

INTRACELLULAR SYNTHESIS, TRANSPORT, AND PACKAGING OF PROTEINACEOUS YOLK IN OOCYTES OF *ORCONECTES IMMUNIS*

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ABSTRACT

The incorporation of leucine-³H into either ovarian or oocyte proteins occurs throughout vitellogenesis, but is at a maximum during early phases of this process. The labeling of ovarian and oocyte proteins is inhibited with cycloheximide. Oocytes are permeable to actinomycin D, and this drug does not affect the incorporation of amino acids into oocyte proteins but does block oocyte RNA synthesis. By means of both light microscope and high resolution radioautography, it has been demonstrated that the initial incorporation of leucine-³H under both in vitro and in vivo conditions occurs in elements of the rough-surfaced endoplasmic reticulum in the oocyte. Under pulse-chase conditions, the label subsequently becomes associated with intracisternal (precursor yolk) granules now aggregated within the cisternae of the connected smooth-surfaced endoplasmic reticulum. By 7 days, mature yolk globules are extensively labeled. The results of experiments designed to assess the possible contribution of maternal blood proteins to yolk deposition indicate that such a contribution is minimal. It is concluded that the crayfish oocyte is programmed for and capable of synthesizing the massive store of proteinaceous yolk present in the egg at the end of oogenesis.

INTRODUCTION

The process by which the female germ cell is prepared for fertilization involves a long and complex series of activities leading to a progressively larger and more specialized cell. In nearly all organisms, the cytoplasm of the developing oocyte becomes engorged to such an extent with stored macromolecules that these cells at the termination of oogenesis are among the largest known. Vitellogenesis is only one of several basic biological activities (e.g. ribosomal RNA synthesis) amplified during oogenesis. The type of food reserves (yolk) found in the ooplasm varies considerably among organisms. In general, yolk may include lipid, glycogen, as well as membrane-bounded inclusions

rich in protein or in protein complexed with either polysaccharide or lipid. All three classes of reserves are present in some organisms, but their relative proportion varies.

In a previous morphological study of the developing crayfish oocyte, Beams and Kessel (4, 5) described the formation of an extensive system of endoplasmic reticulum in the ooplasm before vitellogenesis. This organelle was observed as a massive network of interconnected smooth and ribosome-coated cisternae. Coincident with the formation of proteinaceous yolk, numerous granules appeared within the cisternae of the rough-surfaced endoplasmic reticulum. These intra-

cisternal granules subsequently appeared to be transported into connected elements of the agranular endoplasmic reticulum where they aggregated and transformed into definitive yolk globules. These morphological details were compatible with the view that in these oocytes an elaborate system of endoplasmic reticulum is differentiated for the purpose of (a) synthesis of yolk protein, (b) intracellular transport of the precursor yolk materials, and (c) packaging of the newly synthesized protein into large, membrane-bounded yolk globules. This ultrastructural study constitutes the best morphological example to suggest that an oocyte may possess the necessary biochemical machinery to synthesize the massive stores of proteinaceous yolk present at the end of oogenesis.

It should be emphasized, however, that no direct experimental evidence currently exists to support the sequence of activities as postulated by Beams and Kessel (4, 5) on the basis of their morphological observations. Due to the present lack of critical information regarding the ooplasmic synthesis of yolk protein and since the crayfish oocyte is unquestionably a very favorable cell with which to study this event, an experimental analysis of this system was undertaken.

MATERIALS AND METHODS

Animals

The crayfish used in this study, *Orconectes immunis*, were either obtained from a commercial source, Fin and Feather, (Iowa City, Iowa), or collected in nature at the Iowa Lakeside Laboratory during the months of June, July, August, and September. Animals were fed beef liver biweekly and maintained at 21°C in aerated tubs exposed to natural light.

Staging of Oocytes

Deposition of proteinaceous yolk begins when oocytes are approximately 260 μ in diameter. At the end of vitellogenesis, oocytes measure approximately 700 μ in diameter. Furthermore, the vitellogenic oocytes in a single ovary are fairly synchronous in their development. Therefore, in subsequent descriptions, it is convenient to classify the oocytes arbitrarily as in early (260–400 μ), mid (400–520 μ), and late (520–700 μ) stages of vitellogenesis.

Culture Methods

Several culture conditions were used to study the intrinsic synthesis of ovarian proteins. Portions of ovaries from either one or several females were

cultured at 21°C for 1–2 hr with Leucine-4,5-³H (SA 40 Ci/mole; Schwarz Bio Research Inc., Orangeburg, N. Y.) at a concentration of 25 μ Ci/ml in crayfish Ringer's and sampled at various times during the culture period. In other experiments, ovarian fragments were first preincubated for 10–40 min at room temperature in Ringer's containing either cycloheximide (Sigma Chemical Co., St. Louis, Mo.) or actinomycin D (Merck & Co., Inc., Rahway, N. J.) in concentrations of 1–100 μ g/ml before labeling with leucine-³H. Controls were preincubated in the absence of the antibiotics and subsequently incubated with labeled substrate. The samples were assayed by liquid scintillation counting and radioautography.

To investigate the origin and deposition of proteinaceous yolk, animals were injected with leucine-³H (8–15 μ Ci/g body weight). At different times (15 min–19 days) following the injection, the ovaries were removed and prepared for radioautography. In some cases, a second 50–150 μ l injection of 0.01 M l-leucine was administered 10–15 min after the initial injection of label. Small pieces of ovaries were also cultured in vitro with leucine-³H for intervals of 10 min and 2 hr. Pulse-labeling procedures were also used. To effect a longer chase period, portions of the ovary were pulse-labeled in vitro with leucine-³H for 6 min and then injected with a pasteur pipette into the hemocoel of adult females at the junction of the cephalothorax and abdomen. After 1, 3, and 7 day intervals, the transplanted oocytes were removed and prepared for radioautographic analysis.

In other experiments, ovarian fragments were cultured in crayfish blood labeled with leucine-³H. Maximum incorporation of leucine-¹⁴C into blood proteins occurs within 24–36 hr (16). Thus, oocyte clusters from a single female were inserted by a pasteur pipette into the hemocoel of female crayfish which had been injected 24 hr earlier with 50 μ Ci of leucine-³H. A second 500 μ l injection of 0.1 M leucine was administered to dilute the unincorporated label in the hemolymph. The transplanted oocytes were collected at 24 and 48 hr intervals and prepared for radioautography.

Assay Procedures

LIQUID SCINTILLATION COUNTING: After a brief rinse in 0.1 M l-leucine, ovarian samples labeled with leucine-³H were homogenized in 300 μ l of 0.1 M l-leucine with a Teflon homogenizer. Duplicate or single 100 μ l samples of the homogenate were applied to 2.5 cm discs of Whatman #2 filter paper and prepared for liquid scintillation counting according to the method of Mans and Novelli (31). The discs were treated with 5% trichloroacetic acid (TCA) at 90°C for 30 min, rinsed in fresh 5% TCA, and incubated at 37°C for 30 min in an absolute

ethanol/ether mixture (1:1, v/v). The dried filter-paper discs were placed in 10 ml of scintillation fluid and counted twice by a Nuclear of Chicago Unilux II liquid scintillation spectrometer (Nuclear-Chicago Corporation, Des Plaines, Ill.) with a counting efficiency of 40% for tritium. Background was determined on filter-paper discs carried through the washing process. After counting, the TCA-precipitable proteins absorbed to the discs were solubilized at 21°C in 0.8 ml of 1 N NaOH for 24 hr and measured by the Lowry method (29). Protein standards of bovine serum albumin (Sigma Chemical Co.) as well as ovarian samples were read on a Beckman DU spectrophotometer at 750 m μ (Beckman Instruments, Inc., Fullerton, Calif.) After protein elution, the discs were recounted. These data were used to calculate specific radioactivities of ovarian proteins and are expressed in counts per minute (cpm)/ μ g protein.

RADIOAUTOGRAPHY: The incorporation of tritium-labeled materials into ovarian tissues was demonstrated cytologically by light and electron microscope radioautography. For light microscope studies, the ovarian fragments were fixed in either Ammerman's fluid, Bouin's, 3% glutaraldehyde, or 1% OsO₄ for 2 hr and appropriately embedded in either paraffin or Epon 812. Oocytes cultured with actinomycin-D-³H were prepared according to the method of Anderson (1). These oocytes were fixed with 10% formalin in 0.05 M phosphate buffer (pH 7.3) for 30 min and then incubated at 37°C for 12 hr in 0.5% DNase (Worthington Biochemical Corp., Freehold, N. J.). The DNase was dissolved in a solution of 0.0025 M MgSO₄ and 0.1 M Tris-HCl buffer at pH 7.3. Some of the oocytes were cultured in the absence of DNase. After a 20 min rinse in 5% TCA at 4°C, the tissues were fixed in 1.5% glutaraldehyde, postfixed in 1% OsO₄, and embedded in Epon. Thick sections were cut and mounted on gelatin-coated slides (9). Deparaffinized and hydrated sections of oocytes were treated with 5% TCA at 90°C for 15 min or with 0.5% RNase (Worthington Biochemical Corp.) in 0.1 M Tris-HCl buffer of pH 7.3 for 6 hr at 37°C and processed together with control slides. The slides were stained with either Delafield's hematoxylin or eosin and coated with Kodak NTB-2 emulsion by the method of Kopriwa and Leblond (28). The slides were stored at 4°C in light-proof boxes for a period of 3–6 wk, developed in Dektol, and subsequently processed by the method of Kopriwa and Leblond (28). Thick Epon sections of oocytes were similarly processed and stained with basic fuchsin (18) after radioautography.

In addition, labeled oocytes were fixed for 1–2 hr at 4°C in either 1% phosphate-buffered OsO₄ or 3% glutaraldehyde (pH 7.3), embedded in Epon, and processed for high resolution radioautography. Silver-

gold sections were transferred by a wire loop to celloidin-coated slides (38). The sections were stained for 10 min with 2.5% aqueous uranyl acetate (47), for 1 min in lead citrate (37) and coated with an ultrathin layer of carbon (38). The sections were then coated with dilute Ilford L-4 emulsion by the method of Kopriwa (27), which insures a single layer of closely packed silver bromide grains. The slides were then stored at 4°C for durations of 1–8 months in light-proof boxes. After exposure, the sections were developed in Kodak D-170 for 2.5 min and subsequently processed by the method of Kopriwa (27). The areas of the celloidin film containing the sections were scored with a razor blade and floated onto distilled water for transfer to electron microscope grids. The dried radioautographs were viewed in a RCA EMU-3F electron microscope. In some cases, the radioautographs were treated with 0.5 N NaOH for 5–15 min to remove excess emulsion.

RESULTS

Leucine-³H Incorporation by Ovarian Fragments

The synthetic capability of isolated ovarian fragments was initially tested by determining their ability to incorporate leucine-³H into proteins. Ovaries in different stages of vitellogenesis were cultured in vitro with leucine-³H for 20, 40, and 60 min periods. The radioactivity of ovarian samples was assayed by liquid scintillation counting and the results are illustrated in Fig. 1. Incorporation of labeled leucine into proteins occurs at all stages of ovarian development and vitellogenesis. However, the most extensive labeling of ovarian proteins occurs in those ovaries containing oocytes in early stages of vitellogenesis. Moreover, the rate of incorporation of the isotope progressively decreases as vitellogenesis proceeds and is lowest in ovarian fragments with nearly mature oocytes.

Radioautographs of oocytes cultured in vitro in leucine-³H for 1 hr are illustrated in Figs. 4–6. From these figures, it is clear that the isotope is extensively incorporated by the large, vitellogenic oocytes (Figs. 5, 6). In contrast, comparatively few silver grains are observed in the ooplasm of young, previtellogenic oocytes (Figs. 4, 5). Within the cytoplasm of some vitellogenic oocytes, label is especially concentrated at the periphery of yolk spheres (Figs. 4, 6). It is also apparent that the single layer of follicle cells investing the oocyte is intensely labeled (Figs. 4–6).

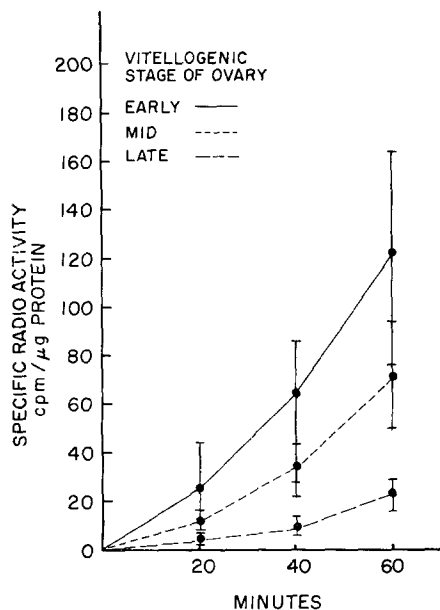


FIGURE 1 The incorporation of leucine-³H into ovarian proteins at different stages of vitellogenesis. Each point represents the average of three or more separate experiments. The bars denote the variability between ovarian samples.

Effect of Cycloheximide on Leucine-³H Incorporation by Ovarian Fragments

In an attempt to determine if, in fact, the incorporation of labeled amino acids by the crayfish ovary represents actual protein synthesis, portions of ovaries from several animals were pooled and incubated in Ringer's containing both leucine-³H and cycloheximide. The results are illustrated in Fig. 2 where it is clearly demonstrated that cycloheximide effectively inhibits the incorporation of leucine-³H into ovarian proteins at all concentrations used. Moreover, within the range of concentrations tested, the degree of inhibition is not dependent upon drug concentration.

The action of cycloheximide on leucine-³H incorporation by oocytes is illustrated by the radioautographs (Figs. 7, 8). Those oocytes cultured *in vitro* for 40 min in isotope, but without cycloheximide, actively incorporate leucine-³H (Fig. 7). Silver grains are especially concentrated over the perinuclear ooplasm as well as the follicular envelope. As illustrated in Fig. 8, however, this incorporation is greatly inhibited by the presence of cycloheximide at a concentration of 10 μg/ml.

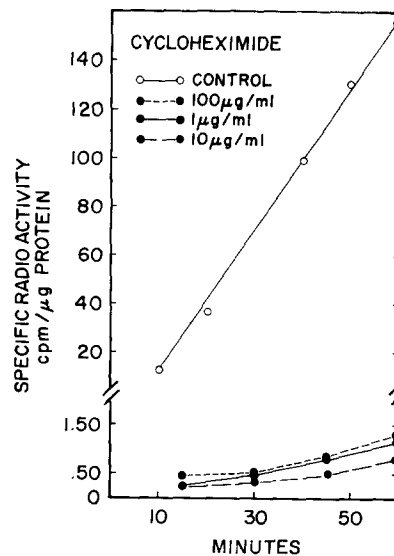


FIGURE 2 Effect of cycloheximide on incorporation of leucine-³H into ovarian proteins.

Thus, the incorporation of leucine-³H into TCA-precipitable material by oocytes is sensitive to cycloheximide and thus appears to represent intrinsic protein synthesis.

Effect of Actinomycin D on Leucine-³H Incorporation by Ovarian Fragments

Small pieces of ovaries from individual females were preincubated in actinomycin D and subsequently cultured in leucine-³H and actinomycin D at concentrations of 10 and 100 μg/ml. The results are illustrated in Fig. 3. As demonstrated previously, these results show that leucine-³H incorporation by single ovaries in early stages of vitellogenesis is markedly greater than that for those ovaries in mid vitellogenesis. Regardless of concentration, however, actinomycin D has no demonstrable effect on the level of incorporation at either stage of ovarian development. Thus, ovarian protein synthesis does not appear sensitive to actinomycin D.

The action of actinomycin D on leucine-³H incorporation by individual oocytes was examined by means of radioautography. Oocytes were cultured for 40 min in medium containing leucine-³H and actinomycin D (20 μg/ml). Control oocytes were incubated in labeled medium lacking actinomycin D. Examination of the radioautographs revealed no appreciable difference in level of

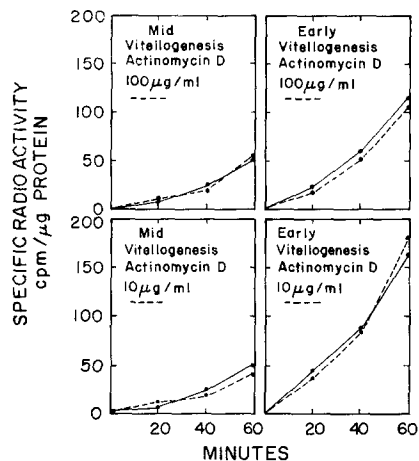


FIGURE 3 Effect of actinomycin D on incorporation of leucine-³H into ovarian proteins during early and mid vitellogenesis.

leucine-³H incorporation by the experimental and control oocytes. These observations indicate that the synthesis of oocyte proteins is unaffected by actinomycin D.

Oocyte Permeability to Actinomycin D

To investigate the permeability of developing oocytes to actinomycin D, oocytes were exposed to actinomycin-D-³H for 10 min and processed for radioautographic study. In such preparations silver grains are predominantly localized over oocyte and follicle cell nuclei, with fewer grains present over the ooplasm (Fig. 9). With longer periods of exposure to actinomycin-D-³H (45 or 60 min), labeling is principally restricted to the chromatin of the oocyte and to follicle cell nuclei while little is associated with oocyte nucleoli (Figs. 10, 13, and 14). Most of the silver grains in the oocyte cytoplasm appear to be associated with yolk bodies (Figs. 11, 12, and 14) and mitochondria. The labeling obtained with actinomycin-D-³H can be removed by DNase digestion or by extraction with hot TCA.

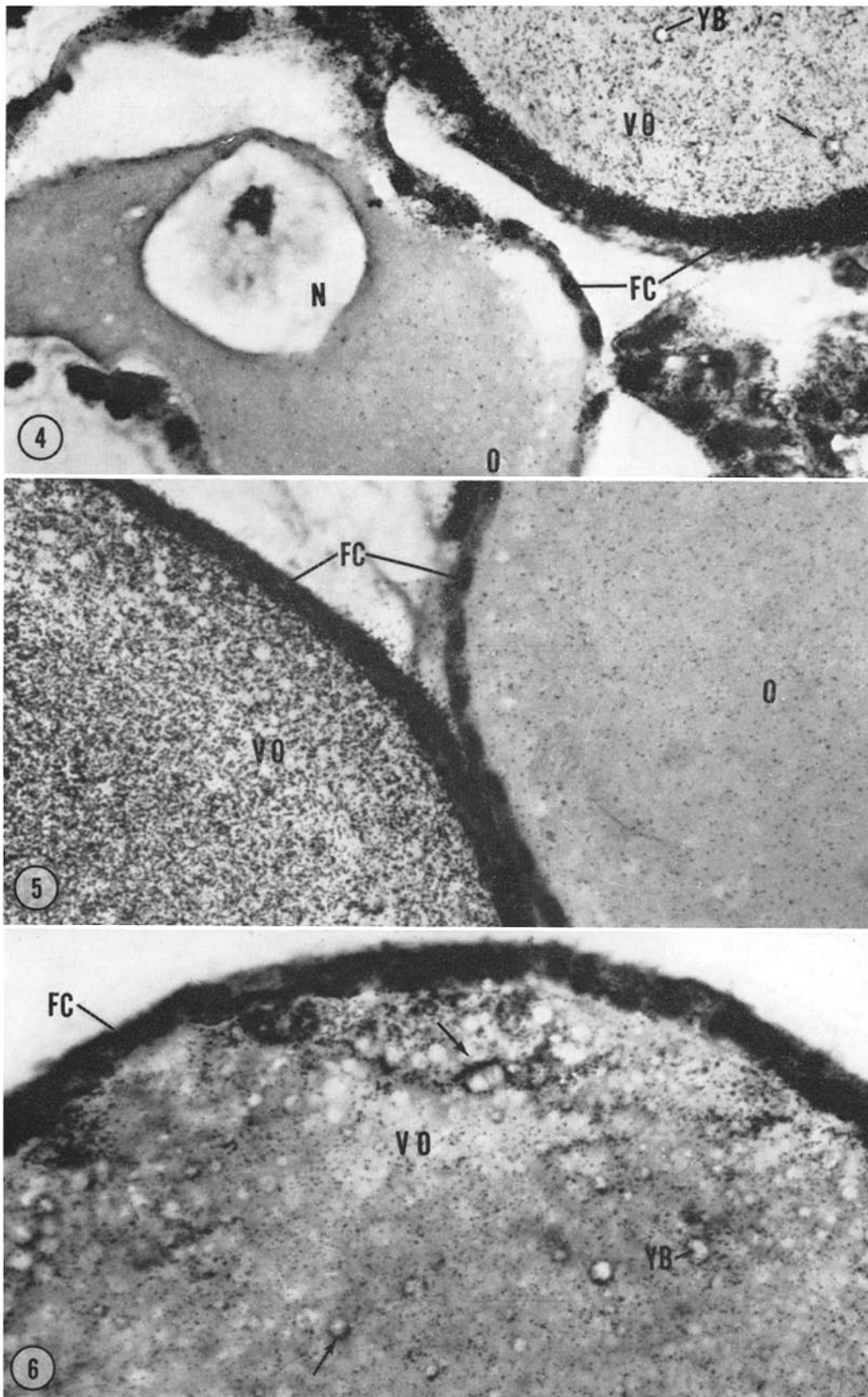
As another means to determine if the crayfish oocyte is permeable to actinomycin D, the effect of this drug on the incorporation of uridine-³H into RNA was investigated by radioautographic techniques. Oocytes which had been precultured for 40 min in actinomycin D at a concentration of 50 µg/ml were then incubated in Ringer's containing both actinomycin D and uridine-³H for 40 min. Under these conditions, the oocytes do not become

labeled with uridine-³H. In contrast, those oocytes not incubated in actinomycin D do become labeled and the silver grains are primarily localized over the oocyte nucleus and perinuclear cytoplasm. The label is removed when sections are treated with ribonuclease. These results not only suggest an inhibition of oocyte RNA synthesis by actinomycin D, but further establish that the crayfish oocyte is permeable to this drug.

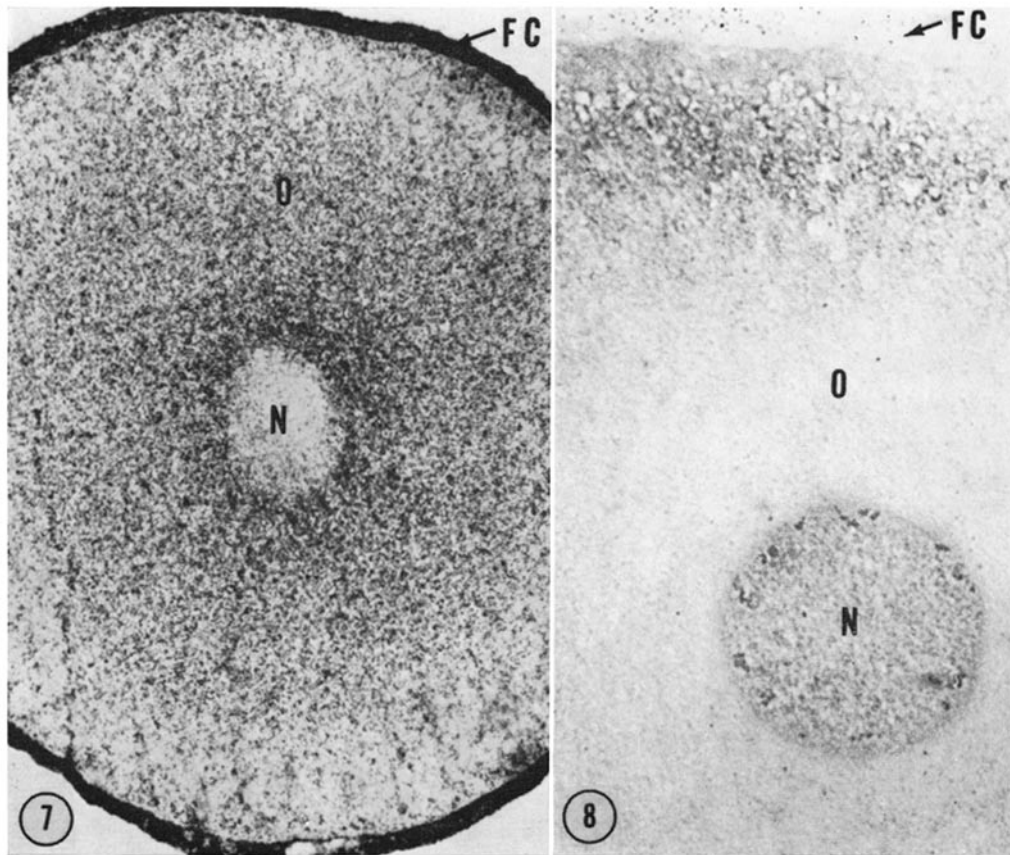
Intracellular Incorporation of Leucine-³H and Migration of Labeled Proteins

IN VITRO EXPERIMENTS: The capability of the rough-surfaced endoplasmic reticulum in the crayfish oocyte to synthesize intracisternal granules was investigated by means of light and electron microscope radioautography. High resolution radioautographs of oocytes cultured in vitro with leucine-³H are illustrated in Figs. 15 and 16. After 6 or 10 min exposure to the isotope, ooplasmic label is primarily localized in regions occupied by elements of the rough-surfaced endoplasmic reticulum and the contained intracisternal granules. After 2 hr the silver grains increase in number, but are still concentrated over the cisternae of the rough-surfaced endoplasmic reticulum (Figs. 17 and 18).

To determine the fate of the newly synthesized intracisternal granules, oocytes were pulse-labeled for 6 min with leucine-³H and subsequently inserted into the hemocoel of unlabeled, adult females. After a chase period of 1, 3, and 7 days, the transplanted oocytes were removed and processed for radioautographic studies. Light microscope radioautographs of pulse-labeled oocytes are illustrated in Figs. 19-21. Silver grains are present in the perinuclear ooplasm of these oocytes and frequently are clustered over striated, cytoplasmic structures which represent stacks of granular endoplasmic reticulum (Fig. 15). Silver grains are also present over single lamellae of rough-surfaced endoplasmic reticulum. After a chase period of 1 day, the label is primarily localized over aggregates of intracisternal granules contained within expanded elements of agranular endoplasmic reticulum (Fig. 25), but only a few silver grains are encountered over forming yolk bodies. More mature yolk spheres lack label at this time. Oocytes processed for radioautography 7 days after transplantation are illustrated in Figs. 22-24. By this time, labeled yolk bodies are extremely



FIGURES 4-6 Light microscope radioautographs of crayfish oocytes cultured in vitro with leucine- ^3H for 1 hr. Vitellogenic oocytes (*VO*) are extensively labeled with silver grains concentrated at periphery of some yolk bodies (*YB* and arrows). In contrast, previtellogenic oocytes (*O*) are not as intensely labeled. Note concentration of silver grains over follicle envelope (*FC*). *N*, oocyte nucleus. Exposure time, 6 wk. $\times 1500$.



FIGURES 7-8 Light microscope radioautographs of crayfish oocytes labeled with leucine- ^3H for 40 min either in presence (Fig. 8) or in absence (Fig. 7) of cycloheximide. Oocyte nucleus (*N*), ooplasm (*O*), follicle cell layer (*FC*). Exposure time, 31 days. Fig. 7, $\times 750$; Fig. 8 $\times 1250$.

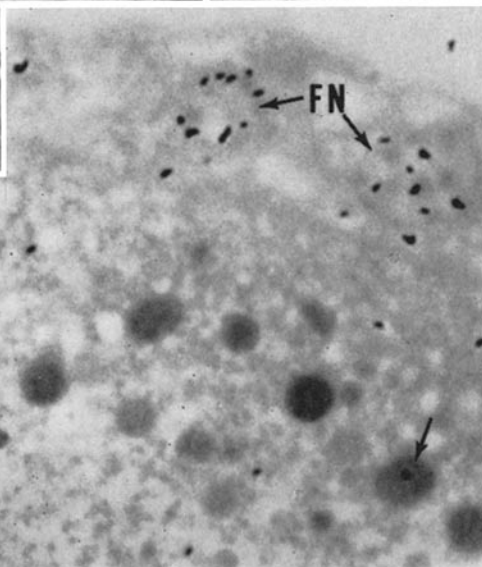
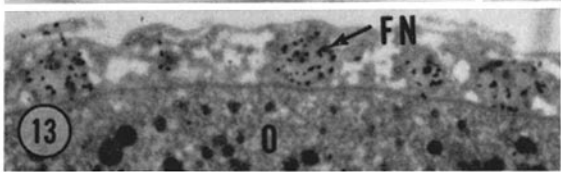
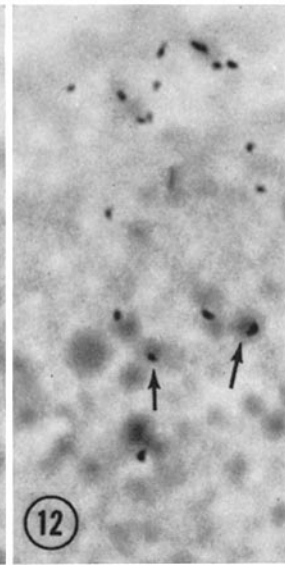
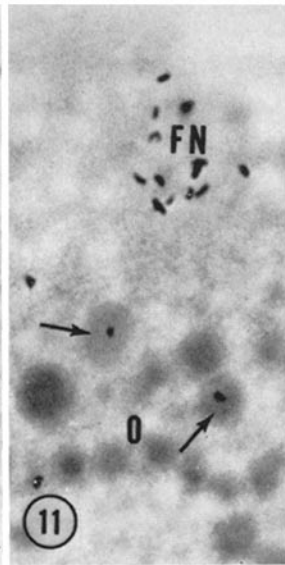
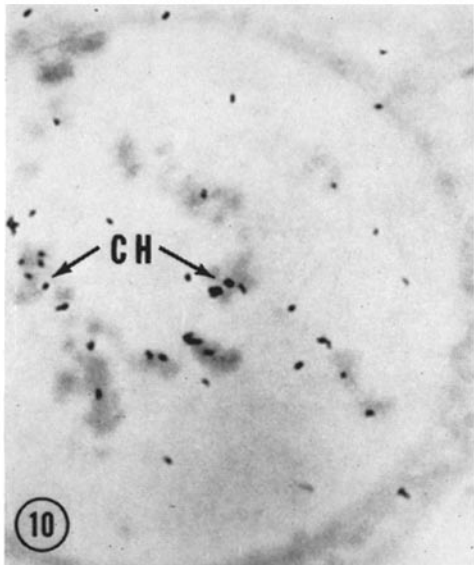
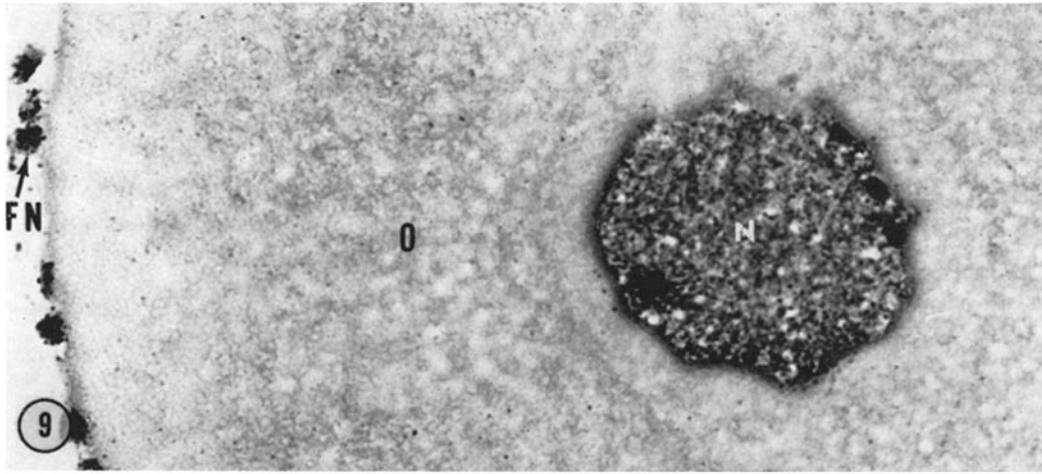
abundant in the cortical ooplasm. These experiments suggest that those intracisternal granules initially synthesized within the rough-surfaced cisternae of the endoplasmic reticulum in the perinuclear ooplasm are transported to forming yolk bodies which first appear in the cortical ooplasm.

Intracellular Incorporation of Leucine- ^3H and Migration of Labeled Proteins

IN VIVO EXPERIMENTS: The synthesis and deposition of proteinaceous yolk was also studied in oocytes cultured with leucine- ^3H under in vivo

FIGURE 9 Portion of oocyte exposed to actinomycin-D- ^3H for 10 min. Label is particularly concentrated over the oocyte nucleus (*N*) and follicle cell nuclei (*FN*). Fewer grains are localized over the oocyte cytoplasm (*O*). Exposure time, 35 days. $\times 1125$.

FIGURES 10-14 Light microscope radioautographs of oocytes cultured for 45 min in actinomycin-D- ^3H . Chromosomes (*CH*) in early diplotene oocyte nucleus are labeled in Fig. 10. Label is associated with the peripheral yolk spheres (arrows) in the ooplasm and with the follicle cell nuclei (*FN*) in Figs. 11-14. Oocyte cytoplasm (*O*). Exposure time, 43 days. Fig. 9, $\times 1800$; Fig. 10, $\times 4000$; Figs. 11, 12, and 14, $\times 5000$; Fig. 13, $\times 1125$.



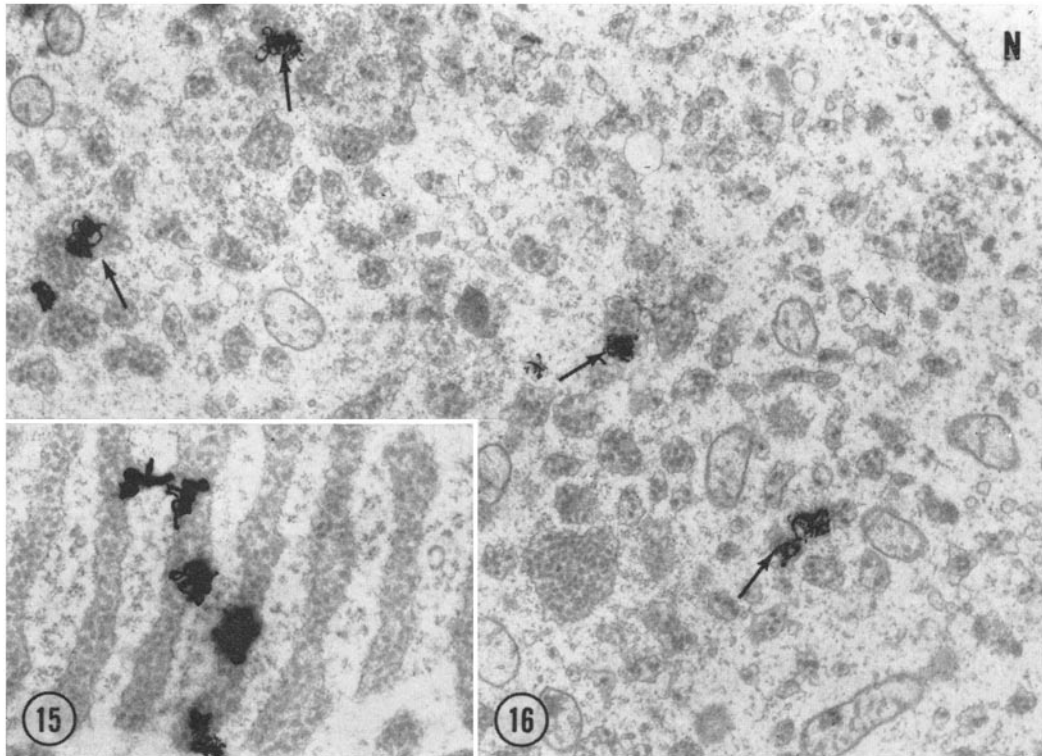


FIGURE 15 High resolution radioautograph of perinuclear ooplasm from oocyte cultured in vitro with leucine- ^3H for 6 min. Developed silver grains are associated with the stacked cisternae of the rough-surfaced endoplasmic reticulum and intracisternal granules. Exposure time, 12 wk. $\times 22,800$.

FIGURE 16 Electron microscope radioautograph of a vitellogenic oocyte fixed 10 min after in vitro incubation in leucine- ^3H . Silver grains are predominantly associated with elements of rough-surfaced endoplasmic and intracisternal (precursor yolk) granules (arrows). Nucleus (N). Exposure time, 5 months. $\times 16,400$.

conditions. In these experiments, oocytes were removed at various time intervals after injection of females with leucine- ^3H and processed for radioautography. After 12 hr of incubation, silver grains are predominantly associated with the rough-surfaced elements of the endoplasmic reticulum. After 3 days exposure to the isotope, silver grains continue to be associated with the stacked form of rough-surfaced endoplasmic reticulum (Figs. 26, 28), but are also evident over forming yolk bodies and adjoined smooth-surfaced cisternae (Figs. 27, 29). By 19 days, many silver grains are localized over mature yolk spheres (Figs. 30–32) while some grains are still observed over elements of the endoplasmic reticulum (Fig. 30). The in vivo experiments thus substantiate the results obtained from the in vitro experiments.

Oocyte Incorporation of Leucine- ^3H -Labeled Blood

To investigate the possible contribution of blood proteins to yolk deposition, ovaries from unlabeled females were transplanted to female crayfish which had been injected with leucine- ^3H 24 hr earlier. Female blood proteins become maximally labeled within 24–36 hr after the injection of leucine- ^{14}C (16). After 24 and 48 hr, the oocytes were removed from the female host and prepared for radioautographic study. At either time interval, the level of ooplasmic labeling is very low (Figs. 33, 34). Furthermore, the label is not specifically localized over yolk bodies at the oocyte periphery, but dispersed throughout the ooplasm. These experiments indicate that the contribution of maternal blood proteins to the vitellogenic process is slight.

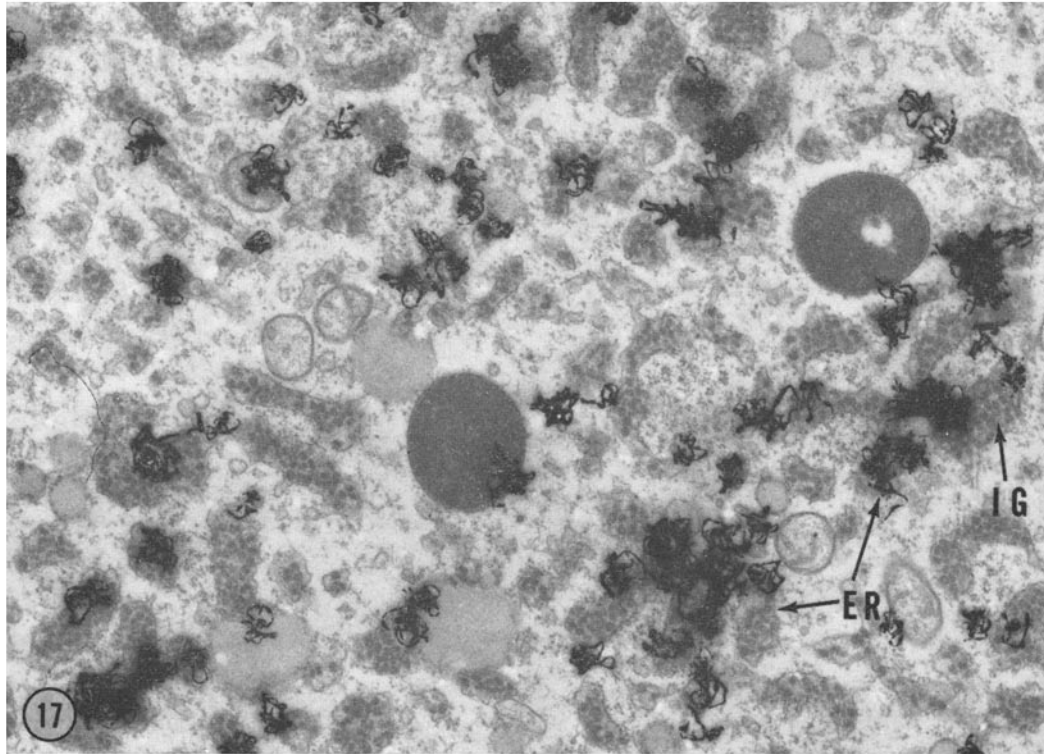


FIGURE 17 High resolution radioautograph of oocyte cultured for 2 hr in Ringer's containing leucine- ^3H . Most of the label is localized over the cisternae of granular endoplasmic reticulum (ER) and contained intracisternal granules (IG). Exposure time, 5 months. $\times 16,400$.

DISCUSSION

Numerous studies have dealt with elucidating the mechanisms whereby yolk is synthesized and packaged, but for the most part these studies are based on fine structural changes of ooplasmic organelles occurring at different periods of oocyte growth. From information currently available, it is apparent that considerable variability exists among different animal species in the extent to which the oocyte is able to synthesize the large quantity of food stores present by the end of oogenesis. For example, yolk has been described as originating, and perhaps being synthesized, within a variety of ooplasmic cytomembranes including mitochondria, endoplasmic reticulum, Golgi apparatus, yolk precursor sacs, and a combination of the endoplasmic reticulum and Golgi apparatus (cf., 3, 24-26, 32, and 46 for review). Conversely, in other species proteinaceous yolk is synthesized at a site external to the ovary (e.g. liver in amphibians, fat body in insects), the products being transported via

the blood to the oocyte and subsequently incorporated into the oocyte by a process of micropinocytosis (cf. 23, 43, and 45 for review). Finally, evidence exists to suggest that in some forms a portion of the yolk may be made within the ooplasm while other deutoplasmic materials are derived from an external source (cf. 13 and 32 for review).

The results of the present study, when considered together, demonstrate that the crayfish oocyte is capable of synthesizing its own yolk proteins. Thus, maximal labeling of ovarian proteins in the crayfish occurs at a time coincident with the deposition of proteinaceous yolk. Furthermore, leucine- ^3H incorporation into acid (TCA) insoluble proteins of isolated ovaries is drastically curtailed in the presence of cycloheximide. Moreover, as determined by radioautographic means, the incorporation of leucine- ^3H by vitellogenic oocytes is markedly inhibited by cycloheximide. Since this drug is known to interfere with the transfer of

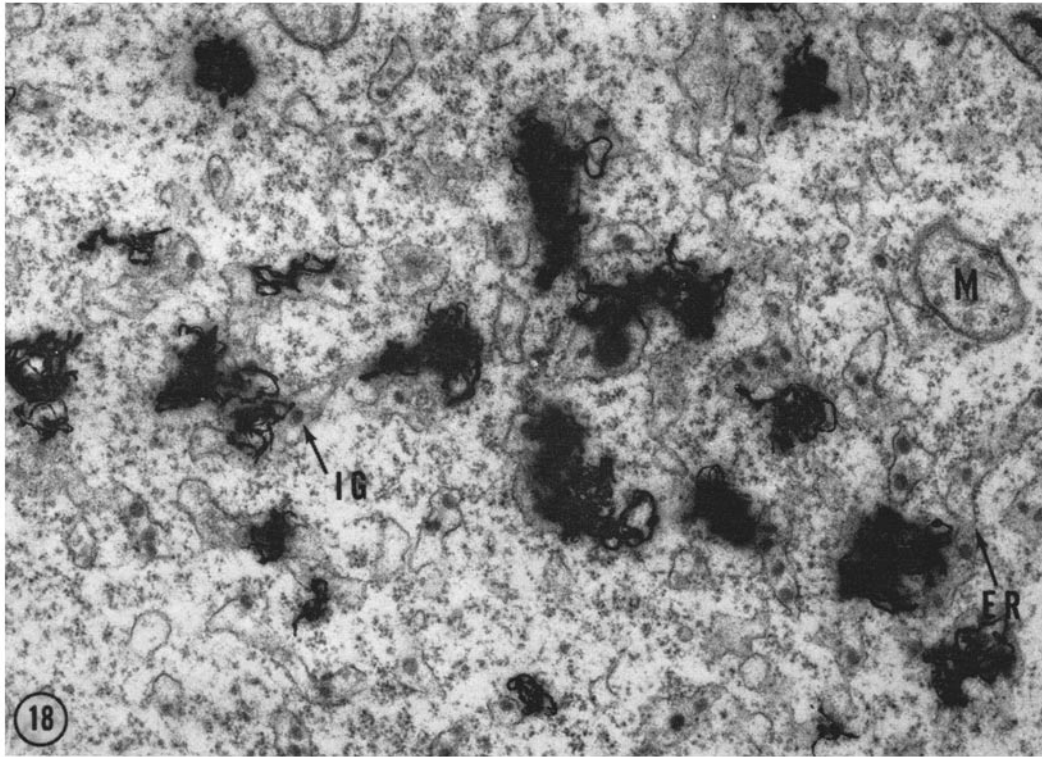


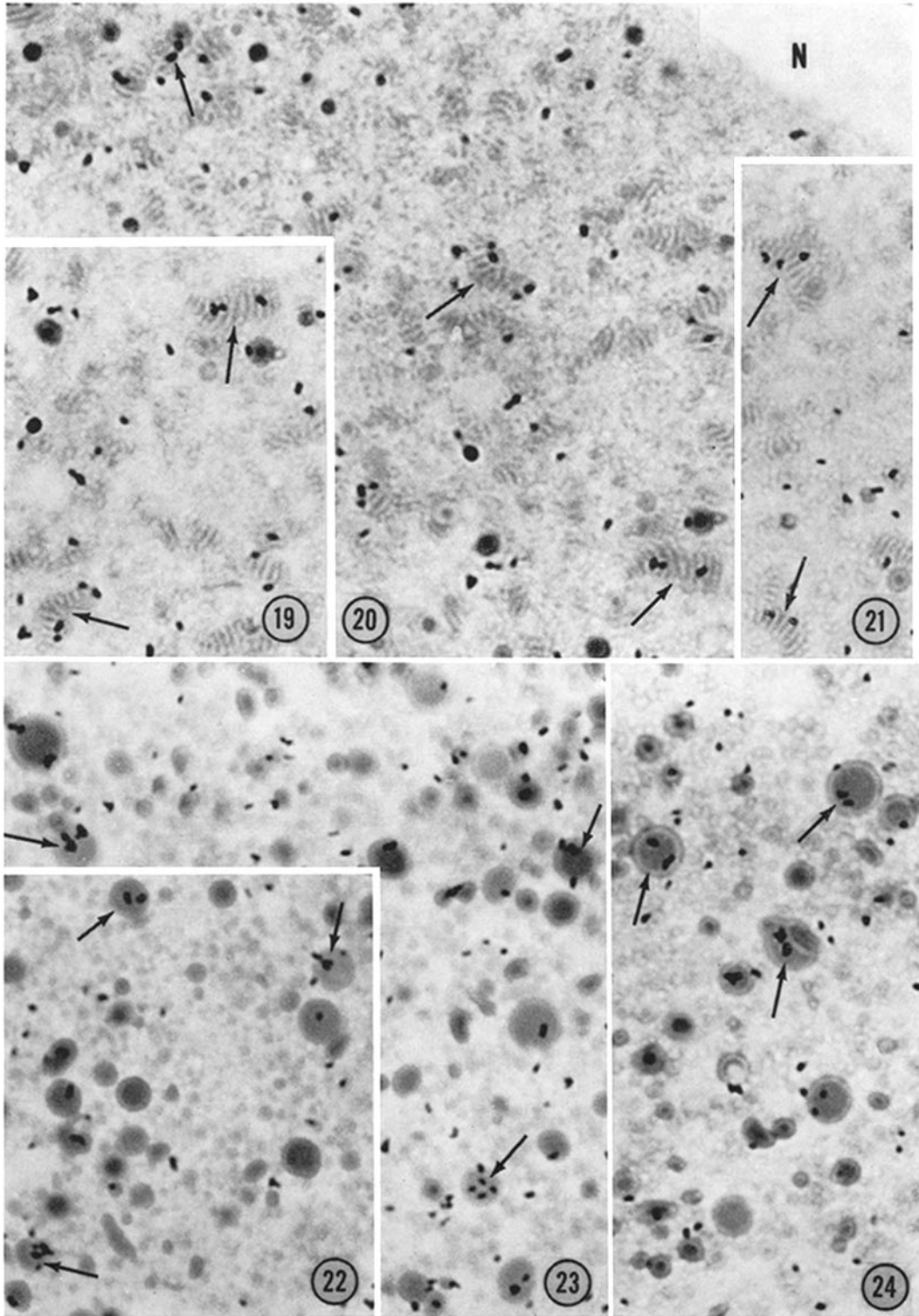
FIGURE 18 Electron microscope radioautograph of oocyte exposed to leucine- ^3H in vitro for 2 hr. Within the ooplasm, silver grains are localized over cisternae of the rough-surfaced endoplasmic reticulum (ER). Intracisternal granules (IG); mitochondria (M). Exposure time, 5 months. $\times 22,800$.

amino acids from aminoacyl-tRNA to elongating polypeptide chains at the site of the ribosomes, it can be concluded that the incorporation of leucine- ^3H by extirpated ovaries results, at least in part, from the synthesis of oocyte proteins.

Actinomycin D has no effect on the incorporation of leucine- ^3H into proteins by crayfish oocytes. Thus, protein synthesis in the crayfish oocyte does not appear to be dependent upon the continued synthesis of RNA. Crayfish oocytes are permeable to the drug since the germinal vesicle as well as the ooplasm become labeled with actinomycin-D- ^3H after 10 min, and the incorporation of uridine- ^3H into oocyte RNA is effectively blocked by actinomycin D. Likewise, it has been demonstrated that developing oocytes of the echinoderm, *Lytechinus pictus*, undergo a considerable amount of intrinsic protein synthesis which is insensitive to actinomycin D (35).

In *Triturus* oocytes, actinomycin-D- ^{14}C is incorporated into oocyte germinal vesicles, ooplasm,

and follicle cell nuclei (8). Steinert and Van Gansen (42) have reported the binding of actinomycin-D- ^3H to amphibian yolk platelets. Thymidine- ^3H incorporation into yolk platelets has also been described (1, 14), and Baltus et al. (2) have extracted DNA from yolk platelets of *Xenopus* oocytes. Because yolk spheres in the crayfish oocyte become labeled with actinomycin-D- ^3H , which reportedly binds specifically to DNA, the presence of DNA in yolk is suggested, but its functional significance is unknown. Brachet (6) has suggested that the DNA in yolk may be transformed into chromosomal DNA during early cleavage and utilized by developing nuclei during embryonic development. More recently, it has been postulated that the DNA contained within yolk platelets may possess information necessary for organ development during embryogenesis (14). Furthermore, Baltus et al. (2) have proposed that this DNA may play a role in the mobilization of yolk-catabolizing enzymes. The actinomycin-D- ^3H incorporation



FIGURES 19-21 Thick epon sections of oocytes cultured in Ringer's containing leucine- ^3H for 6 min. Silver grains are associated with striated cytoplasmic structures (arrows) in the perinuclear ooplasm which represent the stacked form of the rough-surfaced endoplasmic reticulum. Nucleus (N). Exposure time, 43 days. $\times 5000$.

FIGURES 22-24 Light microscope radioautographs of oocytes pulse-labeled for 6 min with leucine- ^3H and transplanted to unlabeled females. After 7 days, many of the mature yolk spheres are labeled (arrows). Exposure time, 43 days. $\times 5000$.

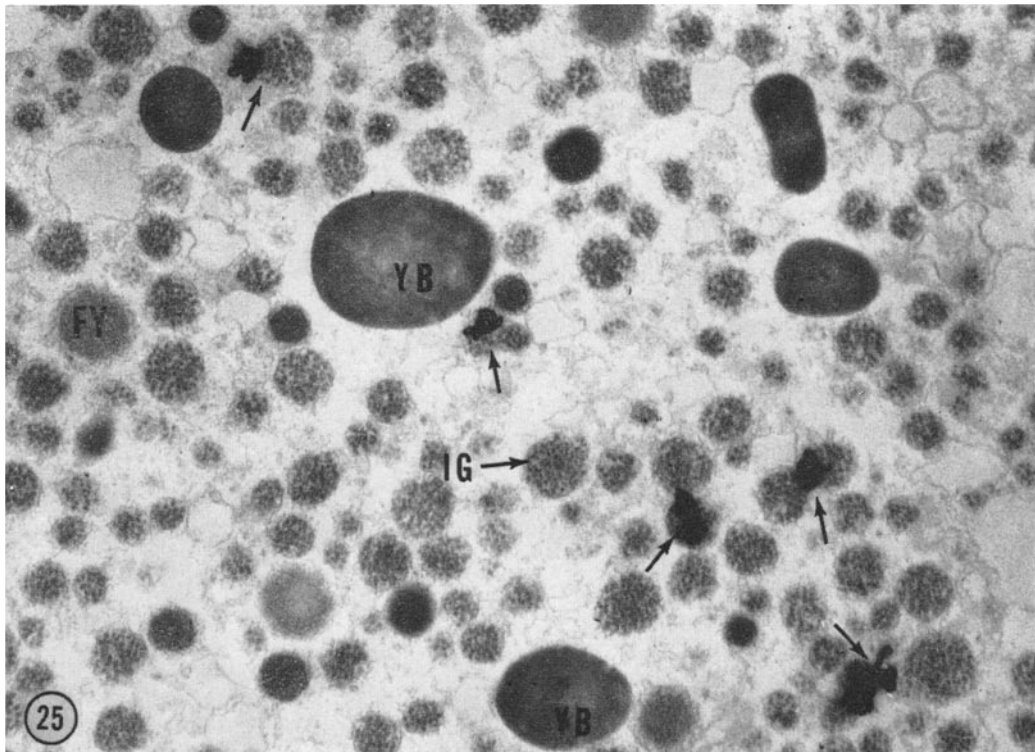


FIGURE 25 High resolution radioautograph of a developing oocyte pulse-labeled for 6 min with leucine- ^3H and transplanted to an unlabeled female for 1 day. Label (arrows) is associated with intracisternal granules (*IG*) aggregated within the single, intercommunicating cisternae of agranular endoplasmic reticulum. Forming yolk bodies (*FY*); mature yolk spheres (*YB*). Exposure time, 12 wk. $\times 16,400$.

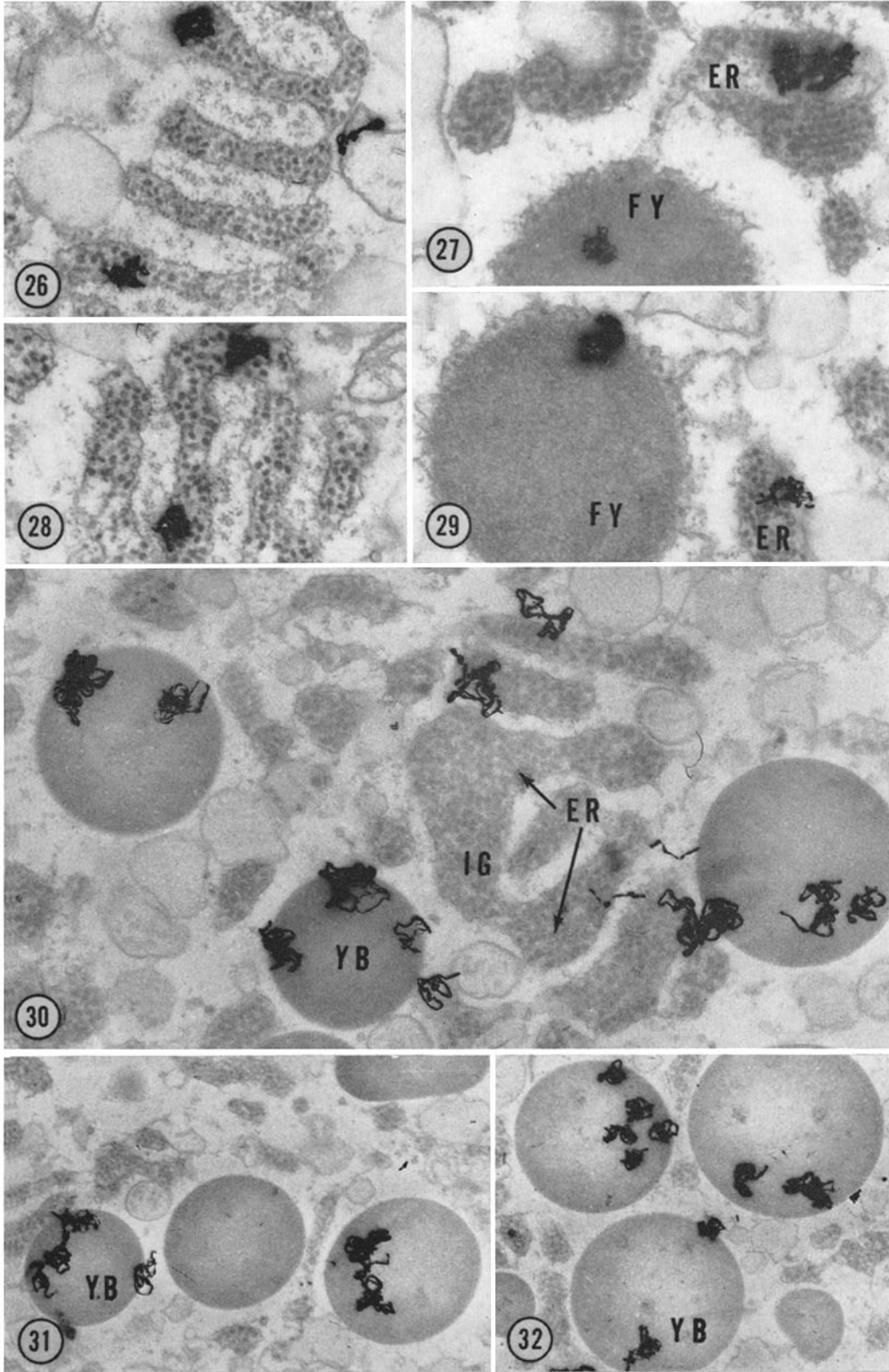
within the ooplasm of the crayfish oocyte not specifically localized over yolk bodies may result from the labeling of mitochondrial DNA.

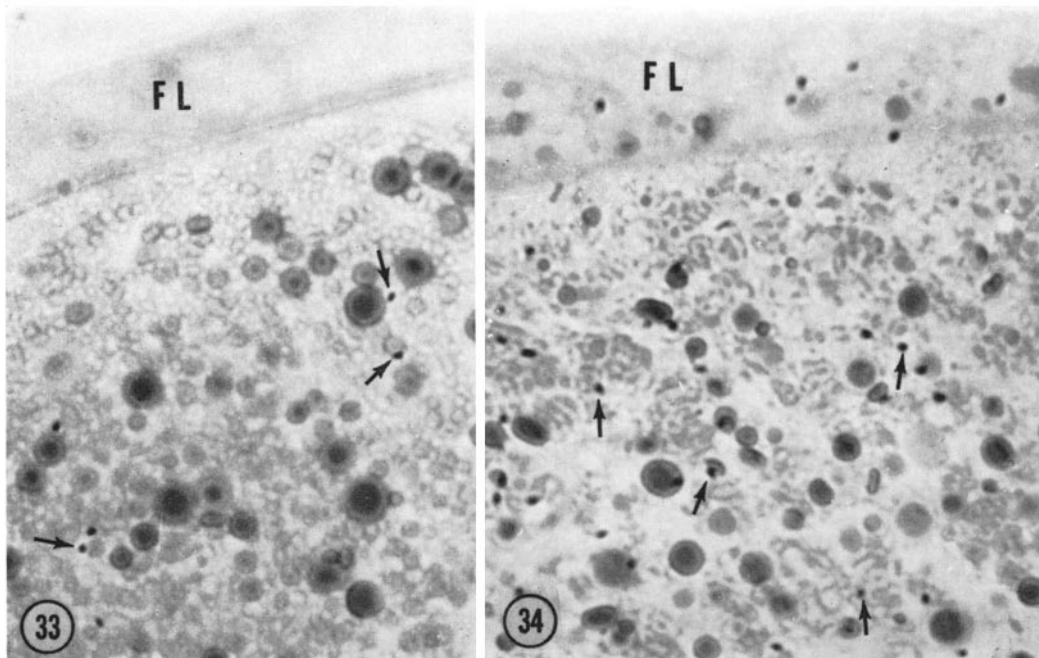
The inhibition of uridine- ^3H incorporation by actinomycin D indicates that some DNA-dependent RNA synthesis occurs during vitellogenesis in crayfish oocytes. Studies on developing oocytes of a sea urchin and an amphibian (15, 19, 35) have also revealed a suppression of RNA synthesis with

actinomycin D. Since actinomycin D does not inhibit the incorporation of leucine- ^3H into crayfish oocyte proteins, it seems likely that this synthesis is not dependent upon the immediate synthesis of RNA; a condition which suggests that a fairly long-lived messenger RNA is present in the crayfish oocyte. The existence of template-active RNA in unfertilized amphibian and echinoderm oocytes has been established (11, 30, 40). Furthermore,

FIGURES 26-29 Electron microscope radioautographs of oocytes incubated in vivo with leucine- ^3H . By 3 days, label occurs over forming yolk bodies (*FY* in Figs. 27 and 29) and adjoined cisternae of endoplasmic reticulum and intracisternal granules (*ER* in Figs. 27 and 29). Stacks of rough-surfaced endoplasmic reticulum are also labeled at this time (Figs. 26 and 28). Exposure time, 8 months. Figs. 26 and 29, $\times 16,400$; Figs. 27 and 28, $\times 22,800$.

FIGURES 30-32 Portion of oocytes cultured with leucine- ^3H for 19 days under in vivo conditions. By this time most of the label is localized over yolk spheres (*YB*), but a few silver grains still occur over branching elements of endoplasmic reticulum (*ER* in Fig. 30). *IG*, intracisternal granules. Exposure time, 61 days. Fig. 30, $\times 22,800$; Figs. 31 and 32, $\times 16,400$.





FIGURES 33-34 Light microscope radioautographs of oocytes cultured for 24 hr (Fig. 33) and 48 hr (Fig. 34) in female hosts containing leucine- ^3H -labeled blood. Note the dispersed and low-level incorporation (arrows) in the cortical ooplasm. Follicle cell layer (*FL*). Exposure time, 4 wk. $\times 5000$.

enucleation and actinomycin D studies on these oocytes have provided information to suggest that this maternal RNA becomes functional upon fertilization and serves as a template for the initial synthesis of embryonic proteins (7, 12, 41). Therefore, the messenger RNA made early during crayfish oogenesis may become activated and functional in the synthesis of proteins during yolk deposition.

The results of the radioautographic studies clearly illustrate that the endoplasmic reticulum plays an active role in protein yolk formation in oocytes of the crayfish. In oocytes cultured *in vitro* for brief intervals (6 and 10 min), leucine- ^3H incorporation was first observed over the stacked and dispersed profiles of rough-surfaced endoplasmic reticulum. While these results do not clearly demonstrate the specific site of protein synthesis, it seems likely that the initial incorporation of leucine- ^3H into proteins must occur on ribosomes of the rough-surfaced cisternae, since cycloheximide drastically inhibits ooplasmic protein synthesis. The synthesis and subsequent segregation of proteins within the ribosome-studded cisternae of

endoplasmic reticulum is further suggested by the increased density of labeled intracisternal granules observed in oocytes cultured for 2 hr with leucine- ^3H . Radioautographic results from a study on the lobster oocyte have also provided evidence that the ribosomes attached to the endoplasmic reticulum are active in the synthesis of proteinaceous yolk (23). Since these activities occur under *in vitro* conditions, the possibility that the newly synthesized proteins are derived from an extraoocyte source is eliminated.

Electron microscope radioautographs of transplanted oocytes pulse-labeled with leucine- ^3H reveal that after 1 day of chase the labeled proteins are predominately localized within the single and expanded smooth-surface cisternae of the intermediate and cortical regions of the ooplasm. In oocytes 7 days after transplantation, silver grains are evident over forming as well as mature yolk bodies, and in some instances are extremely numerous over the central portion of the yolk sphere. A similar labeling pattern is also observed in oocytes cultured *in vivo* with leucine- ^3H . These data suggest that the disc-shaped, intracisternal gran-

ules are transported through the extensive network of tubular endoplasmic reticulum to smooth-surfaced cisternae of the cortical ooplasm, where they form large aggregates which subsequently transform into proteinaceous yolk bodies. Furthermore, the increased intensity and number of labeled yolk platelets suggest that the additional influx of intracisternal granules from the attached smooth-surfaced cisternae ensures the continued growth of the forming yolk body.

The initial events leading to yolk deposition in the crayfish oocyte are strikingly similar to those described during the secretory process in the guinea pig pancreatic exocrine cell (cf. 33). In the initial stage in the secretory process in this exocrine cell, the digestive enzymes or zymogens (e.g. chymotrypsinogen) are synthesized upon the ribosomes attached to the rough-surfaced endoplasmic reticulum and enter into the enclosed cisternae (36). The secretory proteins are then transported from the cisternal compartments of the endoplasmic reticulum via small vesicles to the Golgi region where they are packaged into zymogen granules (10, 20, 21, 39).

Similarly, in the crayfish oocyte it would appear that the synthesis of yolk proteins occurs on the ribosomes attached to the granular form of the endoplasmic reticulum. The newly synthesized yolk proteins are initially visualized as disc-shaped, intracisternal granules (cf. 5). However, unlike the proteins in the exocrine pancreas, yolk proteins are not shuttled to the Golgi region but instead are transported through the massive network of tubular endoplasmic reticulum to regions where they are packaged into yolk bodies, and thus stored within the ooplasm. The limiting membrane of the mature yolk body is thus a derivative of the endoplasmic reticulum. The transport of yolk granules through the cisternae may result from the continuous delivery of newly synthesized proteins from the attached ribosomes of the rough-surfaced endoplasmic reticulum. However, on the basis of the studies on the exocrine pancreas (22), the possibility exists that the intracellular transport of newly synthesized yolk proteins may be an energy dependent process. While the growth of the forming yolk body is dependent upon the accumulation of intracisternal granules from the adjoined smooth-surfaced endoplasmic reticulum, the physiological factors responsible for the maturation of the yolk sphere and its subsequent detachment from the cisternal ele-

ments are not known. Since 2-3 months are required for the crayfish oocyte to reach maturity, yolk formation ensues at a relatively slow rate as compared to the 60-min secretory cycle of the pancreatic exocrine cell. The high resolution radioautographic results suggest that the rough-surfaced endoplasmic reticulum in the crayfish oocyte is continually engaged in the synthesis of yolk proteins during vitellogenesis.

In a number of different organisms, there is now evidence to indicate that yolk proteins made outside of the ovary are carried by the blood to the oocytes, where they subsequently become incorporated into yolk via micropinocytosis (cf. 3, 32, 34). While micropinocytosis occurs in crayfish oocytes at the time of vitellogenesis (5), the results of the present study indicate that this activity plays little, if any, role in the deposition of proteinaceous yolk. The radioautographic results obtained from oocytes cultured in adult crayfish containing leucine-³H-labeled blood further suggest that blood proteins do not become incorporated into forming yolk bodies under the experimental conditions employed. After 24 and 48 hr intervals, transplanted oocytes exposed to labeled female blood demonstrate extremely low levels of incorporation. Furthermore, the label is evenly dispersed throughout the ooplasm. While some labeled yolk platelets are encountered, they are not specifically restricted to the oocyte periphery as might be expected if yolk proteins were derived from an extraoocyte source. For example, Telfer and Melius (44) and Hausman et al. (17) demonstrated that labeled female blood is extensively incorporated into cortical yolk spheres of *Cecropia* oocytes. Since kinetic studies on the incorporation of leucine-¹⁴C into crayfish blood proteins indicate that unincorporated label persists for several days, it is possible that this label represents the incorporation of free leucine-³H into oocyte proteins or the incorporation of labeled proteins required to sustain the general cellular metabolism during oocyte growth. Horseradish peroxidase, when injected into the hemocoel of female crayfish, migrates through the intercellular spaces of the follicle envelope and is incorporated into the oocyte by micropinocytosis (16). However, the exogenous protein appears to be sequestered in ooplasmic dense bodies rather than into developing yolk spheres (16). Furthermore, trypan blue does not become incorporated into crayfish oocyte yolk

globules under either in vitro or in vivo conditions (16).

The intense incorporation of radiochemicals by the crayfish follicle envelope is of interest. An experimental analysis of the functional activity of these cells during oogenesis, including their role in the formation of the vitelline envelope, is in progress.

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This article is respectfully and affectionately dedicated to Professor H. W. Beams on the occasion of his sixty-eighth birthday and his contributions to the field of biology during the past 42 years.

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