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Edward Michael Wolin
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YOLK FORMATION IN CRUSTACEAN EGGS

EDWARD MICHAEL WOLIN
B. A., Reed College, 1970

A thesis submitted in partial fulfillment

of the requirements for the degree of

Doctor of Medicine

Department of Anatomy, Yale University School of Medicine

New Haven, Connecticut

1974

This thesis is dedicated to the memory

of Professor Gabriel Lester, Reed College,

beloved teacher, scientist, and humanitarian.

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Yolk Formation in Crustacean Eggs

Edward M. Wolin

Abstract

A variety of cytochemical techniques were used to demonstrate how crustacean lipovitellin accumulates within the egg. It was found that a protein serologically identical to the lipovitellin of yolk spheres was present in the hemolymph of vitellogenic crustaceans, but was absent from the hemolymph of males and immature females. In the three crustacean species studied (*Uca pugilator*, *Cambarus clarkii*, and *Libinia emarginata*), pinocytosis of fluorescein-conjugated lipovitellin and trypan blue occurred only during those periods when oöcytes were accumulating yolk. It may be concluded from the present studies that yolk spheres develop in crustacean eggs primarily through micropinocytotic uptake of lipovitellin from the hemolymph, although other oöcyte proteins appear to be made *de novo*.

CHAPTER I

HISTORICAL BACKGROUND - Vitellogenesis in Organisms Other than Crustacea

For many years, as a result of studies made on developing oöcytes using light microscopy, it was believed that yolk arose from a special body in the oöcyte called the "yolk nucleus" or the "yolk nucleus of Balbiani", first observed in spider oöcytes in 1845 by Wittich (Raven, 1961; Ward, 1962). Although yolk nuclei have been seen in many kinds of eggs and even assumed to be an essential component of developing oöcytes in general, in every case they have turned out to be other cytoplasmic organelles present in the periphery of the cell where yolk was being most actively accumulated. In the years since yolk nuclei were first described, oöcytes of a great variety of animals have been observed, first with light microscopy, and then in more recent years with electron microscopy. The task of yolk synthesis has been attributed at various times to any of the many cell organelles which happened to be in the same part of the oöcyte as the developing yolk granules. The situation rapidly became so confused the E. B. Wilson (1925) was prompted to write this despairing summary of the problem:

> In spite of numerous researches on yolk-formation, extending over a period of more than fifty years, the subject still remains in so confused a state that all statements in regard to it must be made with considerable reserve. Even in the recent literature we find the origin of the yolk-spherules ascribed to chondriosomes, to Golgi-bodies, to chromidia, extruded nucleoli or nucleolar fragments; while some observers consider that the yolk arises de novo in the cytoplasmic substance without discoverable relation to the other formed elements. We must, therefore, conclude either that there is no general uniformity in the mode of yolk-formation, or that many of the existing accounts of the subject are erroneous.

His words remained accurate until very recently, when modern techniques of biochemistry and immunochemistry combined to shed new light on vitellogenesis. Oögenesis in insects, chickens, and frogs has been most intensively studied with these modern methods. In every case, it is clear that the yolk has an origin external to the ovary, and is incorporated into the developing oöcyte by micropinocytosis.

Wigglesworth (1943) provided the first proof that blood proteins are incorporated into developing oöcytes of insects and arachnids by noting that, after a meal of blood, hemoglobin could be found in the oöcytes. A few years later, Telfer (1954) demonstrated that in the pupal Cecropia, an antigen is present in the blood of female silkworms at a concentration one thousand times as great as its concentration in male blood; that during oögenesis, the concentration of the antigen decreases in the blood while increasing in the oöcytes, until it is twenty times as concentrated in the mature egg as in blood at the conclusion of oögenesis; in ovaries transplanted into males, this antigen was found not to be present unless female blood was transfused. It was then found that each of seven proteins immunologically detectable in the blood of the Cecropia moth has an antigenic counterpart in the developing oöcytes of the same animal, although the female-specific protein is much more avidly taken up than any other blood protein (Telfer, 1960). In order for the Cecropia oöcyte to increase in volume 91% per day through the accretion ot yolk spheres (Telfer and Rutberg, 1960), there must be an efficient and selective mechanism for removing proteins from the blood, if this is indeed the mechanism by which yolk spheres form. Telfer (1961) found that such a selective mechanism for protein uptake does actually exist, by staining 2μ sections of ovary with fluorescein-labeled antibodies to blood proteins, as a very sensitive indicator

of the presence of blood proteins. Blood proteins were detected in histological sections in the intercellular spaces of the follicle-cell layer, in association with a brush border at the surface of the oöcyte, and in the yolk spheres within the oöcyte. The existence of blood proteins within the yolk spheres was confirmed by analyses of isolated yolk spheres. Telfer and Melius (1963) proved through the use of radioautography that blood proteins could reach the oöcyte after passing through the layer of follicle cells, as well as through the cellular envelope on the outside of the follicle cells and through the PAS-positive basement membrane which was tightly apposed to the inner surface of the follicle cells. If ovaries were placed in ^{3}H-labeled serum so that the intercellular spaces became filled with radioactive protein, and then were transferred to unlabeled medium, a rapid exchange of radioactive and non-radioactive protein occurred. Electron microscopic studies of the developing oöcytes of the Cecropia moth by Stay (1965) confirmed the free access of blood proteins to the oöcyte surface and demonstrated that micropinocytosis was the mechanism by which blood proteins were taken up by the oöcyte. Electron-dense material was found in the interfollicular cell space and adsorbed to the outer surface of the highly folded oöcyte membrane. The pits in the oöcyte membrane and vesicles immediately under it were lined with the same material, and had the appearance of yolk spheres. Most of the blood protein in the interfollicular space was flocculent, but that in the ovariole wall was granular and evenly distributed, suggesting that a change had taken place. To confirm that the material entering the egg of micropinocytosis was actually blood protein, horse spleen ferritin was introduced into the blood of the Cecropia moth (Stay, 1965). The presence of this ferritin in the intrafollicular cell spaces and in the crypts of the oöcyte surface confirmed the fluorescent antibody and radioauto-

graphic studies (Telfer, 1961; Telfer and Melius, 1963) which indicated that proteins could readily penetrate all of the extracellular spaces and membranes of the follicle, and also indicated that the material in the crypts on the oöcyte surface and in the newly formed yolk spheres was blood protein. That rapid micropinocytosis was occurring during Cecropia oögenesis could be seen from extensive 0.1μ wide and 3-4μ deep crevices which formed a brush border on the oöcyte surface (Stay, 1965). Micropinocytotic pits occurred on the sides and inner ends of the crevices. The pits were of the same 0.13-.17μ diameter as many vesicles in the cortical oöplasm. The pits and vesicles were lined with a layer of dense granular material derived from blood proteins, and were coated on their cytoplasmic surface with 200Å-long fibrils or bristles, which could conceivable be a cytoplasmic contribution to the yolk sphere.

An electron microscopic examination of mosquito oögenesis by Roth and Porter (1964) showed exactly the same morphology and suggested the same mechanism for the uptake of yolk protein. In fact, the uptake mechanism proposed by Roth and Porter (1964) appears to hold for all insects which have been examined so far: 1) blood passes through the basement layer, 2) after passing through the basement layer, the yolk protein precipitates into aggregates which prevents its diffusing out of the interfollicular space, presumably initiated by a factor produced by the egg or follicle cells, 3) these small aggregates condense on the surface of the egg, 4) micropinocytosis occurs (pits form which close off to form vesicles), and 5) the vesicles fuse to form yolk spheres. This model of micropinocytotic uptake of yolk protein is sufficient to account for its selectivity, since the material adsorbed on the cell membrane is taken up selectively and the material in the lumen of the vesicle is taken up non-selectively. Bennett (1956) made these suggestions to

account for selective protein uptake. As the proportion of the cell membrane covered with protein becomes large and the internal volume of the vesicle formed becomes small, the selectivity of uptake becomes greater; making the selectivity of micropinocytosis greater than if the vesicles formed were large, as for example in the amoeba. In the micropinocytic vesicles of the Cecropia moth oöcyte, the volume of the lumen was minimized by having a very thick coat of protein bound to the surface of the oöcyte. After crossing the vitelline membrane, extracellular materials were adsorbed in 10μ sheets to the deeply folded oöcyte surface, with adjacent folds of the oöcyte bound together by the sheet of material between them (Telfer and Smith, 1969). Anderson and Telfer (1969) found that there was an extracellular mechanism for sorting out proteins and concentrating vitellogenin in the Cecropia moth follicle. Two blood proteins, vitellogenin and a carotenoid protein, not readily taken up by the oöcyte, were found by immunochemical measurements to be 2-4 times more concentrated in the spaces between follicle cells than in the blood; the carotenoid did not form aggregates that were adsorbed and pinocytosed by the oöcyte. When vitellogenin uptake by the oöcyte was prevented with dinitrophenol, the carotenoid no longer was more concentrated than in the blood, suggesting a balance between the intercellular concentration mechanism and the removal of vitellogenin by the oöcyte itself (Anderson and Telfer, 1969). Telfer and Smith (1969) found that the affinity of the oöcyte for extracellular materials was increased by the presence of micropinocytotic pits, since all fixable material, except for that in the pits, was removed by soaking the follicle in physiological saline.

Telfer and Anderson (1968) found that blood protein uptake stopped 24 hours before the formation of the chorion began, and when the oöcyte was only two-thirds of its final volume. The size increase appeared to be due to hydration

and was accompanied by the following changes: closure of intercellular channels across the follicular epithelium, the loss of an extracellular material that binds trypan blue into aggregates and may be involved in blood protein uptake, a thickening of the vitelline membrane, the production of highly refractile spherical bodies in the egg cortex, and an increase in the internal osmotic pressure in the yolk spheres.

Telfer and Melius (1963) have accumulated some experimental evidence favoring the idea that the yolk spheres grow by the fusion of micropinocytotic vesicles, rather than by the addition of individual molecules transported across the cell membrane: 1)yolk spheres of the Cecropia oöcyte could be made to fuse experimentally if the yolk spheres were pushed together with sufficient force by placing them adjacent to each other on a microscope slide and gently pressing down the cover glass, or by centrifuging at 10,000xg, 2)yolk spheres had a fine structure indicative of a crystalline arrangement, thus when yolk spheres were found with three regions differing in the orientation of the crystalline axes, this suggested that three yolk spheres had fused, 3) in the periphery of the oöcyte, yolk spheres ranged in size from less than 1μ to more than 20μ , but in deeper layers, the yolk spheres were of a constant large size, and 4)centrifugation of eggs at 20,000xg for 1 minute left the cortical yolk spheres unmoved whereas the deeper ones formed a cap, indicating that the forces necessary to fuse the yolk granules were probably present in the cortex.

Yolk formation by the uptake of a vitellogenin from the blood by micropinocytosis appears to occur by the mechanisms outlined earlier in every type of insect ovary which has been examined by electron microscopy: in the Cecropia moth (Stay, 1965; Telfer and Anderson, 1968; Telfer and Smith, 1969; Anderson and Telfer, 1969), Saturniid moth (Telfer, 1961), mosquito (Aedes aegypti) (Roth

and Porter, 1964), milkweed bug (Kessel and Beams, 1963a), roach (Periplaneta americana (Anderson, 1964), Panorpa communis (Mecoptera) (Ramamutty, 1964), silverfish (Cone and Scalzi, 1967), and in the dragonfly (Beams and Kessel, 1968).

Beams and Kessel (1968) found that although the follicle cells did not seem to play a role in yolk formation, they appeared to manufacture the vitelline membrane and the chorion. The vitelline membrane presecretion seemed to be synthesized by the rough endoplasmic reticulum, where it first appeared as intracisternal granules. These granules moved to the Golgi complex, and were then secreted into the previtelline space and oriented into columns which fused to form a complete envelope. The chorion was made by the follicle cells after the vitelline membrane had been finished. The presecretion first appeared as dense elongate bodies in the Golgi cisternae, which later coiled up unlarging the Golgi cisternae, and were finally secreted to be added to the developing chorion.

Determining the organ that, in insects, secretes vitellogenin into the blood has long been a rather intractable problem. The follicular epithelium does not seem to be involved in protein secretion except in the formation of extracellular membranes. Nurse cells and oöcytes do not have machinery for protein secretion, and the only organs outside the ovary which have an ultrastructure compatible with a secretory function are the midgut and the fat body. Roth and Porter (1964) found that in the mosquito, after a meal of blood containing ^{3}H-leucine, there was an accumulation of grains in the midgut, and then in the oöcytes, when autoradiographs were made. Hagedorn and Judson (1969), also studying the mosquito (Aedes aegypti), removed the fat body, the midgut, and the ovaries at various intervals after a meal of blood (which initiates

vitellogenesis), reacted these with anti-yolk antibodies, then with goat anti-rabbit gamma-globulin complexed with horseradish peroxidase, and finally incubated in a peroxide solution containing 3,3'-diaminobenzidine. Using light and electron microscopy to determine reaction sites, it was determined that the first tissue to react with antibodies was midgut epithelium. But in an effort to determine which organ in the insect was actually able to secrete vitellogenin into the blood and not merely sequester it, as the fat body is known to do, Pan, Bell, and Telfer (1969) incubated several organs from the Cecropia moth (Hyalophora cecropia L.) in an organ culture medium containing ^{3}H-leucine, to see which organ would incorporate the labeled amino acid into a substance precipitable by antibodies to the sex-limited blood proteins of these species. The female fat body, midgut, and wing sacs were tested, as well as male fat body and midgut; these organs coming from animals in various stages of development. The results of this experiment indicate clearly that the fat bodies of females of the species used were the only organs capable of secreting vitellogenin. The fat bodies of females before the appearance of vitellogenin in the blood. and the fat bodies of males, failed to secrete vitellogenin (yolk protein). So, it appears that the yolk protein is secreted by the fat body in insects, at least in the species which have been investigated so far.

In vertebrates as well, yolk protein appears to be synthesized outside the ovary and to be incorporated into the developing oöcyte by micropinocytosis.

Radioisotopic and serological data accumulated by Flickinger and Rounds (1956) suggested that in the frog, Rana pipiens, yolk proteins are synthesized by the maternal liver and then transported to the ovarian oöcytes by way of the blood stream. In both Rana pipiens and Xenopis laevis, micropinocytosis has been implicated as the mechanism of uptake of yolk proteins from the

blood. Glass (1959) treated sections of Rana pipiens ovary, containing oöcytes at various stages of growth, with fluorescein-labeled antibodies to the frog serum proteins. In early vitellogenic oöcytes, one could see bright masses of fluorescent material at cell periphery, and lesser amounts between yolk platelets and around the nucleus. As vitellogenesis progressed, the fluorescent peripheral clumps were replaced by large yolk granules which fluoresced in toto, although there were also traces of fluorescence around the nucleus and nucleoli. More direct evidence for the pinocytotic uptake of yolk proteins in the frog came from the studies of Dumont and Wallace (1968). Human chorionic gonadotropin was administered to female Xenopis laevis to cause ovulation of the mature eggs, and to stimulate vitellogenesis in the ensuing thirty to forty days, during which time serum lipophosphoprotein was secreted by the liver and simultaneously taken up by the vitellogenic oöcytes at an average rate of 7.7 mg/day. The uptake of serum lipophosphoprotein (SLPPP was very selective, since it was taken up at a rate fifty times that of other serum proteins. After uptake into the oöcytes, the SLPP was transformed into the two frog yolk proteins characterized by Flickinger and Schjeide (1957): lipovitellin and phosvitin. The electron microscopic observations of Dumont and Wallace (1968) reveal that the micropinocytotic uptake of yolk proteins occurs by a mechanism very similar to that found in insects: the micropinocytotic pits develop from the lateral walls of deep crypts which penetrate the oöcyte surface and from spaces between microvilli. Micropinocytotic vesicles containing a core of dense material are then internalized, and they fuse with each other allowing the contents to crystallize out into small by typical yolk platelets. There was no evidence of organelle synthesis of yolk in this electron microscopic study of Xenopus oögenesis.

The amphibian SLPP is synthesized and secreted by the liver under the

influence of estrogen. This was first demonstrated in slices of Xenopus liver in organ culture in a medium containing ^{14}C-sodium carbonate (Rudack and Wallace, 1968). Phosvitin was found to be synthesized by the liver of vitellogenic females and estrogen-treated males. A similar result was obtained in vivo by Wallace and Jared (1969), who injected ^{14}C-leucine into Xenopus and followed its fate. In estrogen-treated males and in vitellogenic females, there was rapid incorporation of label into liver protein, followed by an increase in the specific activity of the serum, with most of variable labeling (not found in non-vitellogenic controls) occurring in SLPP, and finally an uptake of SLPP into the oöcytes. At the time of this uptake, pinocytosis was occurring at a high rate, as indicated by trypan blue uptake and by electron microscopy. Wallace and Jared (1969) also showed that once incorporated into the oöcyte, SLPP splits into the two proteins of amphibian crystalline yolk, phosvitin and lipovitellin, which then crystallize into a yolk platelet. This was shown by injecting vitellogenic female Xenopus with SLPP that was doubly labeled with radioactive phosphate and leucine, and finding that by twenty hours after injection, virtually all leucine label was found in lipovitellin and most of the protein phosphorus label was associated with phosvitin. Since the crystalline part of the amphibian yolk platelet contained exclusively lipovitellin and phosvitin (Wallace, 1963b; 1965), and made up 98% of the total yolk platelet protein in Rana pipiens (Wallace, 1963a), it appeared that the micropinocytosis of SLPP from the blood was sufficient to account for the formation of amphibian yolk platelets. The studies of Dumont and Wallace (1968) and of Wallace and Jared (1969) both showed that no oöcyte organelle system appeared to be involved in yolk platelet formation.

Although it now seems clear that amphibian yolk enters the oöcyte by

pinocytosis, it had been argued rather unconvincingly by earlier authors (Lanzavecchia, 1960; Ward, 1962; and Balinsky and Devis, 1963) that amphibian yolk was synthesized intramitochondrially, as a result of having found that in the mitochondria of young oöcytes there are hexagonal crystalline bodies having a crystalline structure resembling that of yolk platelets. There are several serious objections to this conclusion drawn from these observations. First, as Ward (1962) pointed out himself, it has proved impossible so far to follow a sequence of steps of yolk platelet formation beginning with a yolk crystal within the mitochondrion, and "until the steps are followed sequentially our synthesis must remain inferential and based on circumstantial evidence." Secondly, it is not clear that the oöcytes in which mitochomdria containing crystals were found were actually vitellogenic. Balinsky and Devis (1963) found what they believed were yolk platelet precursonrs in Xenopus oöcytes that were smaller than 580μ , and Ward (1962) found similar structures in the oöcytes of newly metamorphosed Rana pipiens. Thirdly, it has not been demonstrated that the crystalline morphological configuration observed inside mitochondria is chemically related to yolk rather than to pigment granules, cortical granules membrane material, or other cellular component. Fourthly, there is no reason to believe that just because yolk is associated with a particular cellular organelle (i.e. mitochondria) it is synthesized there, and not merely assembled there from proteins or aggregates of proteins made elsewhere. In fact, the yolk crystals found inside mitochondria could easily have arisen by the fusion of pinocytotic vesicles containing yolk with mitochondria. The first appearances of crystalline yolk in amphibian mitochondria occurs at the periphery of the oöcyte between tortuous convolutions of the cell membrane and where pinocytotic vesicles are being formed. It is also a property of pinocytotic vesicles to fuse with mitochondria as

well as with other pinocytotic vesicles. Brandt (1958) found that if amoebae were fed proteins that were labeled with fluorescein, the proteins were taken in by pinocytosis, and that fluorescent granules could then be detected in the mitochondria three days later. Pinocytotic vesicles could be seen to fuse with mitochondria in tissue-cultured cells as well. It is possible then that the peripherally located mitochondria serve as nuclei for the condensation of micropinocytotic vesicles into yolk platelets.

As in the frog, the yolk of the chicken egg originates in the maternal liver and passes into the vitellogenic oöcytes by micropinocytosis after being transported through the circulatory system. Knight and Schechtman (1954) determined serologically that heterologous proteins, e.g. rat serum, crystalline bovine albumin, bovine gamma-globulin, and lobster serum injected into laying hens, found their way into the saline-soluble fraction of the yolk of the eggs that these hens laid. A few years later, pinocytosis was discovered in developing avian eggs (Press, 1959), and this was postulated as a means of taking up proteins from the blood. The yolk protein of the chicken contains a phosphoprotein and a lipoprotein, the former present in the serum in a form heavily complexed with calcium (Schjeide and Urist, 1956; 1959). Inside the yolk granule, these two substances are complexed in the form of an insoluble precipitate (Schjeide and Urist, 1956), a condition which can be duplicated <u>in vitro</u> by the co-precipitation of the phosphoprotein and lipoprotein (Schjeide and Urist, 1959). Schjeide et al. (1963) found that the yolk proteins, after being synthesized in the liver under the influence of estrogen, are taken up from the blood by vitellogenic oöcytes by micropinocytosis. The first appearances of yolk platelet protein was in the mitochondria located around the periphery of the oöcyte, and some substance in the mitochondria could change the physical properties of the

yolk proteins, allowing them to co-precipitate. Mitochondrial synthesis of yolk in the chicken seems highly unlikely in view of the fact that the influx of yolk proteins from outside is so fast, and for other reasons discussed above in connection with frog vitellogenesis. The increase in mass from 100-200mg to 20g in a period of five to six days is very difficult to explain in terms of any kind of *in situ* synthesis (Schjeide et al., 1963) and no mitochondria or endoplasmic reticulum can be seen in large yolk masses. The presence of both white and yellow yolk spheres in a chicken egg do not reflect spheres of different protein composition, but merely the diurnal variation in carotenol (Schjeide et al., 1963). Using organ culture techniques in media containing radioactive labels, Heald and McLachlan (1965) demonstated the *in vitro* synthesis of phosvitin by slices of liver from laying hens and oestradiol-treated roosters; and found that egg protein was not synthesized by kidney, spleen, muscle, large intestine, small intestine, ovary, or oviduct from laying birds.

The incorporation of nutrients from the blood into developing oöcytes appears very common among vertebrates, and no vertebrate egg which clearly does not do this has ever been found so far. Droller and Roth (1966) found that in guppy fish oöcytes, there was micropinocytosis in which bristle-coated vesicles were formed from bristle-coated pits, which later coalesced into large granules, although before micropinocytosis began there was some activity of the Golgi and endoplasmic reticulum. Mancini et al. (1963) found that circulating labeled serum proteins were transferred to the follicle of the rat ovary (whole rat serum, albumin, globulin, and fibrinogen fractions were fluorescently or radioactively labeled and traced). There was a rapid decline of these fractions in the blood, and a simultaneous appearance extravascularly in the ovary, in the zona pellucida, and in the oöcytes of growing follicles.

CHAPTER II

INTRODUCTION - Crustacean Vitellogenesis

Since many kinds of eggs develop in environments that lack sufficient nutrients to sustain embryonic development, food reserves must be accumulated in the oöcyte protoplasm. In early usage, these food reserves were collectively called "yolk", a morphological term used to describe such food storage substance as proteins, lipids, and phospholipids. In more modern usage, yolk is often taken to mean lipovitellin, a high-density lipoprotein that is unique to the eggs of all animals in which oögenesis has been intensively studied. A large amount of cytoplasm and certain cytoplasmic organelles to be partitioned among the very rapidly dividing cells of a cleaving embryo must also be acquired by the developint oöcyte. Although most of the material inside a mature egg is often yolk rather than cytoplasm, the volume of cytoplasm is still enormously greater than in somatic cells.

Developing eggs are encapsuled within a covering of small follicle cells. In panoistic oögenesis, these follicle cells are the only accessory cells, but in meroistic oögenesis, nurse cells with direct cytoplasmic connections to the oöcyte are included within the vollicle cell layer. All eggs are covered with a cell membrane (plasmalemma or oölemma), and in addition, all eggs except those of sponges and some coelenterates have additional "egg membranes". Primary egg membranes (vitelline membranes) are formed in the ovary by the egg cell by a hardening of the outer layer of the oöcyte cytoplasm (Raven, 1961). Secondary membranes (chorions) are formed in the ovary by the follicle epithel-

ium as a cuticular secretion, which in insects, resembles keratin more closely than chitin (Raven, 1961). Tertiary egg membranes are formed after ovulation in the genital ducts.

The conclusions which previous researchers have reached on the mechanism of crustacean vitellogenesis are as multifarious and conflicting as those to be found in any of the literature on yolk formation. Various authors have ascribed the function of yolk synthesis to 1) the mitochondria (King, 1926; Bhatia and Nath, 1931), 2) the Golgi bodies (King, 1926; Harvy, 1929), 3) the nucleolus (Harvy, 1929; Bhatia and Nath, 1931), 4) to the endoplasmic reticulum (Beams and Kessel, 1962, 1963; Kessel and Beams, 1963b; Kessel, 1968a, 1968b; and Hinsch and Cone, 1969), and 5) to sources outside the oöcyte (Heim, 1892; Smith, 1910, 1911; Robson, 1911; Abeloos and Fischer, 1926; Lwoff, 1927; Green, 1957, 1966; Frentz, 1958; Hinsch and Cone, 1969; Horn and Kerr, 1969; Kerr 1969).

Light microscopy was the primary tool of the earliest investigators, who attempted to discover the origin of crustacean yolk by looking for an association between visible yolk deposits and subcellular structures. King (1926) found that there were two kinds of "yolk" bodies present in the egg of Oniscus asellus, one comprised of fat droplets, and the other histochemically defined as albuminous. Because of the proximity of these bodies to particular cytoplasmic structures, King concluded that the "fatty yolk" developed from the Golgi apparatus, and the albuminous yolk represented swollen mitochondria. A few years later, Harvy (1929) made similar observations on the developing oöcytes of Carcinas moenas but came to quite different conclusions, namely, that the albuminous yolk developed from the Golgi bodies rather than from the mitochondria and that nucleolar extrusions were added to the yolk droplets, a situa-

tion unlike that found in Oniscus (King, 1926) which had no nucleolar activity. Harvy (1929) subsequently reported that in Carcinus, the "fatty yolk" arose de novo in the cytoplasm. Bhatia and Nath (1931) implicated the mitochondria in albuminous yolk synthesis of the prawn Palaemon lamerrei, but their study of the crab Paratalphusa spinigera suggested that the origin of yolk protein came about by the direct transformation of nucleolar extrusions into yolk bodies.

Another mechanism proposed for the origin of crustacean proteinaceous yolk, and the one which has proved to have the most merit, arose out of the observation of many researchers (Heim, 1892; Smith, 1910, 1911; Robson, 1911; Abeloos and Fischer, 1926) that in many Crustacea, carotenoid-containing pigments of the same color as yolk could be found in the blood of females of many species. During vitellogenesis there was such a great concentration of these pigments that it seemed possible that the blood was transporting some substances made elsewhere in the animal to the egg. Later it was found that these pigments were actually carotenoid-protein complexes or lipoproteins (Lwoff, 1927; Green, 1957, 1966; Frentz, 1954, 1958).

Some recent studies on crustacean vitellogenesis involved the use of electron microscopy. Beams and Kessel (1962, 1963) noticed that in some places within the crayfish oöcyte the endoplasmic reticulum was composed of stacks of parallel cisternae covered by large numbers of ribosomes which connected to others having a more random orientation and few ribosomes. Within the stacked cisternae, small granules of 40-60μ could be found, and these were considered by the authors to be precursors of yolk granules, which moved along the unoriented cisternae and were then transformed into "small, granular, proteinaceous yolk granules". Although pinocytotic vesicles were seen, and it was noted that all growing yolk bodies were first observed in the peripheral region of the cytoplasm,

it was concluded that pinocytosis occurred at such a slow rate as to be negligible in yolk formation. Yolk granules were also found on occasion inside of mitochondria, but their importance was minimized. Similar studies were made on the developing oöcytes of the lobster, Homarus by Kessel and Beams (1963b) and by Kessel (1968b). Their analysis of the origin of yolk in the lobster led to the same conclusion as in the crayfish, differing only in small particulars. Again, intracisternal granules were found, and these were believed to fuse in the peripheral region of the cytoplasm to form the small yolk granules seen there. The lobster intracisternal granules did not appear to be conducted along the rough-surfaced endoplasmic reticulum, however, and the form and organization of the endoplasmic reticulum appeared to be less complex that that seen in the crayfish. It was also noted (Kessel and Beams, 1963b) that in the lobster, there seemed to be a greater number of intracisternal granules present after oögenesis began than before.

In an attempt to test the conclusion (Beams and Kessel, 1962, 1963) that the effects of pinocytosis on yolk synthesis were negligible, Kessel (1968a) injected horseradish peroxidase dissolved in crayfish Ringer's solution into the hemocoels of immature female crayfish. After intervals of 15, 30, 45, and 60 minutes, portions of the ovary were fixed and prepared for electron microscopy. Peroxidase is an excellent electron microscopic marker for the occurrence of pinocytosis since the reaction product formed with 3,3'-diaminobenzidine is non-crystalline, insoluble, extremely electron-opaque after fixation with osmium tetroxide, and discrete enough to provide sharp localization (Karnovsky, 1967; Graham and Karnovsky, 1966). The peroxidase reaction product was first localized within the basement lamina, in the invaginated part of the plasma membrane, and in the intercellular spaces between the follicle cells (Kessel, 1968a). There

was also a substantial amount of reaction product present in the perivitelline space. Peroxidase-containing vesicles could also be seen within the follicle cells, which appeared to have arisen from the invagination of pinocytotic vesicles from the plasma membrane, and their subsequent fusion. In addition, the peroxidase reaction product was present in small bristle-coated pits in the oöcyte membrane, which were found to detach to produce peroxidase-containing micropinocytotic vesicles in the cortical oöplasm. The aggregation of granules, considered to be yolk, arising in the endoplasmic reticulum, were free of the reaction product. In spite of the fact that hemocoel proteins appeared capable of entering the oöcyte by micropinocytosis after passing through the intercellular spaces between the follicle cells, Kessel (1968a) still believed that the yolk protein was synthesized endogenously within the endoplasmic reticulum.

A third crustacean ovary examined by electron microscopy was that of the spider crab Libinia emarginata (Hinsch and Cone, 1969). In this organism, previtellogenic oöcytes had large germinal vesicles, and cytoplasm containing ribosomes, Golgi elements, and rough endoplasmic reticulum. The oöcyte membrane (oölemma) was smooth and unspecialized, but there were numerous pores in the nuclear envelope through which nucleolar material was presumed to pass into the perinuclear oöplasm. In vitellogenic oöcytes, there was a striking abundance of endoplasmic reticulum, which, as in the crayfish (Beams and Kessel, 1962, 1963), contained intracisternal granules plus a flocculent material. The granules and flocculent material appeared to aggregate, and were thought to form yolk bodies (Hinsch and Cone, 1969). In more mature vitellogenic oöcytes of the spider crab, the oölemma became irregular with the formation of microvilli and micropinocytotic vesicles. A dense granular material was present between the follicle cells and the oöcyte which collected in the forming micropinocytotic vesicles, and could

also be seen in detached vesicles in the cortical oöplasm. The forming and detached vesicles had "fuzzy coats" on their cytoplasmic surfaces that appeared to correspond to the "bristle coats" that Kessel (1968a) noticed in crayfish oöcytes. Shortly after being internalized in the cortical oöplasm, the micropinocytotic vesicles lost their "fuzzy coats", and appeared to fuse to produce smooth membrane-bounded yolk spheres which appeared different from the "yolk" formed within the endoplasmic reticulum.

Thus, in all crustacean oöcytes examined to date by electron microscopy, there appeared to be synthesis of granules within the endoplasmic reticulum which might be capable of aggregating to form yolk granules; there also appeared to be micropinocytosis occurring in the developing oöcyte followed by a fusion of micropinocytotic vesicles into bodies which have been interpreted by some authors (Hinsch and Cone, 1969) to be a different kind of yolk granule, and by other authors (Beams and Kessel, 1962, 1963; Kessel, 1968a, 1968b; Kessel and Beams, 1963B) as being unrelated to vitellogenesis.

Another approach to the study of Crustacean vitellogenesis has involved the use of biochemical and immunological techniques. Horn and Kerr (1963) measured the hemolymph protein and copper concentrations in 333 adult blue crabs (Callinectes sapidus Rathbun). Within each of the groups of animals tested (female adult, female in sponge, male adult) a wide variation (a 10-fold difference) in the concentrations of serum proteins and copper were found, but there was no correlation between the specimen size and the mean serum protein or copper concentrations. Males showed a distinctly lower mean protein concentration in the serum than the females. In a further study, Horn and Kerr (1969) found through the use of starch gel electrophoresis that two proteins were present in the hemolymph of female Callinectes that could not be detected in male crabs. Of

these two proteins, one was yellow and stained positively for protein, lipid, and oxidase activity. This yellow protein was also present in the hemolymph primarily when maturing oöcytes were in the ovary, suggesting to the authors that the hemolymph might be transporting yolk protein or its precursors synthesized elsewhere to the ovary. Kerr (1969), in a statistical analysis of mean protein and copper concentrations in hemolymph samples from 435 female Callinectes that were grouped according to an arbitrary classification of ovary size, showed that there was a significant increase in protein and copper concentrations during oöcyte maturation. Lipovitellin, the major lipoprotein in mature oöcytes, and the yellow female-specific lipoprotein found in hemolymph were isolated and their physical properties examined (Kerr, 1969). They were found to have identical mobilities and staining reactions when subjected to vertical starch gel electrophoresis, and to be serologically identical. Complete cross-reaction in Ouchterlony two-dimensional immunodiffusion in agar was found. On the basis of these findings, Kerr (1969) suggested that the lipovitellin was made outside the ovary and then transferred to the ovary during oögenesis by being transported through the hemolymph.

In an attempt to determine where in the animal the synthesis of the female-specific lipoprotein was taking place in Callinectes sapidus, Kerr (1968) studied the incorporation of ^{14}C-leucine in short term organ cultures of muscle, heart, hepatopancreas, whole hemolymph, and hemolymph serum. When samples were taken at intervals during incubation at room temperature, the most striking uptake of label ocurred in cultures of whole hemolymph. The incorporation of ^{14}C-leucine into total protein during incubation of a sample of hemolymph from a female with maturing oöcytes was four times greater that that of the hepatopancreas of the same animal. An analysis by paper curtain electrophoresis of the incuba-

tion media showed that 40% of the radioactive leucine incorporated into total hemolymph proteins was present in the serum lipoprotein fraction.

The physical properties of crustacean lipovitellin have been determined by Wallace, Walker, and Hauschka (1967). The single principal protein component of the mature ovaries of six decapod crustaceans Homarus, Pagurus, Uca, Sesarma, Cancer, and Libinia were isolated in pure form and in large amounts, and found in every case to be a lipoprotein (30% lipid), which lacked protein-bound phosphorus, and which had an average molecular weight of 3.5×10^5. The intense chromatic properties of the crustacean lipoproteins were found to be due to a carotenoid non-covalently bound to the lipid and/or protein.

The experiments included in this report were designed to determine how crustacean yolk originates and how it is accumulated within the crustacean egg. Yolk protein in vertebrates appears to be synthesized in the liver and then transported to the ovary where it is pinocytosed by developing oöcytes, but the situation is by no means so clear in the invertebrates, with the notable exception of the insects where yolk protein is also taken up by micropinocytosis. Our knowledge of the origin of crustacean yolk is at present in an especially confused state.

CHAPTER III

MATERIALS AND METHODS

The calico-backed fiddler crabs (Uca pugilator) and spider crabs Libinia emarginata used in this study were obtained from Woods Hole, Massachusetts, or from Panacea, Florida. These crabs were kept in running sea water, or in synthetic sea salts (Aquarium Systems, Inc., Wickliffe, Ohio) which were changed every other day. The animals were fed fresh fish, shell fish or rat food twice a week. Cambarus clarkii, the large Southern crayfish, was obtained from College Biological, Escondido, California, and kept in half an inch or less of running tap water.

Fluorescein isothiocyanate-conjugated sheep anti-rabbit gamma-globulin was obtained from Progressive Laboratories, In., Baltimore, Maryland. Fluorescein isothiocyanate on celite particles was purchased from Calbiochem; a sample was also provided by Wesley Bullock of the University of Oregon Medical School. Trypan blue (diamine blue 38) from the National Aniline Division, Allied Chemical Corp., New York, New York.

When the ovaries of a large number of fiddler crabs were examined, it became apparent that the development of oöcytes in any given animal is quite synchronous and that the ovaries can be placed into one of five arbitrary stages of oögenesis, which were determined on the basis of oöcyte size and pigmentation. The ovaries of Uca were classified into five morphological stages of development as Hard (1942) did for Callinectes:

Stage 1 - The ovaries are thin whitish strands. This stage is seen in small crabs which have just completed

metamorphosis into the adult stage. (.012 mm diametric)

Stage 2 - The oöcytes have now turned light yellow from the beginning of yolk deposition.

Stage 3 - The oöcytes are a yellow-pink to light red color and are larger than those in Stage 2.

Stage 4 - The oöcytes are now enlarged to their maximum size and are a deep red dish purple color. (.34 mm diametric)

Stage 5 - The ovaries are stretched and empty now that the oöcytes have passed to the pleopods.

In Cambarus clarkii, oöcytes in several stages of development are present in the ovaries. The oöcytes in this species were classified in a similar fashion to those of Uca:

Stage 1 - Oöcytes are small, white and previtellogenic, measuring 0.25 mm ± 0.05 mm.

Stage 2 - The oöcytes are bright yellow and have a diameter of 0.36 ± 0.05 mm. These are the smallest vitellogenic oöcytes.

Stage 3 - The oöcytes are deep yellow in color and have a diameter of 0.59 ± 0.07 mm. These are midway in the process of vitellogenesis.

Stage 4 - These large brown oöcytes have a diameter of 0.75 mm ± 0.07 mm and are now fully mature.

As in Cambarus, Libinia has oöcytes in all stages of vitellogenesis simultaneously.

Immunological procedures: Antiserum to female Uca serum was prepared in Freund's adjuvant by four injections two weeks apart each containing 0.25 ml of centrifuged serum. The antiserum prepared against the hemolymph was obtained by bleeding Uca females collected in the spring. An antiserum to uniquely female Uca proteins was prepared by adsorbing exhaustively the antiserum to female Uca blood with male Uca serum. Several immunological techniques were used to investigate the possibility of an extra-ovarian source of lipovitellin. Hemolymph to be used as an antigen in Ouchterlony agar diffusion tests was withdrawn from crabs in Stages 2, 3, 4, and 5 of oögenesis by inserting a sterile syringe into be base of the most posterior walking legs after the area was first sterilized with 70% alcohol. The hemolymph was then transferred to a small test tube containing a crystal of phenylthiourea (PTU) to inhibit tyrosinase (which would otherwise cause the hemolymph to turn black on contact with air because of melanin formation) and a few crystals of streptomycin sulfate. Each tube was centrifuged at 21,000 x g for thirty minutes to remove cells and debris. Extracts of the following organs from crabs in oögenesis stages 2, 3, 4, and 5 were prepared: ovary, hepatopancreas, gills, gut, and finally of all other parts of the animal together. Each of these was washed three times with Homarus Ringer's[1] or Pantin Saline[2], ground with a tissue homogenizer, and centrifuged at 12,000 x g at 4°C for half an hour to remove debris. The supernatant fluids of these extracts were also used as antigens.

Ouchterlony plates were set up with peripheral walls containing male Uca serum, and each of the female Uca antigens. Similar plates were set up utilizing Libinia antigens. Adsorbed antiserum and non-adsorbed antiserum were tested in the central well. The specificity of the adsorbed antibody for Uca yolk spheres was also determined by immunofluorescence, using sections of eggs

cut with a cryostat, and also using whole eggs which had been made permeable to antibodies by glycerination. For glycerination, fiddler crab ovaries of stages 2, 3, 4, and 5 were placed in 50% glycerol in 0.1M saline, and buffered to pH 7.5 with 0.05M tris for 2 days at 0°C to increase cell membrane permeability and allow all soluble molecules to diffuse out of the cell, leaving cell organelles and structural proteins inside. This is a modification of the procedures used by Holtzer, et al. (1957). The glycerination of Uca eggs causes a change in the reddish purple pigment in the cell, the yolk spheres becoming bright yellow. After glycerination, the eggs were placed in depression slides containing a 1:8 dilution of the adsorbed antibody made up in 0.1M saline buffered to pH 7.5 with 0.05M tris and containing 25% glycerol. The depression slides were sealed with coverslips ringed with petroleum jelly and incubated at 0°C for one day. The eggs were then rinsed for two days in 8 changes of buffered saline - 25% glycerol at 0°C and were then placed in 1:2 and 1:32 dilutions of fluorescein isothiocynate-conjugated sheep anti-rabbit gamma-globulin in buffered saline - 25% glycerol and incubated for 24 hours at 0°C. This was followed by 2 days of washing in the buffered saline - 25% glycerol to remove unreacted fluorescent label. The eggs were mounted on slides in buffered saline - 25% glycerol and covered with a coverslip ringed with fingernail polish to prevent evaporation. The following immunofluorescence controls were performed with every preparation: The entire procedure was carried out with control serum from the injected rabbit, and the entire procedure was carried out with adsorbed antiserum which had been further adsorbed with stage 4 ovary extract to remove the antibody to female protein. All preparations were viewed under a Zeiss fluorescence microscope with a BG 12 Zeiss excitation filter (max. transmission at 400 mu and a No. 53 Zeiss barrier filter. An HB 200 mercury arc

light was used for illumination.

In order to localize immunofluorescence on sections of ovary, Uca ovaries of stage 2, 3, 4, and 5 were quick-frozen and sectioned with a cryostat. These sections, approximately 8μ thick, were then incubated for 30 minutes in a 1:4 dilution of antiserum to female Uca blood that had been adsorbed with male blood. After washing for a total of 20 minutes in 4 changes of Pantin saline, the sections were flooded with a solution of 4 mg/ml fluoresein isothiocyanate-conjugated sheep anti-rabbit gamma-globulin in Pantin saline for 20 minutes. The sections were then washed 4 times in a period of 20 minutes in Pantin saline and examined after mounting in this saline containing 25% glycerol. The conditions for viewing and the control experiments used were the same as those described previously for the unsectioned glycerinated eggs.

Experiments with indicators of pinocytosis: In order to test for pinocytosis in the crustacean oöcyte, trypan blue was used as a marker (Telfer, 1965). After purification of the colloidal aggregates by dialysis for 24 hours against distilled water, particles remain which mimic the physical properties of proteins, and are too big to enter living cells except by pinocytosis. A 1% suspension of dialyzed trypan blue was made up in Pantin saline. Uca ovaries of stage 2, 3, 4, and 5 were dissected out under sterile conditions and were placed in 1% trypan blue in Pantin saline for one hour. These ovaries were then washed for one hour in four changes of Pantin saline. The ovaries were examined microscopically before sectioning, and were freeze-dried and sectioned for further examination. Whole crayfish ovary, containing oöcytes in all stages of vitellogenesis, and 4 mm slices of Libinia ovary were placed in a 1% solution of trypan blue made up in a saline dissecting medium[3] for 1 hour, and then washed 4 times over a period of one hour. The oöcytes were dissected

out of their follicles with watchmaker's forceps and classified as to stage of development and amount of trypan blue taken up. Libinia ovarian fragments were fixed in Bouin's fixative for one hour and processed for standard paraffin embedding. Eight micron sections were prepared and these were observed unstained, or were stained with Eosin only or with Hematoxylin or Eosin. Ovarian slices were apparently viable for the 60 minute duration of the experiment since the incubation in 1% trypan blue did not result in the intense generalized blue cytoplasmic staining which characterizes dead cells, but rather resulted in discrete granules of localized staining.

Uptake of labeled yolk protein by oöcytes: Lipovitellin from Uca pugilator was isolated and conjugated with fluorescein isothiocyanate so that its uptake into developing oöcytes could be visualized. To isolate and purify Uca lipovitellin, the procedure of Wallace, et al. (1967) was used. All extraction procedures were carried out at 4°C with 2 ml of Stage 4 Uca ovaries. The purified lipovitellin was prepared for conjugation with fluorescein isothiocyanate by dialyzing against Pantin saline buffered with $NaHCO_3$ to a pH of 8.5. Male Uca serum, female Uca serum, and purified lipovitellin were each conjugated with fluorescein isothiocyanate according to the procedure of Rinderknecht (1962). The labeled sera and lipovitellin were again dialyzed against Pantin saline to make the medium suitable for short term culture of Crustacean ovaries.

Pieces of fiddler crab ovaries of stage 2, 3, and 4 were incubated in 0.25 ml of fluorescein labeled male serum, female serum, and purified lipovitellin in depressions of a spotplate which were sealed with coverslips ringed with petroleum jelly and put on a rotary shaker for 3 hours. A piece of crayfish ovary was incubated in labeled Uca male serum, female serum, or lipovitellin in the same way. At the end of the incubation, the ovaries were washed

in Pantin saline and examined under a fluorescence microscope, both before and after sectioning with a cryostat.

Saline solutions used:

HOMARUS RINGER'S SOLUTION (after Cavanaugh, 1956): NaCl 26.4 g.L; KCl 15.0 mm/L; $CaCl_2 . 2H_2O$ 25.0 mm/L; $MgCl_2 . 6H_2O$ 4.0 mm/L; $MgSO_4 . 7H_2O$ 4.0 mm/L; NaOH (.5M) 0.96 ml/L; H_3BO_4 (.5M) 17.6 ml/L. Buffer with NaH_2PO_4 to pH = 6.5.

PANTIN SALINE (after Kleinholz, 1969): 0.6M NaCl 100 parts; 0.6M KCl 2.5 parts; 0.4M $CaCl_2$ 3.5 parts; 0.4M $MgCl_2$ 3.5 parts. Bring to pH 7.0 with solid $NaHCO_3$.

VAN HARREVELD DISSECTING MEDIUM FOR CAMBARUS AND ASTACUS (after Walsh et al., (1968): 0.5M NaCl 380 ml; 0.54M KCl 10 ml; 0.36M $CaCl_2$ (add last) 39 ml; 0.36M $MgCl_2$ 7.2 ml; 0.54M $NaHCO_3$ 4.4 ml. Bring to 1 liter with distilled water. pH - 7.5.

CHAPTER IV

RESULTS

Results of Immunological procedures: Serum from female Uca of stages 2, 3, 4, and 5 and ovaries of stages 2, 3, and 4 form four preciptin bands to unadsorbed antibody in Ouchterlony double diffusion plates. Complete cross reaction is exhibited by all four bands. In the former, only three bands reveal complete confluence with the same band in adjacent reactions and are found when male serum, female serum from a stage 1 animal, and an extract of Stage 5 ovaries are used as antigens. All bands formed to serum are completely confluent with adjacent reactions and appear in the position of the female-specific protein when unadsorbed antibody is used. No bands are found when the other organ extracts (hepatopancreas, gills, gut, and residual organs) are used as antigens from nonvitellogenic specimens to either absorbed or non-absorbed serum. However, when the animals are vitellogenic, the hepatopancreas gives a positive reaction with absorbed antiserum.

Experiments with Libinia give the smae results, showing a female-specific protein in vitellogenic animals. In addition, antigenic cross-reaction is seen between hepatopancreas and female protein (Figure 1).

When intact glycerinated eggs are exposed to female specific antibody and fluorescein conjugated anti-gamma globulin, the yolk spheres remain intact and fluoresce brightly against a background of almost non-fluorescing cytoplasm. In slides made with control serum from the rabbit that produced the antiserum, or with antiserum adsorbed further with ovary extract, there is only a barely visable background fluorescence. Sections of ovary cut with a cryostat and

reacted with indirect immunofluorescent labeling procedure also show intensely fluorescent yolk granules and a faintly fluorescent cytoplasm in the background. The controls for this experiment were negative.

Results with trypan blue as an indicator of pinocytosis: Trypan blue was (pinocytotically) incorporated into the developing oöcytes of the fiddler crab, the crayfish, and the spider crab during the period of yolk accumulation (Figs. 2, 3, 4). In Uca, Stage 2 oöcytes stain most intensely with trypan blue, Stage 3 oöcytes stain less intensely, and Stage 5 oöcytes take up no detectable trypan blue. In order to localize the granules of trypan blue within the oöcytes of Uca, sections of freeze-dried ovaries which had been incubated in a medium containing trypan blue were examined. All trypan blue granules which had been taken up were found in clumps in the cortical oöplasm; Stage 2 oöcytes stain darkest blue because they have taken up the largest number of granules of trypan blue.

In the crayfish ovary, no trypan blue is taken up by Stage 1 oöcytes, which have not yet commenced vitellogenesis (hence, their white color), and no trypan blue is taken up by Stage 4 oöcytes, which are fully mature. Stage 2 oöcytes in the crayfish stain most intensely with trypan blue, and Stage 3 oöcytes stain to a lesser degree.

In crayfish, fiddler and spider crab, there is some accumulation of trypan blue by both the follicle cells and the cells of the stroma of the ovary.

Uptake of fluorescein-labeled proteins by oöcytes: In Uca, Stage 2 oöcytes incubated in fluorescein-conjugated lipovitellin are fluorescent, and in Stage 3 oöcytes, the fluorescence is less intense. Almost no fluorescence is observed in Stage 4 oöcytes. The same pattern of uptake results when the ovaries are incubated in fluorescein-conjugated female Uca serum, although the

fluorescence is slightly weaker in all cases than when the only labeled protein is yolk. Fluorescein-conjugated male serum is not taken up to a detectable degree by the oöcytes of any stage. To verify that the fluorescent material is actually inside oöcytes, they were punctured with a glass needle under 35x magnification, and fluorescent protoplasm flowed out. Also, ovaries which had been in the fluorescein-conjugated proteins were sectioned with a cryostat and examined. In every case, fluorescence could be seen just inside the oölemma.

When crayfish ovaries are incubated in fluorescein-conjugated lipovitellin, oöcytes of Stage 2 and 3 fluoresce whereas oöcytes of Stage 1 and 4 do not (Figure 5). Incubation in fluorescein-conjugated female crayfish serum results in the same pattern of fluorescence as in Uca, while conjugated male serum does not.

CHAPTER V

DISCUSSION

The results obtained with the Ouchterlony two-dimensional precipitin reaction indicate that a serologically unique substance is present in the serum and ovaries of vitellogenic crabs. This substance is not detected in the serum of male crabs or in female crabs which are not forming eggs. The finding that ovary and female blood share a common antigen strongly suggests that both substances contain an identical protein (Ouchterlony, 1968). Also, the absence of this substance in male serum suggests that it has a function unique to female animals. A similar female-specific protein was found in Callinectes by Kerr (1969) and by Horn and Kerr (1969).

The experiments using indirect immunofluorescence and labeling of antigens demonstrate this female-specific protein is localized exclusively in the yolk spheres. Both in whole oöcytes and in their frozen sections, the only areas that bind fluorescein-conjugated antibody to the female-specific protein are the yolk spheres of oöcytes.

The existence of a female-specific protein found only in the yolk granules and in the blood raises the question as to where it arises. Either the lipovitellin leaves the oöcyte to enter the blood, or it is accumulated in the egg from the blood. The latter would allow crab eggs to increase in volume to about 20,000 fold in a period of a few weeks, for the radius of an oöcyte increases from 0.006mm to 0.17mm according to measurements in Uca. This study supports the latter alternative since the oöcyte takes up lipovitellin.

Since pinocytosis is the usual mechanism by which a cell can take up

macromolecules, experiments were performed to see whether this process is significant for yolk accumulation by oöcytes. It was found by Telfer (1965) that trypan blue enters insect oöcytes which are active in pinocytosis. The present study shows that this substance is taken up by Crustacean oöcytes and is specifically localized in small granules immediately inside the oölemma, rather than randomly distributed throughout the cell, as would result if they could diffuse through the membrane. All Oöcytes were alive during the course of the experiment, as evidenced by an absence of the immediate and rapid staining by trypan blue, characteristic of dead cells. It is concluded that the uptake of trypan blue occurs by pinocytisis in the same stages of oögenesis in both the crab and the crayfish; there is no pinocytotic activity in eggs which have not started accumulating lipovitellin and in eggs which have finished oögenesis. Rapid pinocytosis occurs in Stage 2 and at a diminished rate in Stage 3 of both organisms. Thus the rate of pinocytosis decreases as the oöcytes mature.

To test whether the oöcyte takes up the protein lipovitellin from the blood, eggs were incubated in male blood, female blood, and lipovitellin, all of which were fluorescein-conjugated. In both the crab and the crayfish, those stages undergoing pinocytosis, (2 and 3), were the only ones accumulating labeled lipovitellin or components from female blood. Other evidence relating this uptake to pinocytosis is 1) the appearance of all fluorescence at the cell periphery and just inside the oölemma (the fluorescent material flowed out when the cell was punctured); 2) the lack of information concerning an alternative way for the cell to take up protein (lipovitellin) with a molecular weight reported by Wallace et al, (1967) to be 3.4×10^5 for Uca; 3) the existence of a tightly bound coating of fluorescent protein on the external surface of the oölemma. The fact that labeled female serum, found earlier in this study by sero-

logical methods to contain a protein which has the same antigenic sites as lipovitellin, and lipovitellin itself is taken up and incorporated into newly forming yolk spheres *in vitro* indicates that this may happen as a part of normal oögenesis. Since male blood, serologically identical to female blood except for the female specific protein, is not taken up in detectable amounts, it suggests that the primary material taken up by the developing oöcyte is lipovitellin

It is concluded from the present experiments that, as in vertebrates and in insects, the lipovitellin in Crustacean eggs has its origin outside the oöcyte and ends up inside the oöcyte as a result of micropinocytotic activity. Although this conclusion appears to conflict with that of the many authors (Beams and Kessel, 1962, 1963; Kessel and Beams, 1963b; Kessel, 1968a, 1968b; Hinsch and Cone, 1969), who contend that Crustacean yolk arises in the endoplasmic reticulum, it can also explain their results. In all of the studies, micropinocytotic pits and vesicles were found in the oöcytes, but it was decided by all but Hinsch and Cone (1969) that they had no importance in yolk accumulation. Furthermore, Kessel (1968a), using horseradish peroxidase as an indicator of pinocytosis in crayfish oöcytes for electron microscopy, found the protein in the perivitelline space, in small bristle-coated pits in the oöcyte membrane, and in micropinocytotic vesicles in the cortical oöplasm, but no peroxidase could be found in the aggregation of granules formed in the endoplasmic reticulum, he concluded that the pinocytosis which was occurring had nearly nothing to do with the formation of yolk granules. Both *in vivo* and *in vitro* experiments were carried out on the crayfish oöcyte of *Orconnectes immunis* by Ganion and Kessel (1972). Their biochemical and autoradiographic evidence seem to challenge our evidence that uptake of exogenous materials is important since they state "these experiments were designed to assess the possible contribution of

material blood proteins to yolk deposition and indicate that such contribution is minimal." But, since it was found in the present series of experiments that lipovitellin is present in the blood and is pinocytosed by developing oöcytes, the reverse must be true; yolk spheres are formed from pinocytosis of lipovitellin from the blood, and possibly by transfer of material from the endoplasmic reticulum. The chemical nature of the granules made in the endoplasmic reticulum has not been determined. The present study does not deal with the problem of transfer of material from the endoplasmic reticulum. There may be additional components in the yolk spheres derived from the endoplasmic reticulum.

Yolk sphere formation by fusion of pinocytotic vesicles containing lipovitellin would explain why there is a gradient of yolk granules of increasing size ranging from the smallest ones at the cell periphery to the largest ones in the interior.

Although the Crustacean egg appears to accumulate lipovitellin by micropinocytosis of this substance from the blood, the origin of material is still not known. Hints of its origin so far is the finding by Kerr (1968) that hemolymph cells and hepatopancreas both secrete protein in short term organ culture and our finding of antigenic material in the hepatopancreas from vitellogenic females. More rigorous experiments remain to be done, such as those of Pan, Bell, and Telfer (1969) on another vitellogenic system.

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Figure 1: Ouchterlony agar diffusion plate showing reaction between antiserum to female *Uca* blood absorbed with male blood, and reacted with several antigenic materials.

Starting at the top, proceeding in clockwise direction the antigens were:

1. *Libinia* ovary
2. *Uca* ovary
3. Female *Libinia* hepatopancreas (from vitellogenic females)
4. Female *Libinia* blood (from vitellogenic females)
5. Female *Uca* hepatopancreas (from *non*-vitellogenic females)
6. Female *Uca* blood (pooled from several individuals including vitellogenic one)

The production of a single band of precipitate by the antiserum with the *Libinia* and *Uca* ovaries is clearly visible; the line of partial identity indicates antigenic similarity between the *Uca* antigen (the homologous reaction) and *Libinia* (the heterologous reaction). The ovarian antigens are similar to antigens present in the blood. This is revealed by the line of partial identity between female *Uca* blood (reservoir 6) and *Libinia* ovary (reservoir 1).

The reactions at reservoirs 3 and 5 are of particular interest. The one at 3 shows that a hepatopancreas antigen shares the same antigenic reactivity with *Uca* and *Libinia* blood proteins and that a possible source of egg proteins is the hepatopancreas facilitated by transport

through the circulatory system. The reaction at reservoir 5 is negative and suggests that ovarian proteins may bot be produced continuously by the hepatopancreas but only at particular stages of ovarian maturation.

PLATE 2 - Uptake of Trypan Blue
and Blood Proteins by the Oöcyte

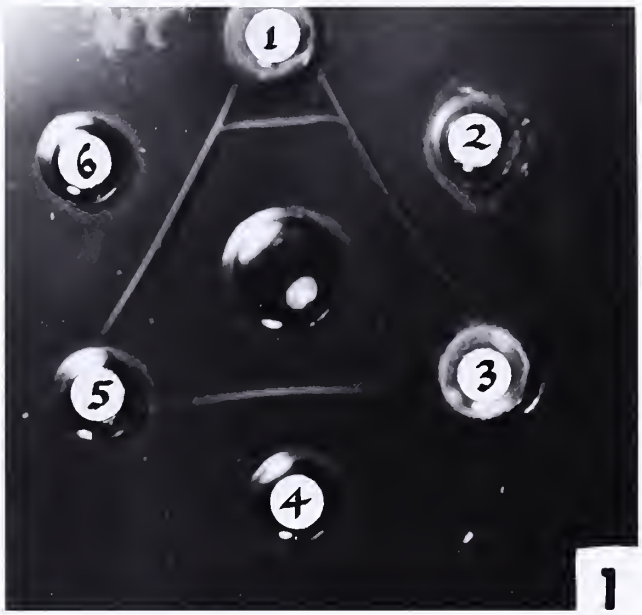
Figure 2: Paraffin section of Libinia ovary one hour after incubation in trypan blue. At the top of this figure is ovarian epithelium showing dye deposits. Just below is the follicular epithelium. Three vitellogenic eggs are seen at the ovarian surface. The dye is accumulated well within their cytoplasm.
See Figure 2 for enlargement (160X, 8 μ thick).

Figure 3: Higher magnification of Libinia ovary after trypan blue incubation. Dye is deposited in small cytoplasmic granules subjacent to the oölemma.

(400X, 8 μ thick, Nomarski optics.)

Figure 4: High magnification of Libinia ovary after a one-hour incubation in trypan blue. The dye is concentrated in the cortical oöplasm of this vitellogenic oöcyte. Accumulation of dye within vesicles is clearly visible.
(400X, 8μ thick)

Figure 5: Cambarus ovary exposed to fluorescein-conjugated yolk protein for 3 hours. The intact ovary is flattened between a slide and coverslip and examined under a fluorescence microscope with dark-field illumination. The previtellogenic oöcytes (stage 1) do not fluoresce. Stages 2 and 3 fluoresced brightly, the intensity of fluorescence decreasing as the oöcytes get larger (stage 4). 1, 2, 3, and 4, represent stages of oögenesis; F = follicle cell layer. (X35. Originally photographed by Polaroid and retouched to remove scratches.)



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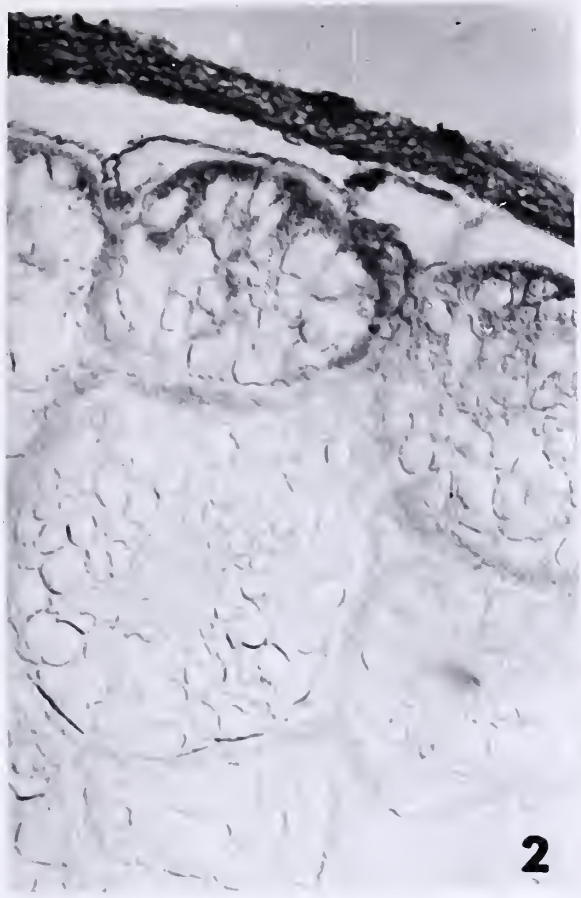
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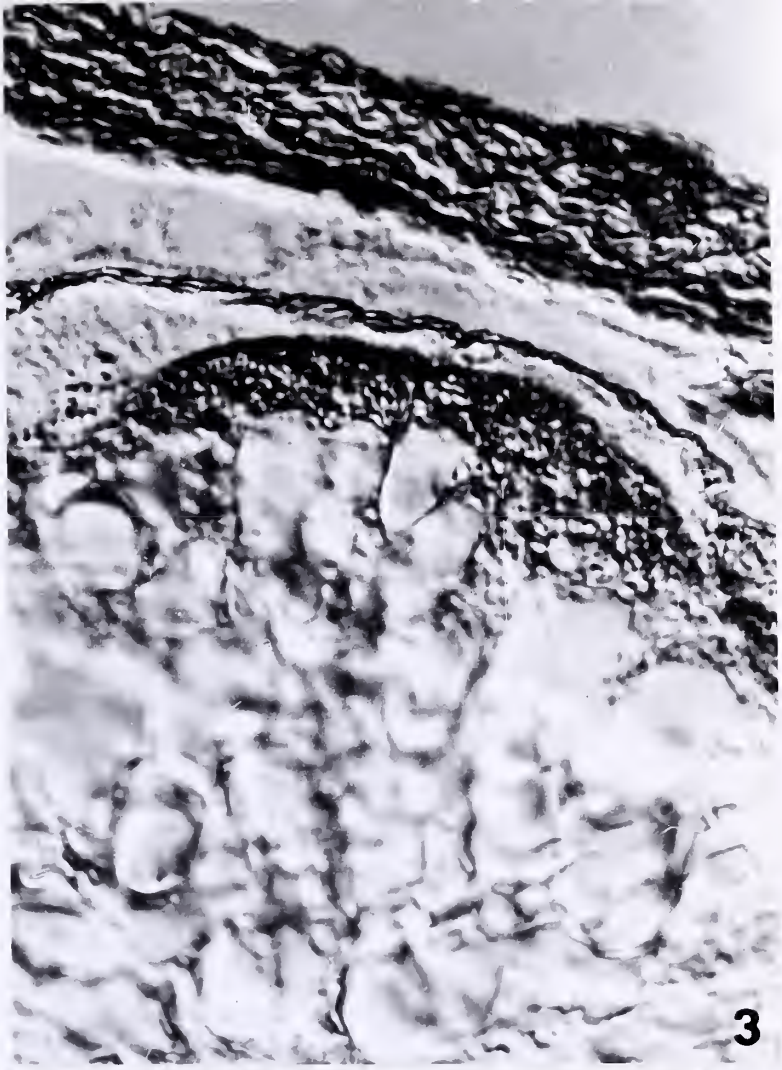
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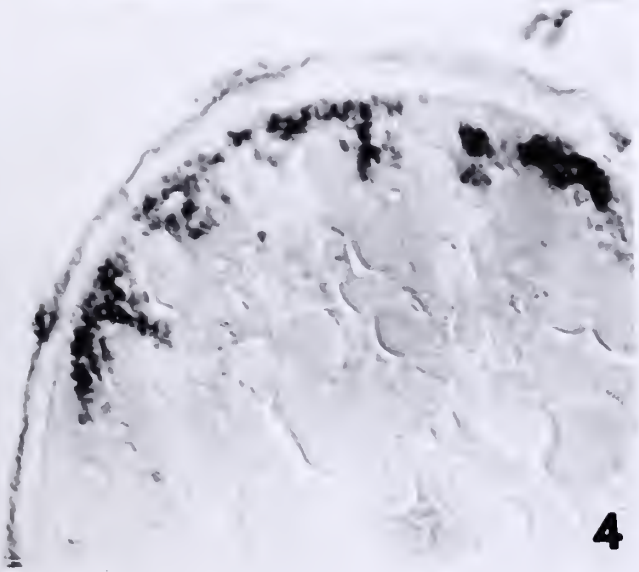
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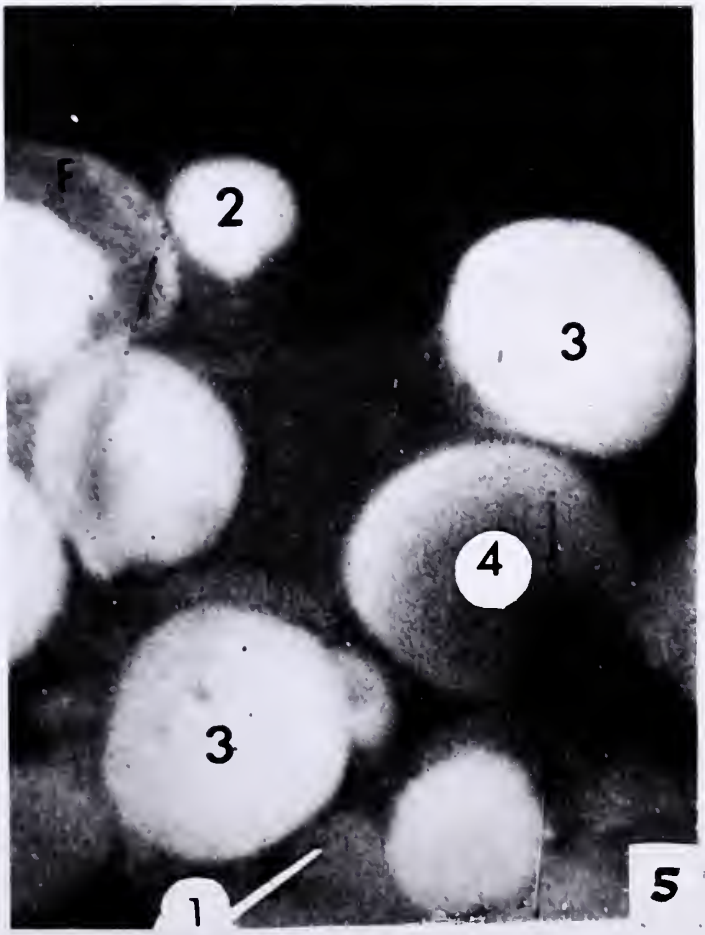
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