine (1.5 μCi/g wet body weight).

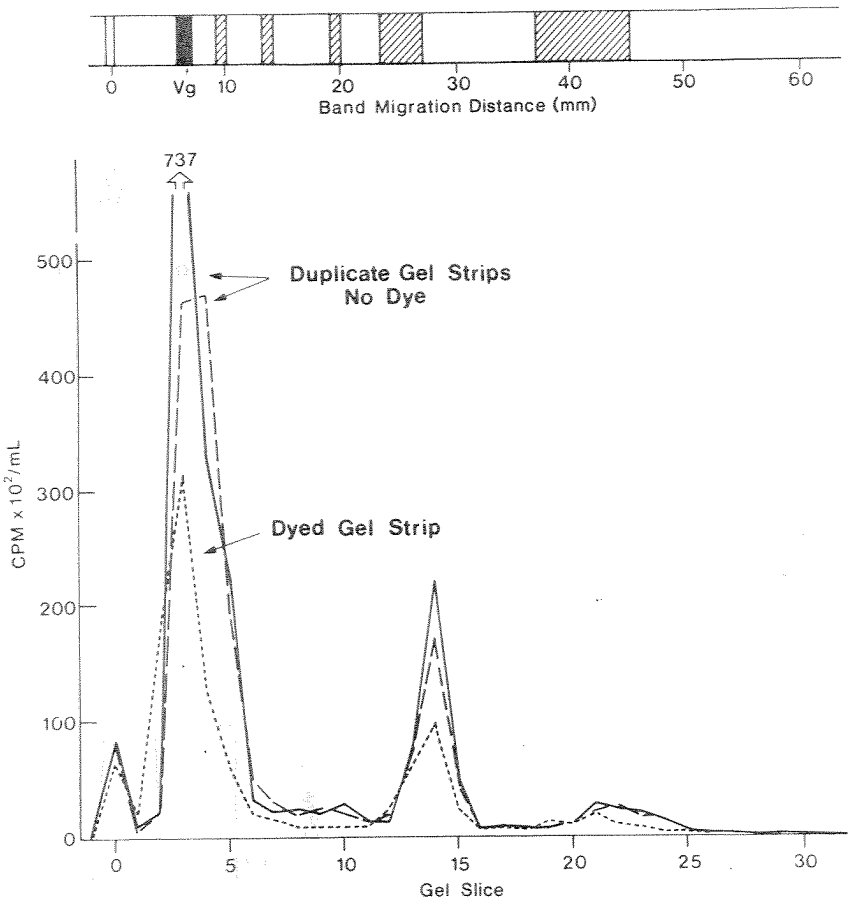

Fig. 3. Representative polyacrylamide gel tracing of serum proteins from a vitellogenic H. americanus 4 d after injection of ^{3}H-leucine (1.5 µCi/g wet body weight). Serum profiles and corresponding CPM/mL are indicated for duplicate undyed and dyed strips. Each gel slice was approximately 2 mm.

with each 2-mm section, and the protein bands with their migration distances. The dye was found to quench (by as much as 40%) the counts within the samples (Fig. 3). As counts were expected to be low for some samples, i.e. in vitro work (p. 10, 20), only undyed gels were used for scintillation counting. Use of undyed gels was not entirely satisfactory for matching radioactive peaks with their corresponding protein band, as variations in protein migration distances were observed in many duplicate samples (Fig. 3). Most of this variation was the result of hand sectioning. This problem was overcome by occasionally sectioning duplicate dyed and undyed strips for corroboration of band migration distances and matching radioactive peaks.

The lipovitellin fraction isolated from the ovary contained radioactivity. Eighty percent of the total CPM/mL or 11% of the CPM/mg total ovarian protein/mL loaded onto the gel was associated with the lipovitellin band (36.7 CPM x 10^2/mg protein). This incorporation may be the result of uptake of label and synthesis of labeled lipovitellin by the ovary and/or the sequestering of labeled serum vitellogenin (see section II. Role of the Ovary, for further discussion).

^{14}C-leucine

Incorporation of label into total TCA precipitable serum proteins had reached somewhat stable levels by 24 h, and slowly decreased by day 7 (Fig. 4). Label was associated with the serum vitellogenin at 24 h, reached highest levels on day 2, and declined through day 7. Figure 5 shows a day 2 gel from one of the females as well as both the counts associated with the first 20 mm of the gel and the protein bands with their migration distances. Levels of total serum protein remained fairly constant throughout the 7-d period, although there was a slight reduction in protein levels from day 1 (69.5±4.7) to day 4 (61.4±5.7), but showed increases by day 7 (66.7±7.3). These were quite small changes well within the normal range of variation (see section III. Endocrine Control, for further discussion), and were most likely due to changes in food intake, as females refused food for the first 24-48 h after injections.

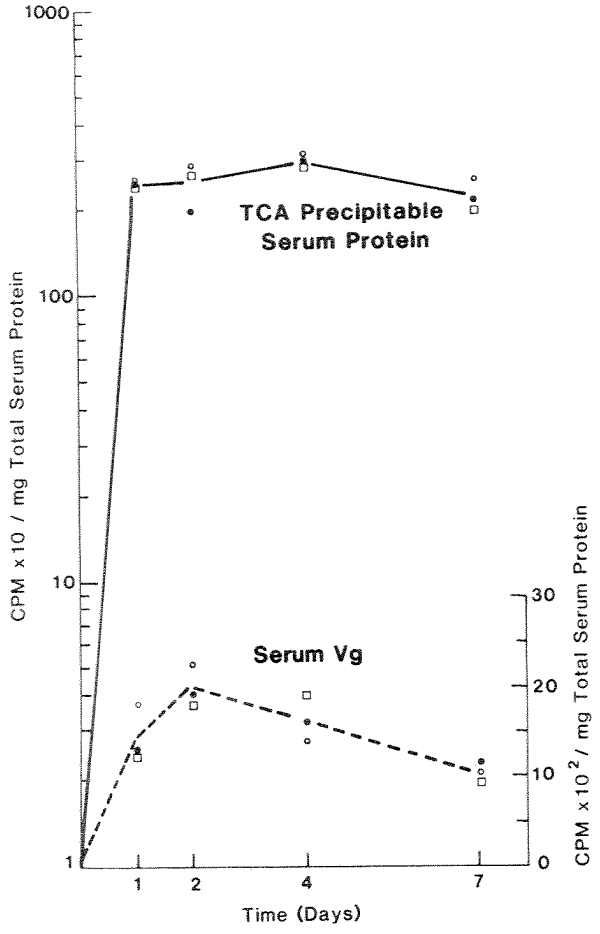

Fig. 4. Time course of incorporation of label into total TCA precipitable serum proteins and serum vitellogenin for vitellogenic H. americanus injected with ^{14}C-leucine (1.5 µCi/g wet body weight). The lines indicate means.

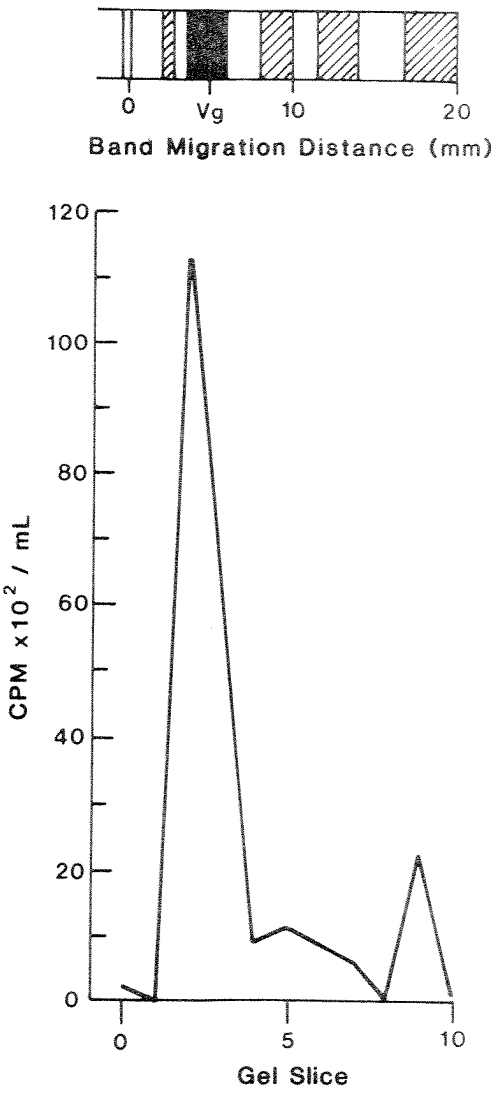

Fig. 5. Representative polyacrylamide gel tracing of serum proteins from a vitellogenic H. americanus 2 d after injection of ^{14}C-leucine (1.5 µCi/g wet body weight). Serum profiles and corresponding CPM/mL are indicated for an undyed strip. Each gel slice was approximately 2 mm.

The lipovitellin fractions isolated from the ovaries contained the label (Table 1). Counts/mg protein associated with both the TCA precipitable proteins and lipovitellin were highest in the crude fractions, indicating the presence of other labeled proteins (non-lipovitellin ovarian proteins). In the purified fractions, 90-98% of the CPM/mL or 12-13% of the CPM/mg total ovarian protein/mL loaded onto the gel were associated with the lipovitellin band. This incorporation may be the result of uptake of label and synthesis of labeled lipovitellin by the ovary and/or the sequestering of labeled vitellogenin from the serum (see section II, Role of the Ovary, for further discussion).

2. FATE OF THE LABELED VITELLOGENIN IN THE HEMOLYMPH DURING SECONDARY VITELLOGENESIS

Incorporation of label into vitellogenin was variable among animals, ranging from 18-52 CPM x 10/mg total serum protein on day 2, and then declined through day 12 (Fig. 6). On July 4th, 7 d after the initial ^{3}H-leucine injection, L-(^{14}C-(U))-leucine (NEN), 1.5 uCi (sp. act. 353 mCi/mmol)/g wet body weight was injected into the ventral abdominal sinus of the three remaining pre-ovigerous females (one female extruded 24 h after the initial injection and no label was found in either the serum vitellogenin or the lipovitellin of the extruded eggs; the other female was sacrificed) to determine whether levels of available label were too low or vitellogenin production was declining. No ^{14}C-label was found associated with vitellogenin, indicating that vitellogenin production had halted by the 7th day (see section above for substantiation that serum vitellogenin can be labelled by ^{14}C). All results are therefore given for ^{3}H-leucine only, even though ^{14}C-leucine counts were taken for all samples following injection of this label.

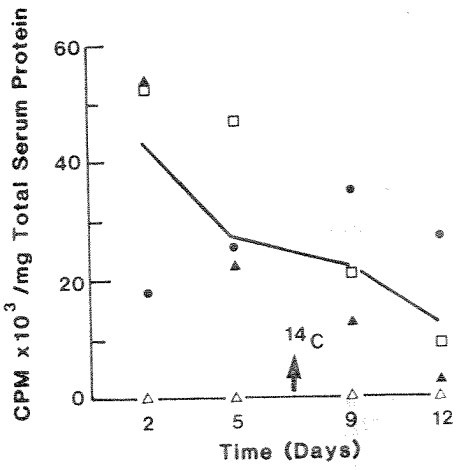

Fig. 6. Levels of incorporation of label into serum vitellogenin from vitellogenic H. americanus females injected initially with ^{3}H-leucine followed 7 d later by ^{14}C-leucine (1.5 µCi/g wet body weight). Data given are for ^{3}H-leucine only, as there were no counts observed for ^{14}C in the vitellogenin band. The lines indicate means.

The remaining females underwent total ovarian resorption 29-33 d after the initial ^{3}H-leucine injection. Levels of labeled vitellogenin decreased prior to resorption, even though levels of label remained high within the serum (Fig. 7). In addition, the electrophoretic bands associated with vitellogenin became quite faint 2-3 wk after the initial injection (Fig. 8a,b), indicating a

Table 1. Protein levels and counts associated with the crude and purified fractions isolated from ovaries of ^{14}C-leucine injected females. Data represent both total TCA precipitable proteins and the lipovitellin which was separated electrophoretically.

	Total TCA precipitation protein			Lipovitellin (Lp)			
Fraction	Protein (mg/mL)	CPM x 10^3/ mL	CPM x 10^2/ mg protein	Protein loaded (mg/mL)	CPM x 10^3/ mL loaded	CPM x 10^3/ mL in Lp band	CPM x 10^2/ mg protein in Lp band
Crude							
1	45.72	104.06	22.76	7.03	26.82	19.58	27.85
2	50.43	115.43	22.89	7.20	24.99	21.06	29.25
3	42.20	84.02	19.91	7.03	23.82	18.28	26.00
Purified							
1	30.67	53.42	17.42	7.67	16.07	15.84	20.65
2	37.02	61.53	16.62	7.40	12.06	10.87	14.69
3	36.08	68.87	19.09	7.22	14.74	13.92	19.28

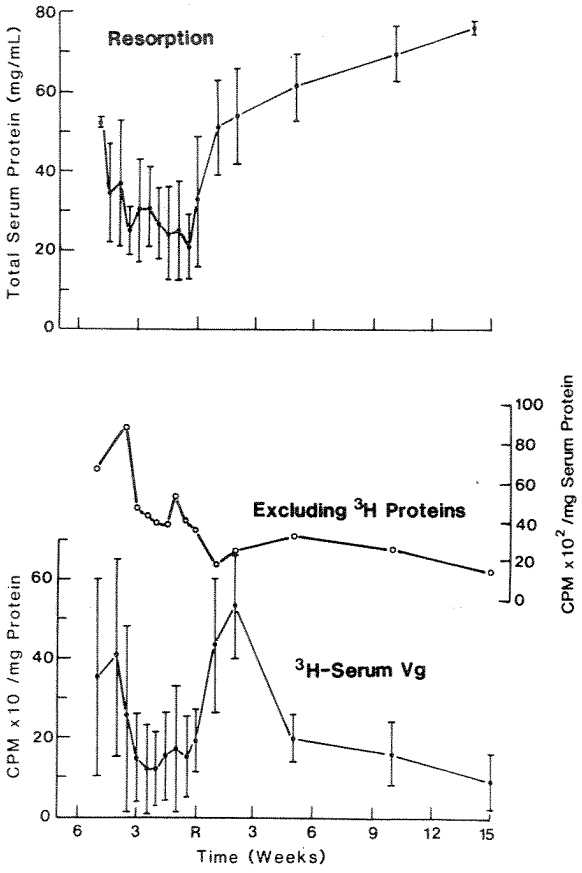

Fig. 7. Mean (± SD) levels of ^{3}H-vitellogenin, levels of total serum protein, and levels of ^{3}H-leucine (excluding all measurable ^{3}H-leucine from polyacrylamide gel strips stained with Coomassie brillant blue) from the serum of vitellogenic H. americanus females which underwent total ovarian resorption 29-33 d after injection with ^{3}H-leucine (1.5 µCi/g wet body weight).

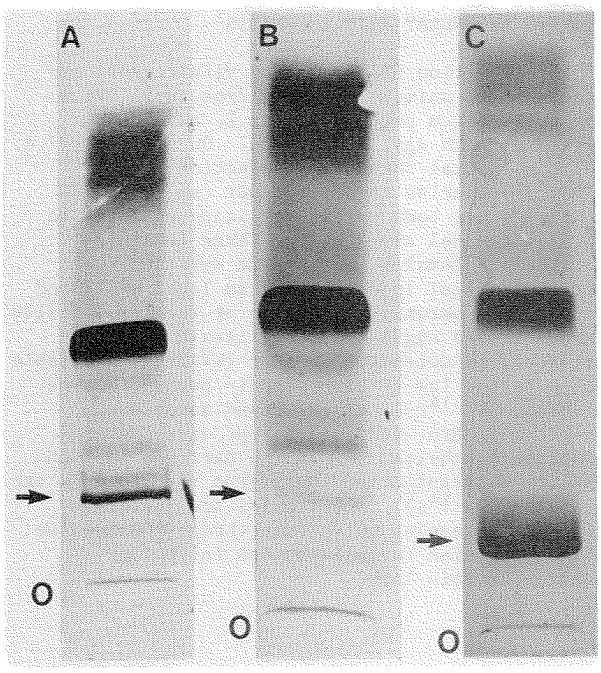

Fig. 8. Representative polyacrylamide gel strips from a single vitellogenic H. americanus that underwent total ovarian resorption 29-33 d after injection with ^{3}H-leucine (1.5 µCi/g wet body weight). A. Initial serum profile the day before injection (5.46 mg/mL loaded onto the gel). B. The serum profile 16 d after the injection (6.39 mg/mL loaded onto the gel). C. The serum profile 51 d after the injection, 18 d after the onset of resorption (6.78 mg/mL loaded onto the gel). Arrows indicate the vitellogenin band and the sample origin is indicated (o). There was some shrinkage of gels due to drying which makes the bands appear to have different mobilities. This is entirely artifactual.

reduction in the total level of this protein in the serum. With the onset of resorption, the electrophoretic bands associated with the vitellogenin became prominent (Fig. 8c), indicating an increase in the total level of this protein in the serum. In addition, levels of labeled vitellogenin increased markedly (Fig. 7). It was assumed that this increase in vitellogenin was the result of protein salvage by the ovary, rather than new protein synthesis, as there were no ^{14}C-leucine counts associated with the vitellogenin, even though this label also was available in the serum. There were no observable differences in protein mobilites of vitellogenin in the serum of normally maturing and resorbing females either before or during resorption, indicating that the vitellogenin going in and coming out was the same in both groups. Total serum protein levels followed the same pattern as vitellogenin: decreasing prior to resorption and then increasing with the onset of resorption (Fig. 7).

The lipovitellin fraction isolated from the one female 7 d after the initial injection contained the label. Approximately 95% of the CPM/mL or 12% of the CPM/mg total ovarian protein/mL loaded onto the gel was associated with lipovitellin band (16.83 x 10 CPM/mg protein). This incorporation may be the result of uptake of label and synthesis of labeled lipovitellin by the ovary, but also must be the result of uptake and sequestering of labeled serum vitellogenin as levels of ^{3}H-leucine labeled vitellogenin declined 2-3 wk after injection then increased markedly with resorption; total levels of vitellogenin also followed the same pattern and no ^{14}C-leucine labeled vitellogenin was observed in the hemolymph either before or after the onset of resorption.

Fyffe and O'Connor (1974) suggested that high serum vitellogenin levels might be the result of protein in transit from the extra-ovarian synthetic site to the ovary or the result of reabsorption of lipovitellin from the egg. They did point out that these processes were not mutually exclusive events. Kerr (1969) has shown that passage of lipovitellin into the hemolymph from a normally maturing egg did not occur. In many crustaceans, a small rise in levels of both blood proteins and serum vitellogenin occurs following the completion of oocyte growth, which may be related to resorption from eggs not extruded and/or to proteins not utilized (Adiyodi and Adiyodi 1974). Under certain circumstances, some or all of the oocytes may be resorbed (see Adiyodi and Adiyodi 1974 for review). Much of the available information associated with resorption comes from work with insects. Bell (1971) has reported a decrease in production of vitellogenin and a marked increase in the concentration of blood vitellogenin during resorption in the insect Periplaneta americana, and suggests that increases in serum vitellogenin are the result of protein salvage from the ovary. Histochemical studies on Schistocera gregaria ovaries indicated that resorption included an enzymatic breakdown of the protein and lipid yolk constituents (Lusis 1963). Bell (1971) suggests that P. americana releases its vitellogenins intact into the hemolymph, as there were no immunochemical differences between vitellogenins derived from normal or resorbing oocytes, or those isolated from the blood of normally maturing or resorbing females.

These data indicate that for Homarus high serum vitellogenin levels can indeed be the result of both protein in transit from the extra-ovarian synthetic site to the ovary and the resorption of lipovitellin from eggs; and with normal maturation the first precedes the second. Although uptake of label and synthesis of labeled lipovitellin by the ovary cannot be excluded (see section II. Role of the Ovary, for further discussion), it does seem that uptake of labeled serum vitellogenin also occurs. In addition, vitellogenin production ceases and levels within the serum decrease prior to resorption; and with the onset of resorption levels of vitellogenin increase in the hemolymph as a result of protein salvage from the ovary. It also appears that vitellogenin may be released intact from the resorbing oocytes into the hemolymph.

ROLE OF THE OVARY IN THE SYNTHESIS OF LIPOVITELLIN AND/OR UPTAKE OF VITELLOGENIN

INTRODUCTION

Ultrastructural evidence indicates that crustacean oocytes have the necessary synthetic apparatus to produce lipovitellin intra-oocytically (Beams and Kessel 1963, 1980; DeLeersynder et al. 1980; Dhainaut and DeLeersynder 1976; Eurenius 1973; Ganion and Kessel 1972; Hinsch and Cone 1969; Kessel 1968; Kessel and Beams 1963, 1968; Schade and Shivers 1980; Souty 1980; Zerbib 1977, 1979). Various incubation studies have shown that isolated oocytes and ovarian fragments are able to incorporate labeled amino acids into ovarian proteins, but specific yolk proteins have not been isolated to observe their incorporation of label (Ganion and Kessel 1972; Kessel 1968). A series of in vitro studies on various crustaceans have shown that the ovary is capable of synthesizing not only lipoproteins, but also at least three of the five subunits of the lipovitellin molecule first described by Wallace et al. (1967) (Lui et al. 1974; Lui and O'Connor 1976, 1977).

Ultrastructural evidence also indicates that crustacean oocytes exhibit extensive micropinocytotic activity (Beams and Kessel 1980; DeLeersynder et al. 1980; Dhainaut and DeLeersynder 1976; Eurenius 1973; Hinsch and Cone 1969; Schade and Shivers 1980; Souty 1980; Wolin et al. 1973; Zerbib 1977, 1979). Micropinocytosis across the surface of the oocyte may act as the major route of entry for the circulating extra-ovarian vitellogenin. It has been shown that no uptake of label occurs in Uca ovaries incubated in vitro, but both female serum and labeled lipovitellin were taken up micropinocytotically (Wolin et al. 1973).

Accumulating evidence from a variety of studies strongly suggests that there is a dual source of lipovitellin in crustaceans, which may be related to the stage of vitellogenesis. Endogenous synthesis of lipovitellin is primarily a function of primary vitellogenesis as the small immature oocytes exhibit extensive granular endoplasmic reticulum, show higher levels of labeling in vitro, and exhibit very weak, if any, micropinocytotic activity (Beams and Kessel 1980; DeLeersynder et al. 1980; Dhainaut and DeLeersynder 1976; Eurenius 1973; Fyffe and O'Connor 1974; Ganion and Kessel 1972; Hinsch and Cone 1969; Kessel 1968; Schade and Shivers 1980; Souty 1980; Varadarajan and Subramoniam 1980; Zerbib 1977, 1979). Uptake of exogenous vitellogenin from the serum is a function of secondary vitellogenesis as micropinocytotic activity increases with maturation and the granular endoplasmic reticulum becomes less well developed (Beams and Kessel 1980; DeLeersynder et al. 1980; Dhainaut and DeLeersynder 1976; Eurenius 1973; Hinsch and Cone 1969; Schade and Shivers 1980; Souty 1980; Zerbib 1977, 1979).

In view of the active discussion and difference of opinion concerning the role of the ovary in the synthesis of yolk protein in Crustacea, it seemed important and of interest to investigate: whether Homarus could synthesize lipovitellin in vitro; whether there were any differences in synthetic function during primary and secondary vitellogenesis; whether the ovary could take up labeled vitellogenin from the serum and/or labeled lipovitellin isolated from the ovary in vitro; and to compare in vitro and in vivo incorporation/accumulation rates, as no kinetic studies have been done previously in any of the crustaceans.

MATERIALS AND METHODS

1. IN VITRO INCORPORATION OF LABEL AND/OR LABELED PROTEIN INTO LIPOVITELLIN

Ovaries were removed as aseptically as possible (animals were surface sterilized with 70% ETOH), and placed in sterilized lobsters Ringers containing 1% antibiotics (10000 U/mL penicillin, 10000 mcg/mL streptomycin, 25 mcg/mL fungizone, Grand Island Biological). Ovaries were cut into small pieces, rinsed a minimum of three times in sterile Ringers and antibiotics, and transferred to sterile vials containing the culture media. All preparative work was done in the cold (4°C).

Several different Ringers solutions were tried. Cavanaugh's (1956) solution (per L: 26.4 g NaCl, 15 mM KCl, 25 mM, $CaCl_2$, 17.6 mL H_3BO_4 (0.5 M), 4mM $MgCl_2$, 4mM $MgSO_4$, 0.96 mL NaOH (0.5 M) adjusted to pH 7.6 with Na_2HPO_4) was used for all the 1981 cultures. As a flocculent precipitate was produced and had to be filtered when adjusting to pH 7.6, normal hemolymph pH, Welsh et al.'s (1968) solution (per L: 462.8 mM NaCl, 15.7 mM KCl, 25.9 mM $CaCl_2$, 8.3 mM $MgCl_2$, 8.4 mM $NaSO_4$, 5 mM D-glucose, 5 mM Tris-HCl giving a final osmolality of 995 ± 5 mOsmol/Kg H_2O, pH 7.4) was adopted for all the 1982 cultures.

Culture media varied, but all contained 1% antibiotics (see above). Ovarian fragments were incubated in male serum and label (^{3}H-leucine, 5 μCi/mL), female serum containing ^{3}H-leucine labeled vitellogenin, or ^{3}H-leucine labeled lipovitellin isolated from ovaries. The labeled serum and ovarian lipoproteins were prepared by injecting a female twice, 3 d apart, with L-(3,4,5,^{3}H)-leucine 1.5 μCi/g wet body weight. Three days after the second injection, serum was collected and the ovary removed for lipovitellin isolation. Following purification (p. 2), the lipovitellin was thoroughly dialyzed against sterile Ringers containing antibiotics.

Ovarian fragments were incubated at 19.5 ± 0.2°C in a Gilson shaking water bath. Following incubation, ovaries were rinsed in a minimum of 200 mL sterile Ringers followed by a minimum of 200 mL of 0.5 M NaCl-5 mM EDTA to remove any excess label adhering to the surface, homogenized, purified, and separated electrophoretically. Protein levels loaded onto the gel for purified fractions were between 7 and 10 mg/mL (0.07-0.10 mg/10 μL) during secondary vitellogenesis and 2 to 7 mg/mL (0.02-0.07 mg/μL) during primary vitellogenesis. Incorporation of label or of labeled protein into total TCA precipitable ovarian proteins and lipovitellin was measured. Levels of total ovarian protein were measured by the biuret method (p. 2).

2. IN VIVO ACCUMULATION OF LABEL AND/OR LABELED PROTEIN INTO LIPOVITELLIN

As results for all in vitro ovarian cultures indicated low levels of incorporation into lipovitellin, it seemed necessary to examine in vivo accumulation rates in order to provide information that incubation times used were satisfactory for monitoring synthetic activities. Five pre-ovigerous females, all in the same approximate reproductive condition (as indicated by cement gland stages), were injected with ^{3}H-leucine (1.5 µCi/g wet body weight) on June 1, 1982. One female was sacrificed each at 5, 12, 24, 48, and 96 h post-injection. Ovaries were removed, washed thoroughly (see in vitro), homogenized, purified, and separated electrophoretically. Incorporation of label and/or labeled protein into total TCA precipitable ovarian proteins and lipovitellin was measured. Levels of total ovarian proteins were measured by the biuret method.

RESULTS AND DISCUSSION

1. IN VITRO INCORPORATION OF LABEL AND/OR LABELED PROTEIN INTO LIPOVITELLIN

Secondary vitellogenesis

During the late summer of 1981, ovarian fragments were incubated in 3 mL of male serum for 24 and 48 h. Levels of labeled TCA precipitable proteins increased from 24 to 48 h in both crude and purified fractions (Fig. 9), indicating the cultures were viable and incorporation was a function of time. Levels of incorporation of label were higher in crude fractions when compared to purified fractions (Fig. 9), indicating the presence of other labeled non-lipovitellin proteins. The percentage of the total CPM/mL loaded onto the gel that was found associated with the lipovitellin band was less than 25% in both cultures (Fig. 9). These counts represented less than 3% of the total CPM/mg protein/mL loaded onto the gel (Fig. 9), indicating that only a very small amount of radioactivity present was associated with lipovitellin. Most of the label was associated with other non-lipovitellin proteins present in the ovary.

Souty (1980) reported that the isopod Idotea balthica basteri showed evidences of endogenously synthesizing yolk proteins early in secondary vitellogenesis. As the previous culture was done mid to late in secondary vitellogenesis, it was decided to repeat the culture early in secondary vitellogenesis and concomitantly examine the uptake of both labeled serum vitellogenin and purified ovarian lipovitellin. During the early summer of 1982, ovarian fragments from the same female were incubated in either 3 mL of male serum and label, label, female serum containing labeled vitellogenin, or purified labeled lipovitellin. Levels of labeled TCA precipitable proteins were highest in crude fractions of fragments incubated in female and male serum when compared to purified fractions (Fig. 10), indicating the presence of other labeled non-lipovitellin proteins. Levels of labeled TCA precipitable proteins were identical in both crude and purified fractions of fragments incubated in lipovitellin (Fig. 10), indicating the presence of only labeled lipovitellin. When examining the CPM/mg total ovarian protein in the lipovitellin band, the highest counts were associated with

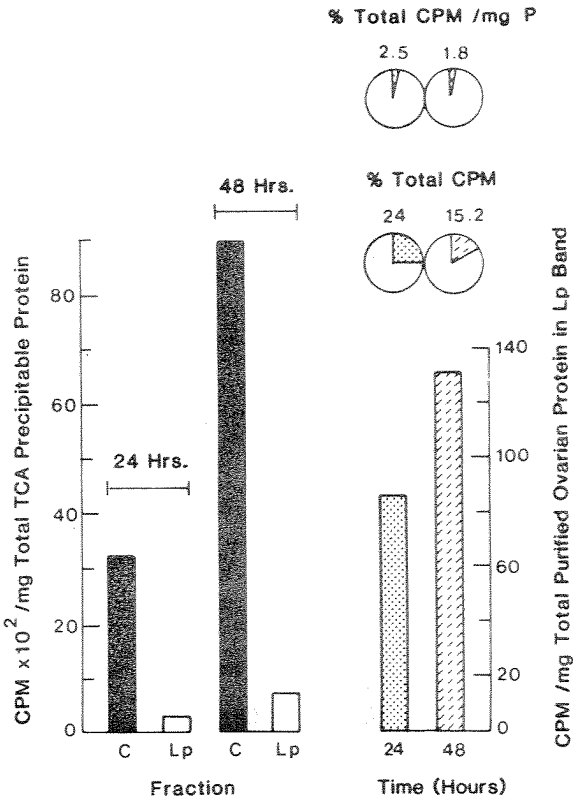

Fig. 9. Levels of label incorporation into the TCA precipitable proteins of crude and puified fractions and levels of label incorporated into purified lipovitellin from an H. americanus ovary incubated in vitro for 24 and 48 h in male serum and ^{3}H-leucine (5 µCi/mL) during secondary vitellogenesis. The percent of the total CPM and total CPM/mg protein loaded onto the gel that was found in the lipovitellin band is indicated also.

fragments incubated in male serum, followed by those incubated in female serum, while the lowest counts were associated with those incubated in purified lipovitellin (Fig. 10). When expressing these counts in terms of the percent of the total CPM/mL or percent of the total CPM/mg/protein/mL loaded onto the gel, the highest percentages were associated with those fragments incubated in purified lipovitellin, followed by those incubated in female serum, and those incubated in male serum (Fig. 10), indicating that the ovary was more efficient in taking up labeled lipovitellin or serum vitellogenin than in converting ^{3}H-leucine into lipovitellin. However, results do indicate that the ovary is capable of synthesizing this lipoprotein.

Primary vitellogenesis

Females used for ovarian cultures had extruded 3-4 to 8-9 wk prior to use, and hence varied in their stage of primary vitellogenic activity (see Aiken and Waddy 1980, 1982, for a review of ovarian condition, size, color, etc. in relation to maturity in Homarus). The earliest ovary (3-4 wk post-extrusion) was light green in color and showed evidences of resorption in the posterior lobes. The middle ovary (6-7 wk post-extrusion) was medium

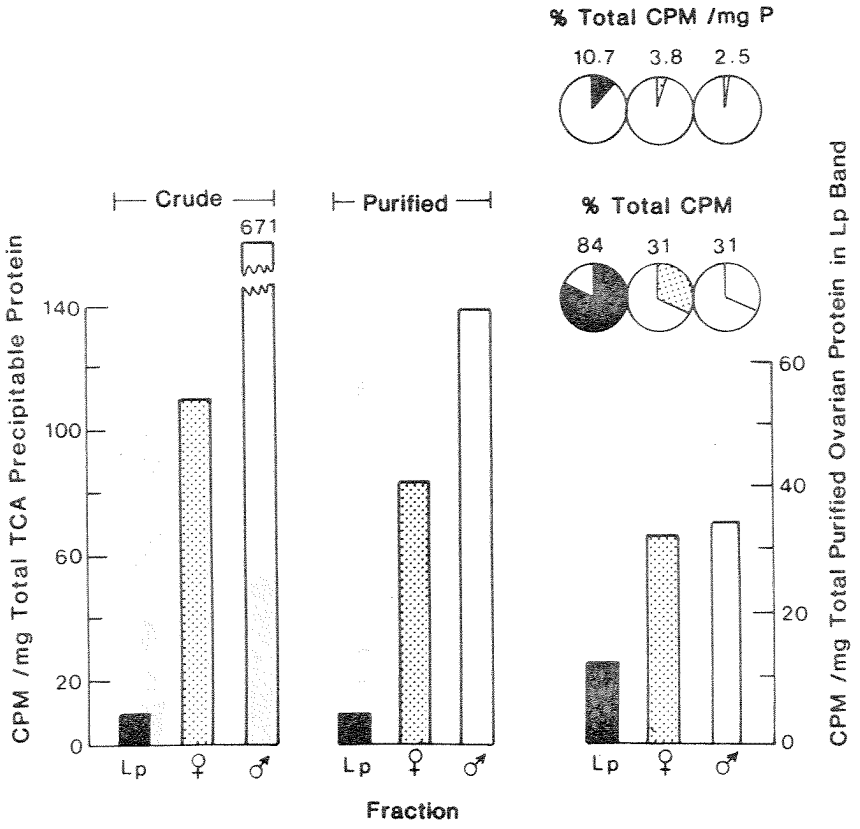

Fig. 10. Levels of label incorporated into the TCA precipitable proteins of crude and purified fractions and levels of label incorporated into purified lipovitellin from an H. americanus ovary incubated in vitro for 24 h in either ^{3}H-lipovitellin (Lp), ^{3}H-female serum (♀), or male serum and ^{3}H-leucine (♂) (5 µCi/mL) during secondary vitellogenesis. The percent of the total CPM and CPM/mg protein loaded onto the gel that was found in the lipovitellin band is indicated also.

green in color and showed small evidences of resorption in the tips of the posterior lobes; and the latest ovary (8-9 wk post-extrusion) was medium green in color and showed no evidence of resorption (it is this ovary stage that remains throughout the winter; (Waddy and Aiken 1983)). Ovarian fragments were cultured for 36 h in male serum and ^{3}H-leucine (5 µCi/mL). Levels of incorporation of label into TCA precipitable proteins were higher in crude fractions when compared to purified fractions (Fig. 11); indicating the presence of other labelled non-lipovitellin proteins. Levels of TCA precipitable proteins increased from the earliest to the middle post-extrusion ovary, then decreased in the latest post-extrusion ovary (Fig. 11), indicating that the production of lipovitellin occurs concomitantly with resorption of relict oocytes during primary vitellogenesis, and is of a short duration in the lobster. The CPM/mg total ovarian protein in the lipovitellin band showed highest levels of labeled protein in the 6-7 wk post-extrusion ovary, which represented 22% of the total CPM/mL or 11% of the total CPM/mg protein/mL loaded onto the gel (Fig. 11). The CPM/mg total ovarian protein found in the lipovitellin band as well as the percent of the total CPM/mL and the percent of the total CPM/mg protein/mL were considerably lower for those ovarian fragments from the earliest and latest post-extrusion ovaries, again indicating that the time course of lipovitellin synthesis during primary vitellogenesis is of short duration.

Ultrastructurally, Eurenius (1973) and Hinch and Cone (1969) have described two very different ways that yolk protein formation occurs in crustaceans. The cisternae of the granular endoplasmic reticulum is involved in one type of yolk formation, while accumulation of material within a limiting membrane of the smooth type, presumably derived from micropinocytosis, accounts for the second type of yolk formation. Zerbib (1977, 1979), DeLeersynder et al. (1980), and Varadarajan and Subramoniam (1980) have added various types of cytochemical and histochemical data that not only substantiate the ultrastructural evidence for a dual origin of yolk formation, but also pinpoint endogenous yolk formation as a product of primary vitellogenesis while exogenous yolk formation is a product of secondary vitellogenesis. Souty (1980) has reported for the isopod Idotea that a small amount of endogenous synthesis of lipovitellin occurs early in secondary vitellogenesis and is replaced by pinocytotic uptake of materials as secondary vitellogensis progresses. She also reported that micropinocytotic activity ceases as ovarian maturation nears extrusion.

In light of this new information, it is of value to try to reinterpret past experimental data

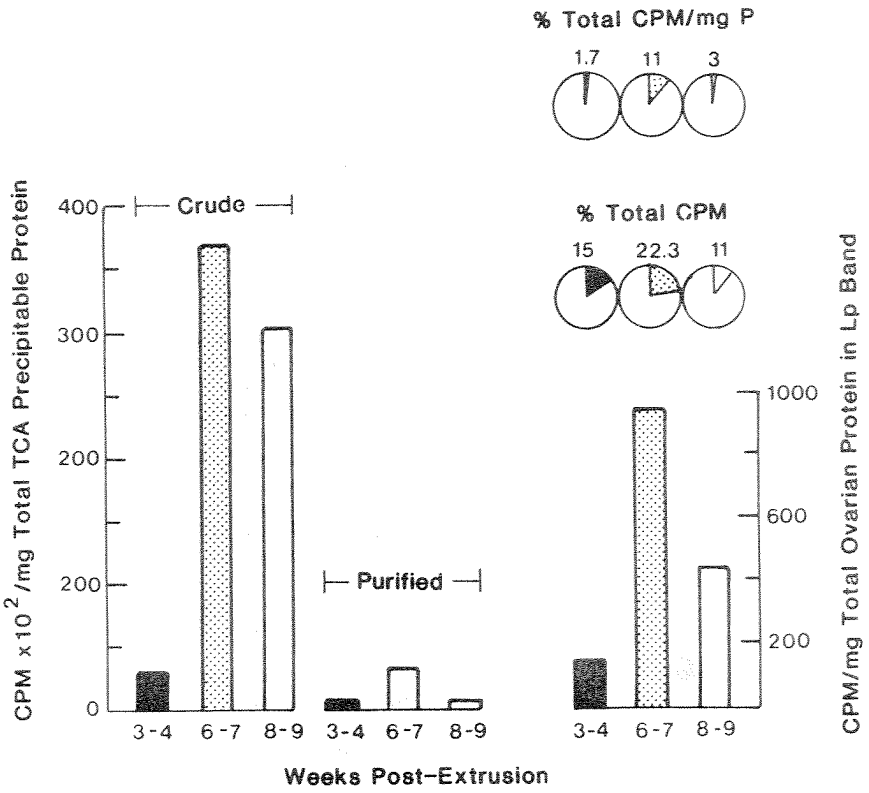

Fig. 11. Levels of label incorporated into the TCA precipitable proteins of crude and purified fractions and levels of label incorporated into purified lipovitellin from H. americanus ovaries incubated in vitro for 36 h in male serum and ^{3}H-leucine (5 uCi/mL) during primary vitellogenesis. The percent of the total CPM and total CPM/mg protein loaded onto the gel that was found in the lipovitellin band is indicated also.

that describes yolk formation as entirely endogenous or exogenous in origin. Ganion and Kessel (1972) showed that the ovaries of the crayfish Orconectes were capable of synthesizing ovarian proteins in vitro, but lipovitellin was not isolated. Using autoradiography to cytologically examine ^{3}H-leucine incorporation, they found that the most extensive labeling of oocytes were those in early stages of vitellogenesis, and that the rate of labeling decreased as vitellogenesis proceeded. One can only presume that these early stages (small oocytes 260-400 μm) were oocytes in primary vitellogenesis, while later stages (medium and large oocytes) were in early and middle to late secondary vitellogenesis, respectively. Lui et al. (1974) showed that the ovaries of the crayfish Procambarus could synthesize lipovitellin in vitro. In a later in vitro study, it was shown that radioactive amino acids were incorporated into three of the five purified subunits of lipovitellin, which strongly suggested that the ovary was capable of endogenous synthesis of lipovitellin (Lui and O'Connor 1976). The reproductive state of these crayfish was not described, but medium size oocytes would indicate that these animals most likely were in early stages of secondary vitellogenesis. Wolin et al. (1973) showed uptake of fluorescein-conjugated serum and ovarian proteins by micropinocytosis. The greatest amount of fluorescence was observed in the earliest stages of secondary vitellogenic oocytes and gradually tapered off to no fluorescence in mature, ready-to-extrude, oocytes. They also reported no uptake of fluorecein-conjugated male serum in any stage examined, but their work did not include primary vitellogenic oocytes. Ganion et al. (1972) incubated ovarian fragments and isolated oocytes of Libinia in isolated ^{3}H-serum vitellogenin and ^{14}C-leucine. They found that the ^{14}C-leucine label was associated with insoluble protein, while all the ^{3}H-label was found in the soluble protein fraction, to which lipovitellin belongs. They concluded that the ovary sequesters lipovitellin and endogenously synthesizes other non-lipovitellin protein(s). Wallace believes that the other protein(s) may be associated with the matrix of the yolk platelets (pers. comm.). It is assumed that these animals were in early to middle stages of secondary vitellogenesis as serum vitellogenin was being synthesized.

These data for Homarus indicate that the ovary is capable of endogenously synthesizing ovarian protein(s), and among them lipovitellin. The greatest amount of synthetic activity for lipovitellin appeared to be associated with ovaries undergoing primary vitellogenesis, followed to lesser degrees by ovaries in early-mid stages of secondary vitellogenesis (Fig. 12). Levels of labeled lipovitellin were minimal, yet levels of labeled TCA precipitable proteins were high, indicating the presence and synthesis of other non-lipovitellin proteins in the ovary. Uptake of purified lipovitellin also occurred during secondary vitellogenesis, indicating that the ovary is capable of sequestering proteins (see I. Role of Serum Vitellogenin, for further discussion). As the

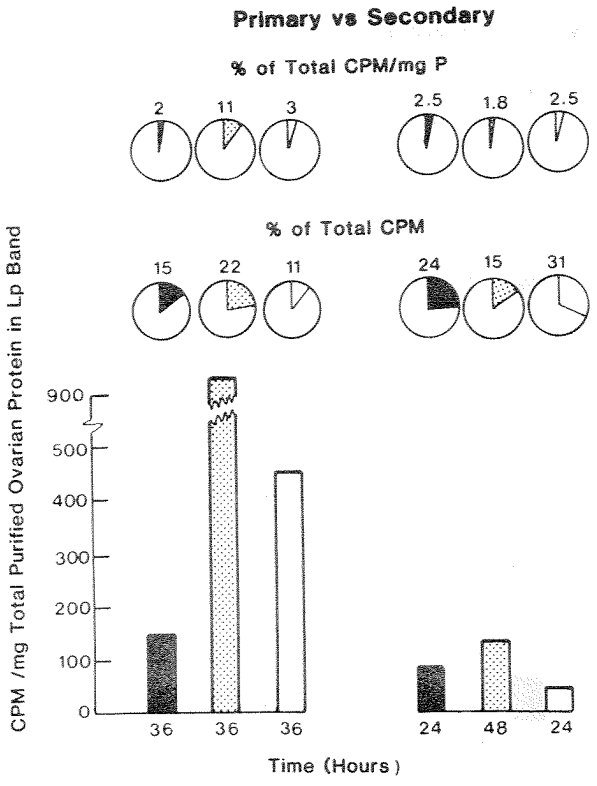

Fig. 12. Comparisons of levels of label incorporated into purified lipovitellin from H. americanus ovaries incubated in vitro in male serum and ^{3}H-leucine (5 µCi/mL) during primary and secondary vitellogenesis.

amount of label associated with lipovitellin was low in all cases examined, and the purified lipovitellin fraction does contain other protein contaminants in small amounts (p. 2), refinement of both purification techniques and methods of quantifying the presence of label in lipovitellin needs to be given considerable attention before additional work is attempted. The counting efficiencies for tritium in polyacrylamide gels were low (20%). It is hoped that gel autoradiography using a film especially designed for tritium will provide more details concerning the presence of label in lipovitellin and the other proteins present in the purified fractions. This is currently under way, but improved purification methods for isolating lipovitellin from the ovary and vitellogenin from the serum are needed to provide conclusive evidence that these proteins are sequestered and/or, in the case of lipovitellin, produced in vitro. It also is hoped that, in the future, investigators will provide definitive information concerning the exact reproductive state of animals used to allow for clarification of the origin of lipovitellin and the role of the ovary as regards protein synthesis during vitellogenesis.

2. IN VIVO ACCUMULATION OF LABEL AND/OR LABELED PROTEIN INTO LIPOVITELLIN

Levels of labeled TCA precipitable proteins increased from 5- to 96-h post-injection in both crude and purified fractions (Fig. 13), indicating incorporation was a function of time. Label was associated with the lipovitellin at 5 h and increased through 96 h in both crude and purified fractions (Fig. 13, 14). Counts were higher in crude fractions, indicating the presence of other non-lipovitellin proteins (Fig. 13, 14). The percentage of the total CPM/mg protein/mL loaded

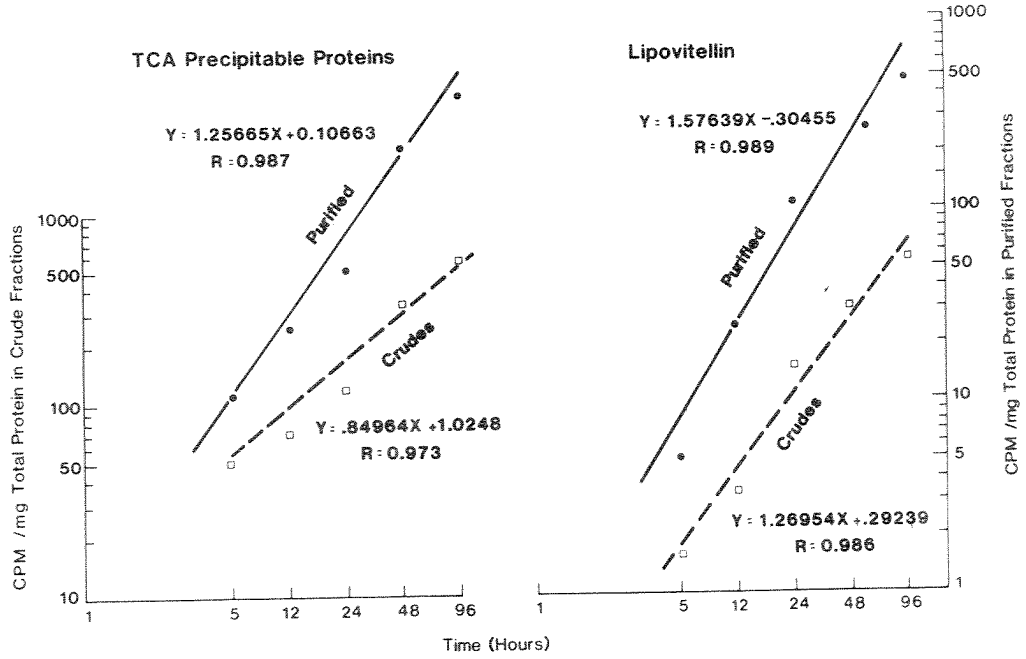

Fig. 13. In vivo incorporation/accumulation rates of label into TCA precipitable proteins and lipovitellin of crude and purified fractions of ovaries from vitellogenic H. americanus injected with ^{3}H-leucine (1.5 µCi/g wet body weight).

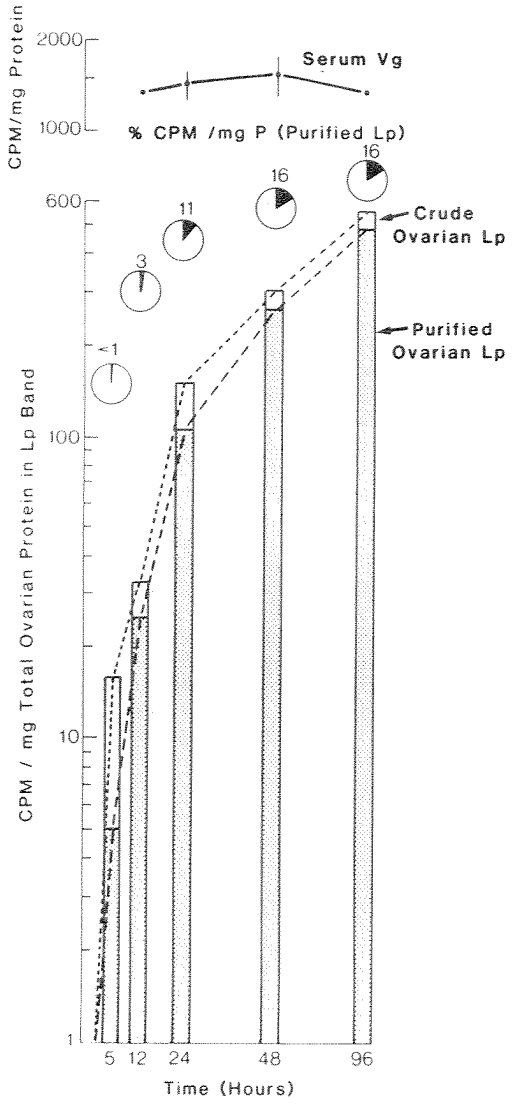

Fig. 14. Time course of incorporation/accumulation of label and/or labeled proteins into the lipovitellin of crude and purified fractions of ovaries and serum from vitellogenic H. americanus injected with ^{3}H-leucine (1.5 µCi/g wet body weight).

onto the gel that was associated with the lipovitellin band increased from less than 1 at 5 h to 16 at 96 h (Fig. 14). Rates of incorporation of label into lipovitellin for animals in early secondary vitellogenesis at 24 h show higher levels, 11% CPM/mg protein in vivo compared with 2.5% CPM/mg protein (p. 11), in vitro. This may indicate either faster synthetic rates in vivo as a result of suboptimal incubation conditions, or additional sequestering of labeled vitellogenin from the serum. At this time, it is not known what accounts for these observed differences. No comparisons are available for other crustaceans undergoing secondary vitellogenesis or for females in primary vitellogenesis.

III. ENDOCRINE CONTROLS OF VITELLOGENESIS

INTRODUCTION

The controls of vitellogenesis are just beginning to be investigated in crustaceans (see Meusy 1980 for review). Figure 15 outlines the hypothetical controls involved in endogenous regulation of crustacean vitellogenesis. It is assumed that the neurosecretory center produces three hormones: gonad inhibitory hormone (GIH), gonad stimulating hormone (GSH), and molt inhibitory hormone (MIH). It has been suggested that GIH inhibits ovarian maturation, GSH stimulates formation and the utilization of yolk proteins, while MIH prevents molting during the reproductive cycle through suppression of the Y-organ which produces the molting hormome (MH)(Adiyodi and Adiyodi 1970; Aiken and Waddy 1980). Evidence from various experimental studies indicate that the Y-organ produces a factor, perhaps low levels of MH, which has a positive effect on both the vitellogenin synthesizing tissues and gametogenesis (Besse 1976; Besse and Maissiat 1971; John and Thampy 1973; Meusy et al. 1977). Experimental evidence also suggests that the vitellogenin synthesizing tissue is stimulated by a hormone, vitellogenin stimulating ovarian hormone (VSOH), produced by the ovary (Junera et al. 1977b). Presumably, synthesis of VSOH would be suppressed by GIH and stimulated by GSH (Aiken and Waddy 1980). The mandibular organ also has been suggested to have a possible endocrine function, as ovaries of immature crabs exhibited signs of precocious vitellogenesis after mandibular organ implants (Hinsch 1980). A progesterone-like substance has been identified in the mandibular organ (Couch et al. 1978), and may play a role in stimulating ovarian activity in conjunction with other neuroendocrine organs. Seasonal effects of eyestalk extirpation have been observed, indicating that various environmental factors may act as proximate controls of endogenous systems (Adiyodi and Adiyodi 1970).

Eyestalk ablation accelerates vitellogenesis in a variety of decapods (Adiyodi and Adiyodi 1970; Anilkumar and Adiyodi 1980; DeLeersynder and Dhainaut 1977; DeLeersynder et al. 1980; Meusy 1980). Eyestalk ablation removes the X-organ sinus gland complex which acts as the neurosecretory center, and presumably removes the effects of GIH. In addition, eyestalk hormones may control the maintenance, resorption, and/or uptake of vitellogenin. Neurosecretory controls have not been demonstrated for any of the crustaceans (Meusy 1980). It seemed of interest to examine the effects of neurosecretory center removal on the vitellogenic activity of females in final stages of ovarian development (secondary vitellogenesis), and more specifically to examine those processes associated with extra-ovarian vitellogenin production, uptake and/or production by ovaries, and to see if the neurosecretory center had a role in ovarian maintenance.

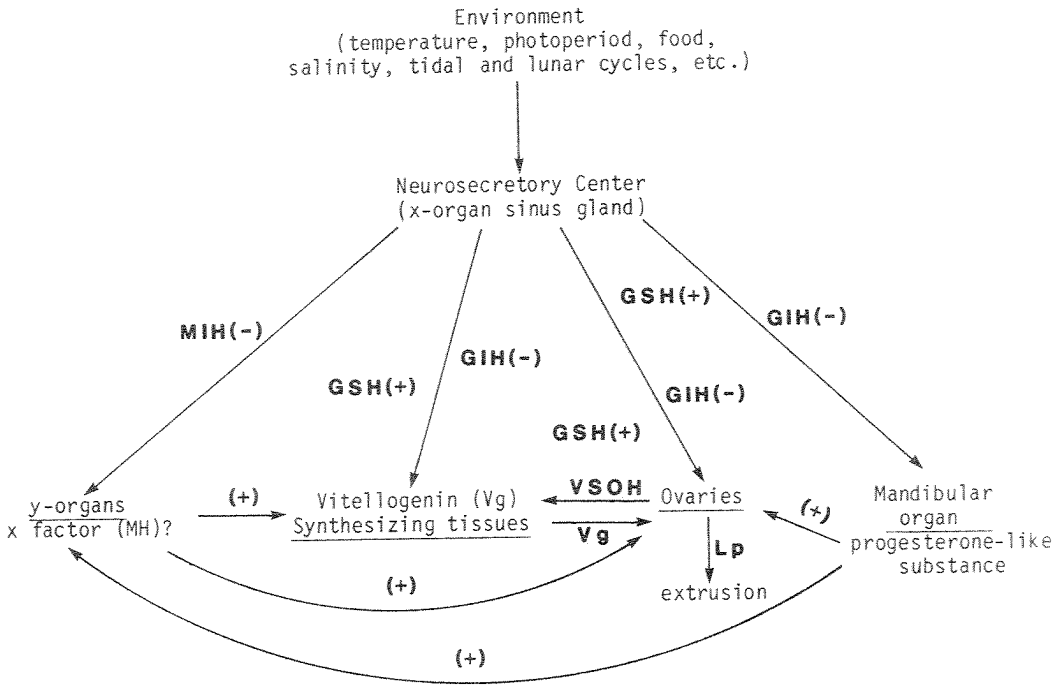

Fig. 15. Hypothetical controls of reproductive activity in crustaceans (modified from Adiyodi and Adiyodi 1970; Aiken and Waddy 1980; and Meusy 1980). MIH - molt inhibiting hormone, MH - molt hormone, GSH - gonad stimulating hormone, GIH - gonad inhibiting hormone, VSOH - vitellogenin stimulating hormone, Vg - vitellogenin, Lp - lipovitellin.

MATERIALS AND METHODS

Five pre-ovigerous females (415-369 g wet weight) were bilaterally eyestalk ablated with a polyethylene thread ligature, while five pre-ovigerous females (419-326 g wet weight) served as controls. On May 28, 1981, both groups were injected with ^{3}H-leucine (1.5 uCi/g wet body weight). Ablations were performed 24 h prior to injections. A 1-mL serum sample was taken from the ventral abdominal sinus at approximately 3- to 4-d intervals for the first 33 d following the initial injection, then once weekly for the 1st month thereafter. Incorporation of label into serum vitellogenin was examined electrophoretically. Levels of serum protein were measured by the biuret method. Protein levels loaded onto the gels were between 5 and 7 mg/mL (0.05-0.07 mg/10 ul). After the 7th day, one female from each group was sacrificed, and the ovary homogenized and purified. The amount of label associated with lipovitellin was examined electrophoretically. The remaining members of both groups were re-injected on the 7th day to determine whether levels of free label available were low or if vitellogenin production was declining, as levels of labelled serum vitellogenin were decreasing. The control group was injected with L-(^{14}C-(U)-leucine (NEN), 1.5 μCi/g wet body weight) while ablated females were injected with ^{3}H-leucine. Ablated females were not injected with ^{14}C-leucine as this marker was being reserved in case eyestalk implants became necesary. Results are given for ^{3}H-leucine label only, as no ^{14}C-label was found associated with serum vitellogenin at any time throughout the day 9-71 observation period.

RESULTS AND DISCUSSION

Incorporation of label into vitellogenin was variable among animals in both control and ablated groups (Fig. 16). Levels of labeled vitellogenin were generally higher in control females, but were statistically significant only on the 1st day examined (2-d post-injection) (Fig. 16). No ^{14}C-label was found associated with serum vitellogenin of control females at any time during the 71 d they were followed, indicating vitellogenin production had ceased by the 7th day; nor were there any appreciable increases in the amount of ^{3}H-labelled vitellogenin in ablated females, again indicating that vitellogenin production was declining or had halted by this time. Levels of ^{3}H-labeled vitellogenin continued to decline in the serum until either extrusion or resorption occurred, after which levels increased (Fig. 17). These results indicate that this protein was being salvaged from the ovary and not synthesized de novo as there was no ^{14}C-label associated with the post-resorption vitellogenin. Increases in levels of labeled vitellogenin were much higher in females which resorbed ovaries (Fig. 17). Three control females resorbed ovaries 29-33 d after the initial injection, and one female extruded 1 d after the initial injection. No label was found in either the serum vitellogenin or the extruded eggs of this female. All remaining ablated females extruded eggs 7-23 d after the initial injection. Digests of extruded eggs indicated variable levels of both radioactivity and total protein (Table 2). Purified fractions of extruded eggs also showed incorporation of label into lipovitellin (20 x 10^{2} CPM/mg protein).

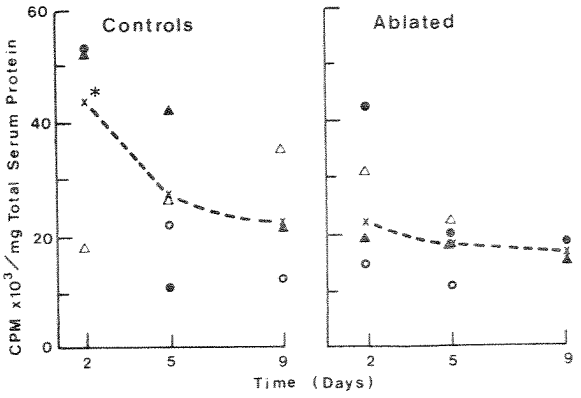

Fig. 16. Levels of incorporation of label into serum vitellogenin of control and bilaterally ablated H. americanus females injected with ^{3}H-leucine (1.5 μCi/g wet body weight). The lines indicate means.

Levels of total serum protein decreased prior to either resorption or extrusion, after which levels increased (Fig. 17). Changes in levels of serum protein had not been examined previously for pre-ovigerous females. It was not known if the observed decrease in protein levels was normal or the result of perturbations induced by the addition of the label. For this reason, changes in serum protein levels were followed in non-radioactive pre-ovigerous females. Four of these females extruded. Levels of total serum protein followed the same pattern as that of radioactive females (Fig. 18), indicating that the radioactivity present did not alter serum protein levels.

The purified fractions isolated from ovaries of females sacrificed 7 d after the initial injection contained the label (Fig. 19). Approximately 95% of the CPM/mL or 12% of the CPM/mg protein/mL loaded onto the gel was associated with the lipovitellin from both control and ablated females (Fig. 19). No observations were made on the levels of label in crude fractions or on the levels of TCA precipitable proteins.

Eyestalk ablation accelerates vitellogenesis during primary vitellogenesis (Adiyodi and Adiyodi 1970; Anilkumar and Adiyodi 1980; Charniaux-Cotton and Touir 1973; DeLeersynder et al. 1980; DeLeersynder and Dhainaut 1977; Descouturelle 1978; Meusey 1980; Schade and Shivers 1980), but results during secondary vitellogenesis are less impressive (Adiyodi and Adiyodi 1970). Ablation had no visible effect on vitellogenin production in H. americanus during secondary vitellogenesis, as ^{3}H-leucine was incorporated into serum vitellogenin of ablated females. That ablation had no effect on vitellogenin production is in agreement with observations of Blanchet et al. (1979) who found vitellogenin synthesis independent of hormonal fluctuations in the amphipod Orchestia. Initial levels of incorporation were higher in the control group, but ablated females extruded 7-23 d after the initial injection. Highest levels of incorporation were associated with females which extruded 23 and 17 d after the initial injection, while lowest levels were associated with the female who extruded 7 d

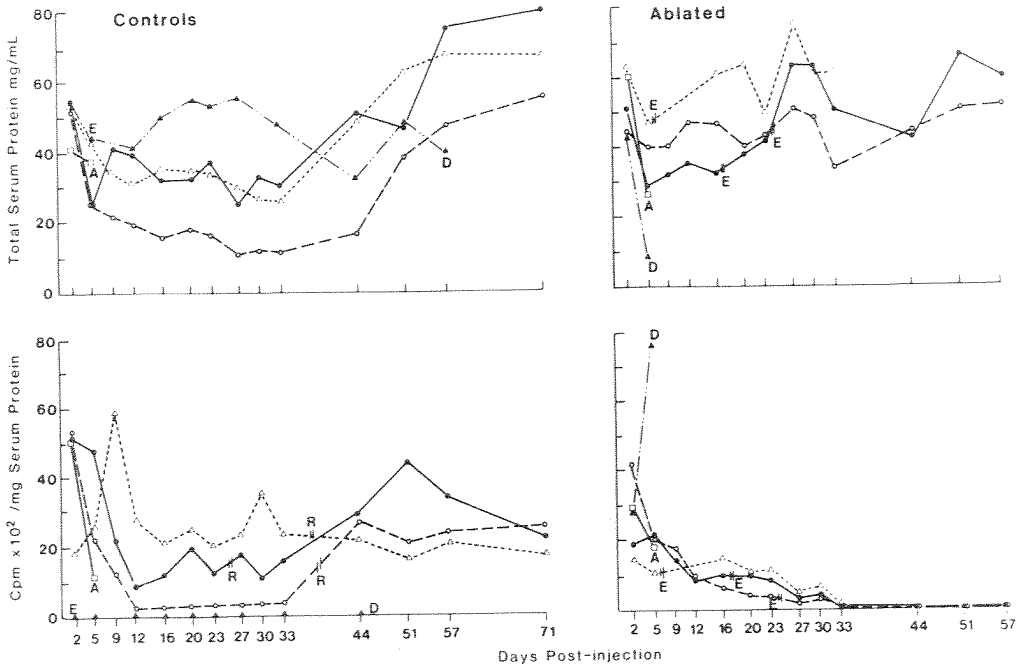

Fig. 17. Levels of ^{3}H-vitellogenin and levels of total serum proteins in control of bilaterally ablated H. americanus females injected with ^{3}H-leucine (1.5 µCi/g wet body weight). R - resorption, D - died, E - extrusion, A - autopsied.

Table 2. Levels of radioactivity and levels of total protein associated with whole egg digests of extruded eggs from ablated female H. americanus injected with ^{3}H-leucine (1.5 µCi/g wet body weight).

Extrusion (Days post-injection)	CPM x 10^2/egg		Protein (mg/mL)	
	X ± SD	Range	X ± SD	Range
17	7.29 ± 2.3	4.5 - 14.9	4.8 ± 0.8	7.5 - 3.9
7	4.8 ± 3.8	0.4 - 13.2	4.6 ± 0.9	7.5 - 2.9

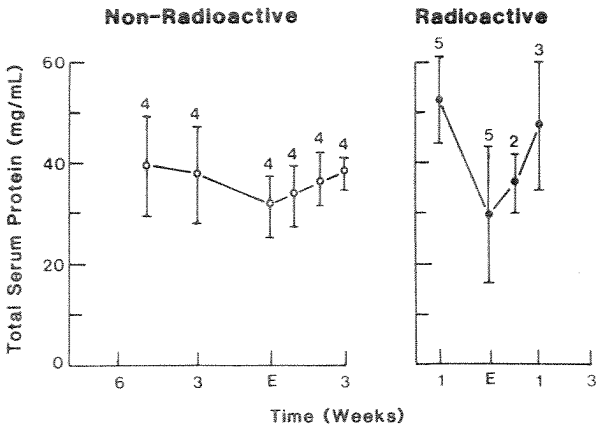

Fig. 18. Levels of total serum protein in radioactive and non-radioactive H. americanus that underwent extrusion.

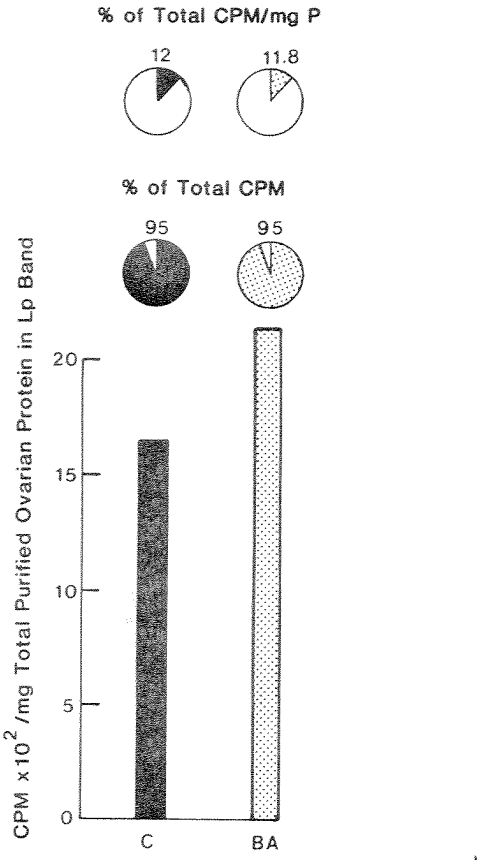

Fig. 19. Levels of label incorporated into purified lipovitellin from ovaries of control (C) and bilaterally (BA) ablated H. americanus females 7 d after injection with ^{3}H-leucine (1.5 µCi/g wet body weight).

after the initial injection. This supports the observation that serum vitellogenin production decreases and stops prior to extrusion and/or resorption (no ^{14}C-label was found associated with the serum vitellogenin of control females). Ablation had no visible effect on vitellogenin uptake and/or the uptake of ^{3}H-leucine, as the label was found associated with the lipovitellin from both ovaries and extruded eggs.

Normally maturing oocytes are ovulated and oviposited but, under certain circumstances, total ovarian resorption may occur (Aiken and Waddy 1980). Resorption of control females most likely was stimulated by differences in the holding conditions of the two groups. Ablated females were held in individual compartments to prevent cannabalism, as ablation results in hyperphagy (Adiyodi and Adiyodi 1974). Control females were held communally with individual shelters into which they might crawl. The lack of individual compartments has been observed to be a possible trigger of resorption in the lobster (Waddy and Aiken 1983).

Changes in total serum proteins of females during the reproductive season previously had not been followed. These data for H. americanus indicate that both serum protein levels and levels of serum vitellogenin decline during secondary vitellogenesis. Following extrusion or resorption, levels of total serum protein and serum vitellogenin increase in response to the resorption of relict eggs and/or protein synthesized but not utilized (see I. Role of the Ovary, for further discussion). The increase in levels of serum protein is greater in females who undergo total ovarian resorption. In many crustaceans, a small rise in levels of both blood proteins and serum vitellogenin has been observed following the completion of oocyte growth, and has been attributed to protein salvage from the ovary (Adiyodi and Adiyodi 1974; Anilkumar and Adiyodi 1980).

IV. SYNTHETIC SITE FOR EXTRA-OVARIAN VITELLOGENIN

INTRODUCTION

Various studies, using electrophoretic, incubation, and immunochemical techniques, have provided confusing evidence for delineating the synthetic site of extra-ovarian vitellogenin in crustaceans. The three suspected sites have been: the hepatopancreas, which is analogous to the liver, the synthetic site of yolk precursors in vertebrates; the hemocytes, which have been suggested to contribute to the production of serum proteins in insects; and the subepidermal adipose tissue, which is analogous to the fat body, the synthetic site of extra-ovarian vitellogenin in insects (see Adiyodi and Adiyodi 1974 and Meusy 1980 for reviews).

The hepatopancreas has been suggested to be the main site of extra-ovarian vitellogenin synthesis (Besse et al. 1970; Ceccaldi 1970; Ceccaldi and Martin 1969; Kessel 1968; Wolin et al. 1973). Besse et al. (1970) and Wolin et al. (1973) reported the existence of a protein substance in hepatopancreatic extracts that was identical to the female-limited serum protein, vitellogenin. Picaud and Souty (1980) demonstrated the presence of vitellogenin in the hepatopancreas, but they did not find any incorporation of label into this material, suggesting that the hepatopancreas may absorb and eliminate excess vitellogenin. Electrophoretic analysis of the hepatopancreas of Paratelphusa showed the presence of none of the major vitellogenins (Adiyodi and Adiyodi 1972).

Preliminary evidence indicated that the hemocytes may synthesize the sex-limited serum protein, as they have been found to produce labeled proteins in vitro (Kerr 1968). However, electrophoretic attempts to demonstrate the presence of vitellogenin in the hemocytes of Paratelphusa were unsuccessful (Adiyodi and Adiyodi 1972). No other attempts have been made to clarify the possible role of the hemocytes in vitellogenin production.

Recently, evidence has been accumulating in support of the subepidermal adipose tissue being the synthetic site of extra-ovarian vitellogenin. During both sexual inversion and normal progression of vitellogenesis, the subepidermal fat has been observed to undergo rapid growth and development (Berreur Bonnenfant 1971). Junera and Croisille (1980) have shown, via autoradiography, that ^{3}H-leucine was incorporated into the subepidermal fat of the thoracic region. In a series of both in vivo and in vitro studies, using immunofluorescence, immunoradiographic, and radioimmunoassay techniques, it has been demonstrated that the subepidermal fat contains not only immunoreactive sites, but also incorporates label into vitellogenin (Picaud 1980; Picaud and Souty 1980; Souty and Picaud 1981).

The purpose of this phase of the work was to examine all previously suspected synthetic sites of the extra-ovarian vitellogenin, using in vitro culture methods in hopes of clarifying the origin of this protein. As all methods used were inconclusive in identifying the synthetic site for vitellogenin, only the basic methods tried and information concerning their usefulness or lack of usefulness will be discussed.

METHODS, RESULTS AND DISCUSSION

Intact hemocytes were gathered as described by Stewart et al. (1966). All equipment including the lobsters were pre-cooled (4°C). The hemolymph was withdrawn from the ventral abdominal sinus into syringes coated with vaseline to prevent clot formation. Samples were transferred to vaseline coated centrifuge tubes and immediately centrifuged at 17000 RPM for 15 min. Following centrifugation, the hemocytes were separated from the pellet in a volume of potassium phosphate buffer (0.21 M, pH 8.0) equal to 1/5 the original hemolymph volume for 24 h. Following extraction and centrifugation, the supernatant was used for culturing.

The hepatopancreas and adipose tissues were removed as aseptically as possible (the carapace was surface cleansed with 70% ETOH), and placed in sterile Ringers containing antibiotics (p. 10). Organs were rinsed twice before they were cut into small pieces. To minimize the lytic action of the hepatopancreatic enzymes and thus slow tissue breakdown, the Ringers solution was removed by pipet with minimal disturbance to the organ pieces. The procedure was repeated 5-6 times until the solution was relatively free of small tissue debris. Organ pieces were then carefully blotted and transferred to sterile culture vials containing the culture media. Adipose tissues were treated in the same manner.

Organ pieces and isolated hemocytes were incubated in the presence of ^{3}H-leucine (5 µCi/mL, sp. act. 115.3 Ci/mmol) at 19.5 ± 0.2°C in a Gilson shaking water bath. Following incubation, media was filtered, 25 mM PMSF added to inhibit enzymatic degradation, and examined for incorporation of label into TCA precipitable proteins and for the presence of vitellogenin. Protein levels were measured by the Lowry method (p. 2).

In the late summer of 1981 organs from a pre-ovigerous female were cultured for 24 h in male serum (100%), antibiotics (1%) and ^{3}H-leucine. All cultures exhibited incorporation of label into TCA precipitable proteins, but vitellogenin was not detected by the Coomassie blue stain, nor were there any appreciable counts present in any of the electrophoretic bands. Levels of labeled protein loaded onto the gel were less than 6% of the total TCA precipitable proteins. It appeared that using male serum as a culture media combined with electrophoretic separation of proteins was not suitable for measuring small levels of radioactivity or for determining the presence of newly synthesized vitellogenin.

A radioimmunoassay was then devised to be used in conjunction with immunoelectrophoresis (Clark and Freeman method), during the 1982 reproductive season, in hopes of increasing the sensitivity of measuring small levels of vitellogenin and insuring that vitellogenin was the only protein in the immunoprecipitate. The basic RIA was modified from those used by Hagedorn et al. (1978) for insects and Souty and Picaud (1981) for isopods. Organs were cultured for 5 h in Ringers (Welsh et al. 1968), label and antibiotics. The RIA consisted of 100 uL

media to which 25 uL of unlabeled ligand (purified ovarian lipovitellin, 1 mg/mL) and 125 uL of anti-serum (1:50 dilution, as determined from a dilution curve) was added. This was incubated for 2 h at 37°C and then placed in the cold (4°C) overnight. After mixing, the precipitate was transferred, without dilution, to a 0.45 uM HAWP millipore filter which had been pre-soaked in sodium phosphate buffer (0.05 M, pH 7.4) containing 0.5% BSA, 1% unlabeled D,L-leucine, and 0.9% NaCl to reduce non-specific protein binding. The incubation tube was rinsed with 4 mL of the phosphate buffer and transferred to the filter which was then washed with an additional 30 mL of buffer, followed by 4 mL (1:1) chloroform: methanol. The filter was allowed to dry then transferred to a toluene-based scintillant, stored in the dark for 24 h, and counted.

Incorporation of label into TCA precipitable proteins was followed in two ways: At hourly intervals, 250 uL of media was removed and centrifuged. Duplicate 50-uL samples were pipeted onto filter paper discs (Mans and Novelli 1961). In this way, incorporation into proteins could be followed throughout the incubation period. At the end of the incubation period after treatment of the media (see above), 100 uL of media to which 25 uL of unlabeled ligand and 125 uL of 10% TCA were added to examine incorporation into TCA precipitable proteins. The precipitate was washed onto a millipore filter, rinsed with 30 mL of 10% TCA, 4 mL chloroform: methanol, dried, and treated as the RIA filters for scintillation counting. Data were expressed as the ratio of label incorporated in immunoprecipitate vs label incorporated into total TCA precipitable proteins, which eliminated variations due to differences in amino acid pools and incorporation rates among different animals.

Incorporation of label into TCA precipitable proteins was linear throughout the incubation period for all cultures, indicating that the cultures were viable. All cultures showed counts associated with the RIA precipitates, presumably indicating the presence of vitellogenin. Results from the immuno-electrophoresis showed the presence of multiple precipitin arcs, indicating that either the antibody was contaminated or the antigenic determinant was a common component among many different proteins.

As the 1982 reproductive season was nearing completion and no other antiserum was available, the culturing protocol was altered in the hopes of simply identifying the presence of vitellogenin. Organs were cultured for 5 h in Ringers. Levels of incorporation into TCA precipitable proteins were followed throughout the incubation period, using the filter paper disc method. Following incubation and treatment of media, the media were thoroughly dialyzed against electrophoresis buffer (p. 2) to remove excess unincorporated label. Electrophoresis was carried out on 5% polyacrylamide gels. Half of the gel was stained with silver stain, the other half with Coomassie blue. Incorporation of label into TCA precipitable proteins was linear for all cultures. Results of silver staining indicated that the adipose tissue culture media contained a protein band in the approximate position of vitellogenin. This band was not visible in any of the other culture media, nor in the half of the gel stained with Coomassie blue. Coomassie-stained gels were dried for autoradiography on LKB-Ultrofilm. Silver-stained gels could not be used for this purpose, as the silver interferes with autoradiography (VanKeuren et al. 1981). Results are not yet complete for this technique.

GENERAL CONCLUSIONS

These data from Homarus americanus tend to support current theories for a dual origin of yolk proteins in crustaceans. Extra-ovarian serum vitellogenin has a role in yolk formation during secondary vitellogenesis, while endogenous synthesis of yolk proteins seems to be more active in primary vitellogenesis.

Secondary vitellogenesis is characterized by high levels of vitellogenin in the serum which are the result of protein in transit from the synthetic site to the ovary. Vitellogenin production declines and ceases at least 24 h prior to extrusion and at least 2-3 wk prior to total ovarian resorption. Total levels of serum proteins decrease prior to extrusion or resorption, after which they increase, and vitellogenin levels follow the same pattern. With resorption, levels of total serum protein and vitellogenin increase markedly and are the result of protein salvage from the ovary. Eyestalk ablation had little effect on vitellogenin production and uptake during secondary vitellogenesis.

The ovary is capable of synthesizing proteins and lipovitellin. The greatest amount of synthetic activity is during primary vitellogenesis. The ovary is also capable of sequestering proteins during secondary vitellogenesis as purified lipovitellin was taken up in vitro.

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