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THE CELL BIOLOGY OF MOSQUITO VITELLOGENESIS

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Insect vitellogenesis involves coordinated activities of the fat body and oocytes. We have studied these activities at the cellular level in the mosquito. During each vitellogenic cycle, the fat body undergoes three successive stages: 1) proliferation of biosynthetic organelles, 2) vitellogenin synthesis, 3) termination of vitellogenin synthesis and degradation of biosynthetic organelles by lysosomes. Analysis with monoclonal antibodies and radiolabelling demonstrated that the mosquito yolk protein consists of two subunits (200-kDa and 65-kDa). Both subunits are glycosylated, their carbohydrate moieties are composed of high-mannose oligosaccharides. The yolk protein subunits are derived from a single 220 kDa precursor detected by an in vitro *translation. Oocytes become competent to internalize proteins as a result of juvenile hormone-mediated biogenesis of endocytotic organelles. The yolk protein is then accumulated by receptor-mediated endocytosis. A pathway of the yold protein and factors determining its routing in the oocyte have been studied.*

In insects, vitellogenesis is a cornerstone of egg maturation. This process requires the coordinated activity of the fat body, which synthesizes a precursor of the yolk protein (YP), vitellogenin (VG), and that of oocytes, which specifically accumulate YP as crystalline vitellin (VT). These activities are controlled by factors at the molecular, cellular and organismal levels. A detailed knowledge of the mechanism governing vitellogenesis is crucial for our understanding of insect reproductive physiology and may eventually lead to new approaches to pest control.

The challenging goals in studying vitellogenesis are to understand mechanisms regulating expression of YP genes in the fat body as well as to elucidate the molecular basis for the specific accumulation of YP by oocytes. An important step in accomplishing these goals is to learn about vitellogenesis at the cellular level. In this report, I summarize our studies in the cell biology of mosquito vitellogenesis.

In addition to the self-evident value in investigating this important vector of numerous diseases of humans and animals, the mosquito has become one of the model systems for studying insect vitellogenesis. This situation is mainly due to the extraordinary developmental synchrony of vitellogenesis in mosquitoes (Hagedorn, 1985; Raikhel & Lea, 1987).

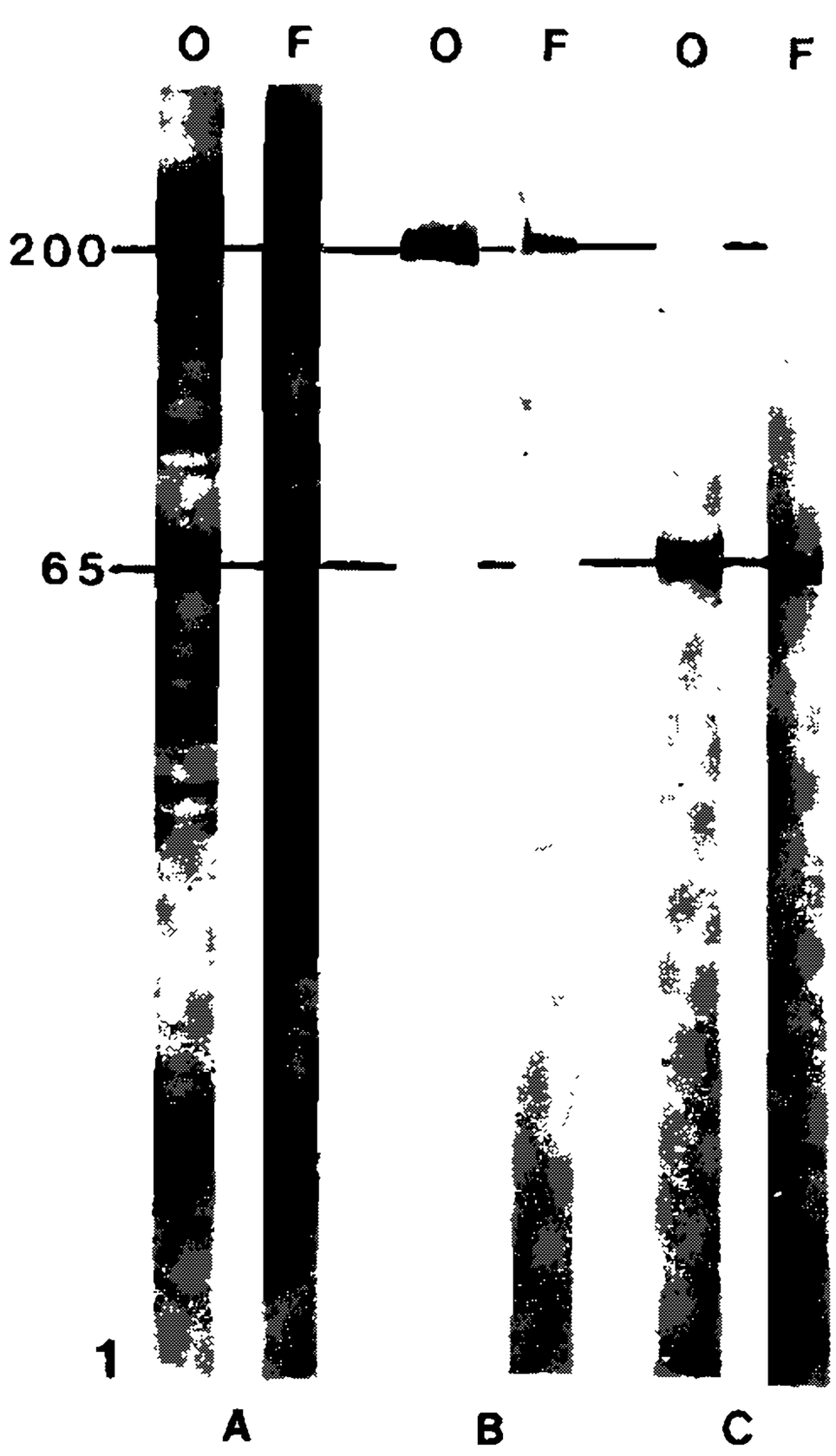

Fig. 1. Immunoblot analysis of monoclonal antibodies directed against the mosquito yolk protein. A – electrophoretic patterns (SDS PAGE) of crude extracts used for immunoblot, of ovaries (O) and fat bodies (F) 24 hr after a blood meal. Numbers indicate molecular sizes in kDa B – immunoblot pattern of a mAB recognizing a 200±3 kDa polypeptide in these tissue extracts. C – immunoblot pattern of a mAB recognizing a 65±3 kDa polypeptide.

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PROPERTIES OF THE MOSQUITO YOLK PROTEIN

Monoclonal antibodies as a tool for analysis of vitellogenesis: In order to develop molecular probes applicable to a wide range of questions regarding vitellogenesis, we produced a library of monoclonal antibodies (mAB) against the yolk protein of the mosquito *Aedes aegypti* (Raikhel et al., 1986; Raikhel & Lea, 1987). These mABs were characterized by enzyme-linked immunosorbent assay, immunoblot and immunocytochemistry. Immunoblot analysis revealed that mABs reacted with polypeptides only in extracts from vitellogenic fat bodies and ovaries and recognized either a 200 KDa or a 65 kDa polypeptide (Fig. 1).

Composition of the yolk protein: It was debated whether mosquito YP was composed of large subunits only, of large and small subunits, or even multiple subunits of different sizes (Atlas et al., 1978; Harnish & White, 1982; Ma et al., 1984; Hagedorn, 1985; Borovsky & Whitney, 1987).

To answer this question, we used a library of mABs against the mosquito YP and radiolabelling experiments. The immunoprecipitation analysis demonstrated that the mosquito YP consisted of two subunits, the 200±3 KDa and 65±3 kDa polypeptides which were recognized by mABs. Labelling experiments showed that during vitellogenesis only these two subunits (200±3 kDa and 65±3 kDa) were synthesized and secreted by the fat body, and were subsequently accumulated by the developing oocytes. In newly laid eggs, VT also consisted of only these two subunits.

Carbohydrate moieties of the mosquito yolk protein: Elucidation of the nature of carbohydrate moieties is important for understanding their possible roles in the processing and secretion of YP by the fat body as well as in YP recognition and specific accumulation by the oocyte.

Experiments with radiolabelled carbohydrate precursors demonstrated that both subunits of the mosquito YP were glycosylated, and their carbohydrate moieties contained mannose and N-acetylglucosamine. The YP carbohydrate moieties were sensitive to treatment with endoglycosidase H (Endo H), an enzyme which specifically cleaved asparagine-linked oligosaccharides (Fig. 2). Treatment with tunicamycin, a drug inhibiting N-linked glycosylation, also blocked incorporation of

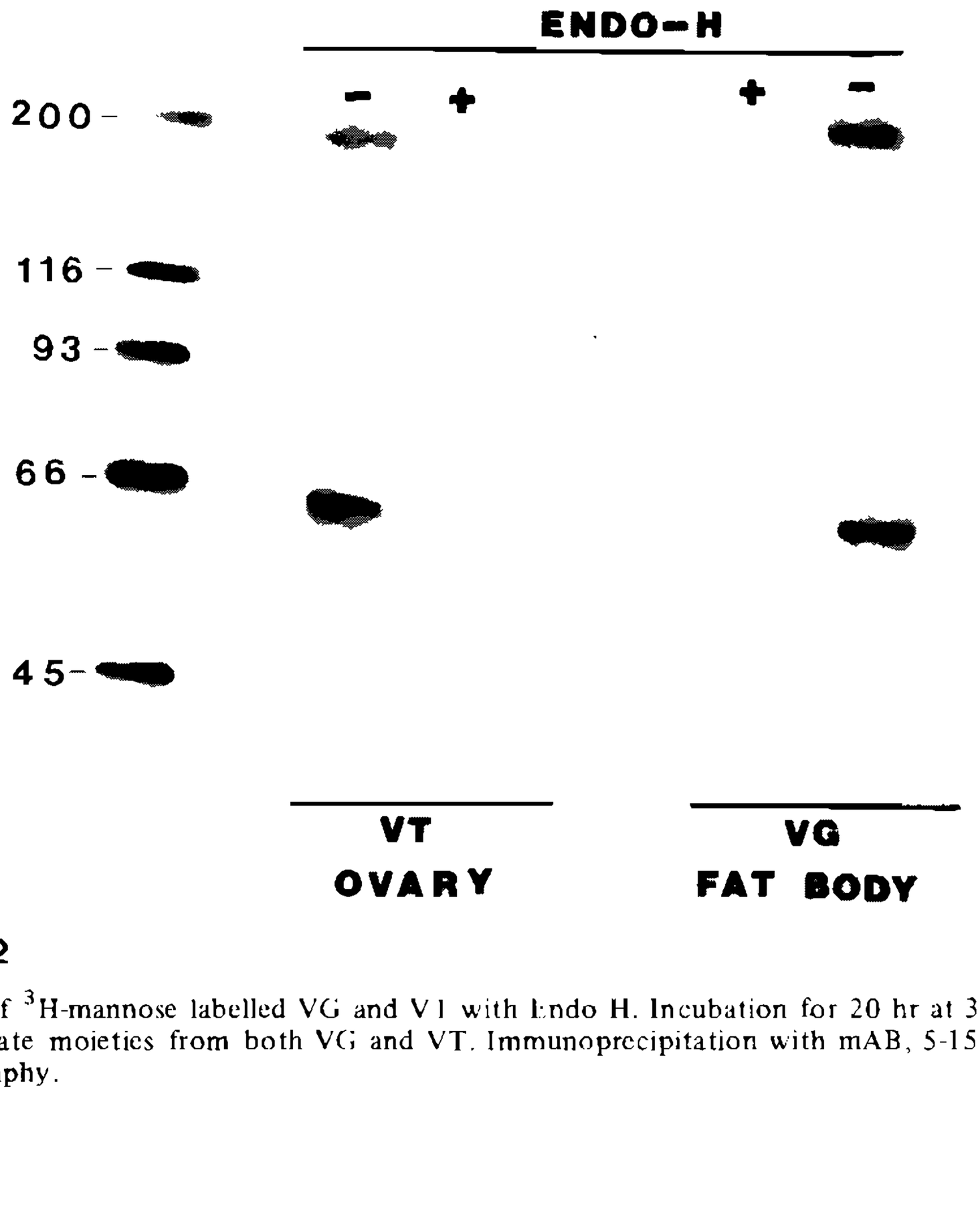

Fig. 2. Treatment of ^{3}H-mannose labelled VG and VT with Endo H. Incubation for 20 hr at 37°C completely removed carbohydrate moieties from both VG and VT. Immunoprecipitation with mAB, 5-15% gradient SDS PAGE and fluorography.

^{3}H-mannose into both YP subunits. These experiments showed that the carbohidrate moieties of mosquito YP were high-mannose oligosaccharides, as in other insects studied so far (Nordin et al., 1984; Osir et al., 1986).

SYNTHESIS AND SECRETION OF THE YOLK PROTEIN IN THE FAT BODY

Synthesis of vitellogenin: A detailed knowledge of primary protein products and their co- and post-translational modifications is an important requirement for the study of YP gene expression. In most insects studied so far, VG is first synthesized as a single large precursor which then undergoes complex processing resulting in smaller subunits of mature VG (Chen, 1980; Harhish et al., 1982; Wojchowski et al., 1986). In contrast, the processing of three *Drosophila* vitellogenins is a simple removal of a 1000-dalton signal peptide from each pre-VG (Warren et al., 1979).

We studied the precursor-product relationship during VG synthesis in the mosquito by using *in vitro* translation, treatment with tunicamycin and pulse-chase lebelling. After *in vitro* translation of poly(A) + RNA from vitellogenic fat bodies, a high molecular weight polypeptide of 220 kDa was detected by immunoprecipitation with mABs. Monoclonal antibodies to both YP subunits, 200-kDa and 65-kDa, recognized the 220-kDa polypeptide (Fig. 3A). These experiments suggested that a high molecular weight polypeptide (220-kDa) was a precursor for both YP subunits. Treatment of the fat body by tunicamycin, an inhibitor of glycosylation, resulted in accumula-

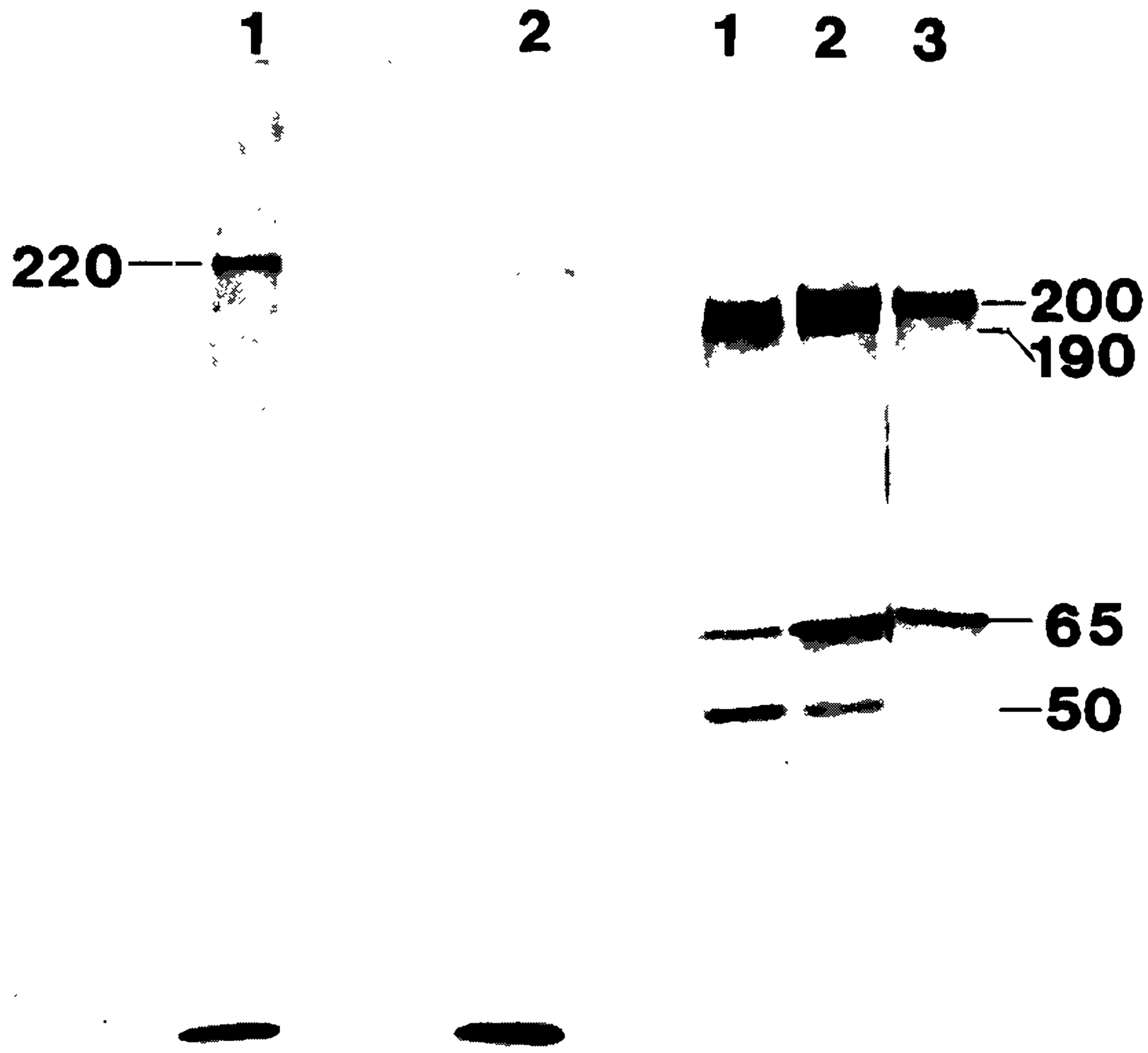

Fig. 3. Analysis of VG processing by *in vitro* translation (A) and experiments with tunicamycin (B).
A. Poly(A)+ RNA from vitellogenin fat bodies was translated in the reticulocyte lysate *in vitro* translation system; ^{35}S-methionine labelled translation products were immunoprecipitated by a mAB to a small YP subunit (lane 1) or by a mAB to a large YP subunit (lane 2). Notice the presence of a 220-kDa polypeptide in both lanes.
B. Inhibition of VG processing by tunicamycin *in vitro*. Vitellogenic fat bodies were first incubated in the medium containing tunicamycin (50 μg/ml) for 30 min (lanes 1 and 2) and then labelled by ^{35}S-methionine: lane 1 – labelling in the presence of tunicamycin, lane 2 – fat bodies were treated by tunicamycin but labelled in the absence of the drug; lane 3-control labelling without the treatment. Notice the accumulation of 190-kDa and 50-kDa polypeptides in tunicamycin-treated fat bodies. 200-kDa and 65-kDa are the mature YP subunits.

tion of 190-kDa and 50-kDa polypeptides (Fig. 3B). Pulse-chase experiments confirmed this step in the VG processing. Thus, in the mosquito, the YP subunits were derived from a common precursor by the posttranslational cleavage.

Cellular aspects of synthesis and secretion of vitellogenin: Immunocytochemistry at the electron microscopic level demonstrated the VG secretory pathway in fat body cells of the mosquito (Raikhel & Lea, 1983). Subunit-specific mABs and double immunolabelling were utilized to investigate the exact intracellular location of the YP subunits (200-kDa and 65-kDa) during their processing in the fat body cells. This study showed that the same secretory pathway was employed by both large and small subunits. The YP subunits were co-localized in all compartments of the secretory pathway, particularly in the Golgi complex and in secretory granules (Fig. 4).

CYCLICITY OF THE VITELLOGENIC ACTIVITY OF FAT BODY CELLS

Mosquitoes are characterized by a highly regulated cyclic egg production. As a consequence, the VG synthesis by the fat body occurs only periodically when oocytes are competent to accumulate VG. Analysis of cellular events occuring in trophocytes of the mosquito fat body showed that during each cycle of egg maturation, these cells undergo three successive stages of activity (Fig. 5): (1) proliferation of biosynthetic organelles; (2) the synthesis of VG; (3) termination of the VG production and breakdown of the biosynthetic apparatus by lysosomes (Raikhel & Lea, 1983; Raikhel, 1986a; 1986b).

Proliferation of biosynthetic organelles: During vitellogenesis, the trophocyte nucleolus undergoes cyclic transformations corresponding to the state of its activity (Raikhel & Lea, 1983). During the previtellogenic phase, a dramatic enlargement of the nucleous, an organelle producing ribosomes, occured and was followed by proliferation of ribosomes. These events were mediated by juvenile hormone (JH). They were blocked by removal of the corpora allata at eclosion and restored by implanting corpora allata from a single donor or application of physiological doses of JH (Raikhel & Lea, unpublished).

The second peak in the activity of the nucleolus and production of ribosomes occurs during the first 12 hr after a blood meal. Between 12 and 36 hr after the blood meal, active multilobed nucleoli regressed into compact bodies, suggesting a cessation in ribosome synthesis (Raikhel & Lea, 1983). These data are consistent with a recent report by Hotchkin & Fallon (1987). These authors found that

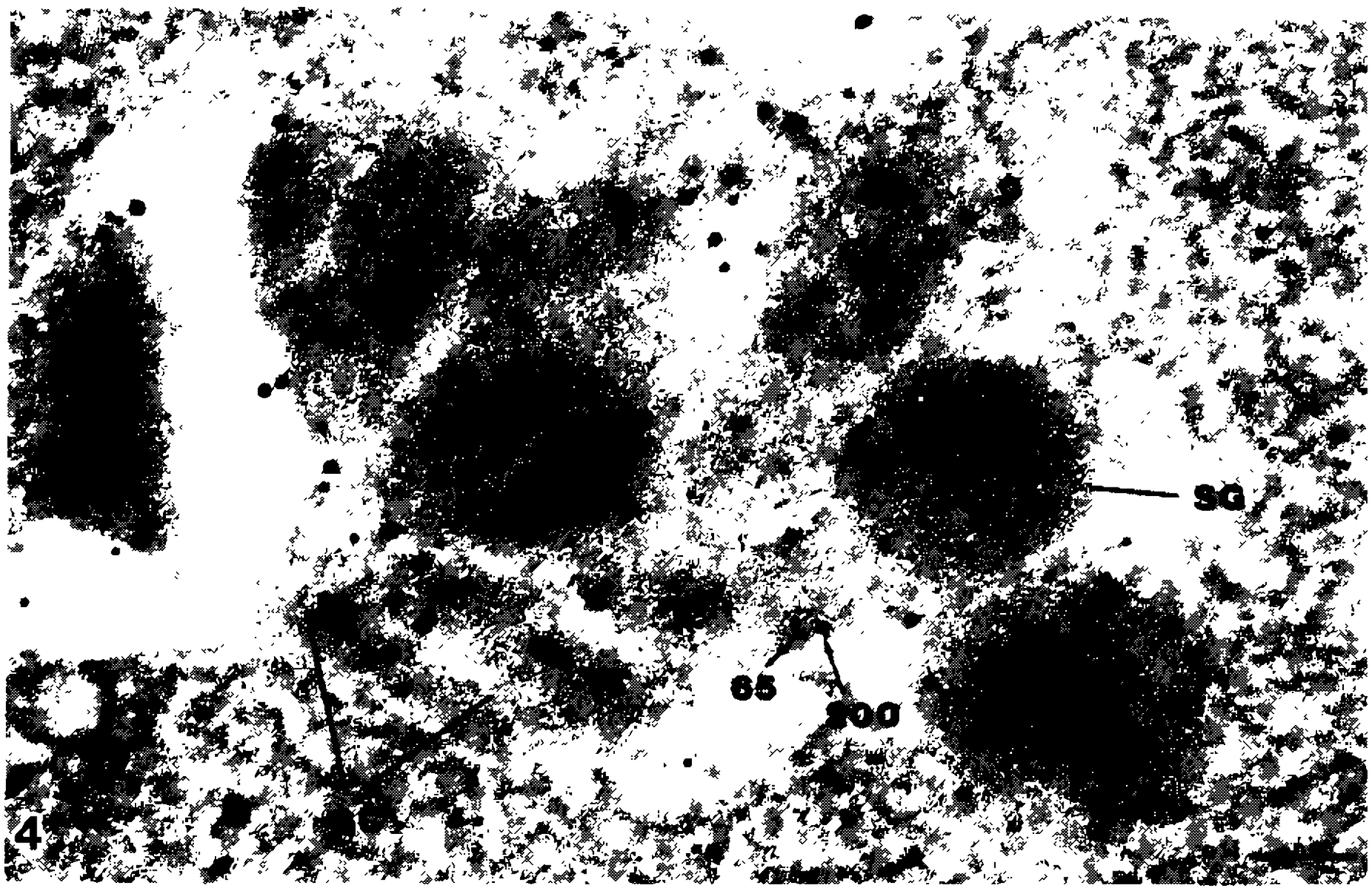

Fig 4. Double immunolabelling of the vitellogenic fat body cells using two subunit-specific mABs (mAB 2G1 to 65-kDa YP subunit and mAB A1D12 to 200-kDa) and protein A – colloidal gold probes of different sizes. Co-localization of the YP subunits in the Golgi complex (GC) and secretory granules (SG) of the fat body cell, a small YP subunit (65) is marked by 10 nm gold, a large YP subunit (200) by 15 nm gold. 110,000 X; bar is 0.1 um

ribosome synthesis and accumulation dramatically increased during the first part of the vitellogenic phase (first 18 hr after a blood meal). By 24-30 hr after the blood meal, the accumulated ribosomes began to be degraded, and the RNA content in the fat body eventually returned to previtellogenic levels. The factors initiating the post-blood meal activation of ribosome production as well as its cessation remain to be elucidated.

Lysosomal regulation of secretory activity of trophocytes: The cessation of VG synthesis and its secretion by trophocytes is coordinated with oocyte development. Attempts to explain the mechanism of this precisely timed event led to several hypotheses: "pre-programmed termination" by Bohm et al. (1978), "a feedback control" by Borovsky (1981). Our study demonstrated that cellular events during the cessation of VG synthesis were far more complex that just an interruption of VG synthesis. We found a novel mechanism which was involved in the regulation of VG secretion, e.g. the lysosomal breakdown of VG.

The rise in specific activities of lysosomal enzymes coincided with a dramatic decline in VG concentration in the fat body (Fig. 5). Further analysis by video-enhanced fluorescent and electron microscopy demonstrated that the lysosomal activity is directed toward a specific degradation of VG-containing organelles (Raikhel, 1986a; 1986b). These studies pointed out two important physiological roles of lysosomes: (1) interruption of the VG secretion by swiching the secretory pathway to a degradative one, (2) degradation of the VG biosynthetic machinery and subsequent remodelling of trophocytes (Fig. 6). Experiments with ovariectomized vitellogenic mosquitoes suggested that a feedback response, to a high concentration of VG in the hemolymph, is the mechanism regulating this specific lysosomal activity in the fat body cells.

Thus, it appears that, in the mosquito fat body, two separate mechanisms are involved in regulation of the cessation of VG synthesis and secretion: one is responsible for turning off the expression of VG genes, and another, the lysosomal activity, for interrupting the VG secretion and organelle degradation.

THE SPECIFIC ACCUMULATION OF YOLK PROTEIN BY DEVELOPING OOCYTES

Hormone-mediated development of oocyte competence for protein uptake: The oocytes of newly-eclosed mosquitoes are not competent for endocytosis of proteins. Oocytes must be exposed to juvenile hormone during previtel-

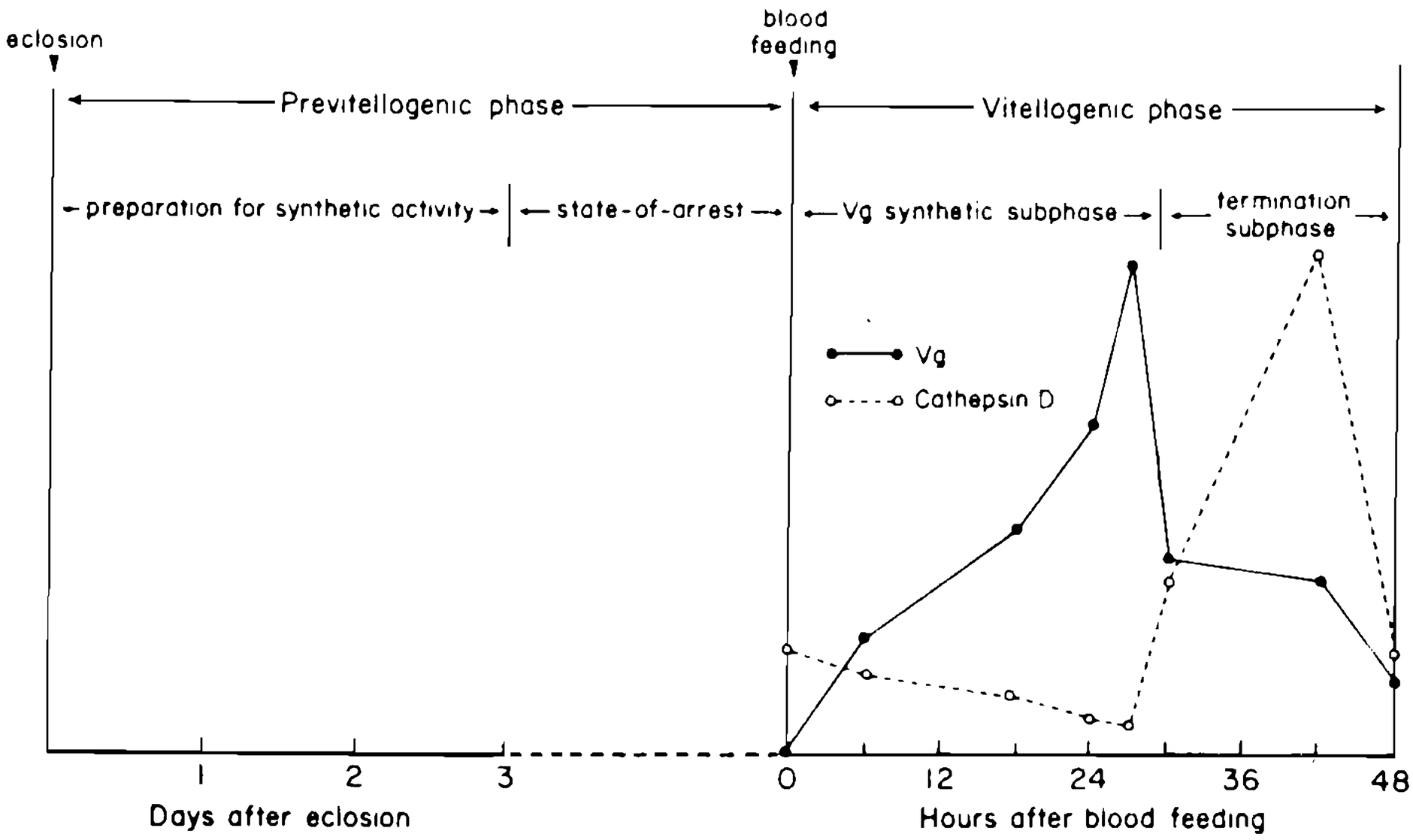

Fig. 5. The time sequence of events in trophocytes of the mosquito fat body during the first cycle of egg maturation. The previtellogenic phase begins at eclosion of the adult female mosquito; during this phase, the fat body becomes competent for subsequent vitellogenesis. The female then enters a state-of-arrest; VG is not synthesized during the previtellogenic phase or arrest. The arrest is terminated when the female takes vertebrate blood. The ingestion of blood stimulates a release of hormonal factors that results in VG synthesis. The termination subphase then follows. It is characterized by a rapid decline of VG synthesis and a sharp rise of the lysosomal activity.
Concentration of VG and specific activity of a lysosomal enzyme, cathepsin D, during the vitellogenic phase are shown (Raikhel, 1986b, with permission).

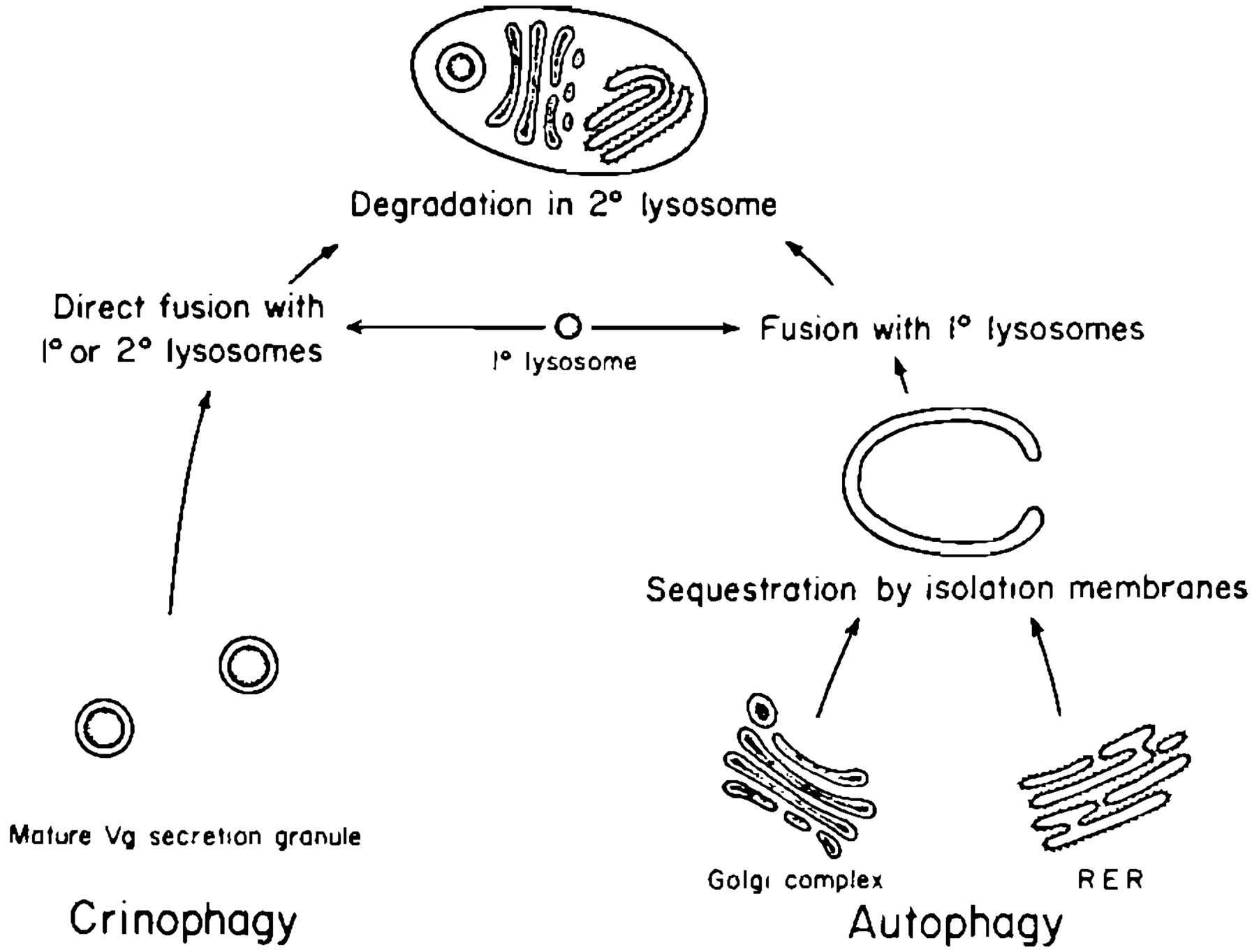

Fig. 6. The schematic representation of the organelle degradation during the termination of VG synthesis in the mosquito trophocyte. A massive and selective degradation of VG-containing Golgi complexes and secretion granules as well as rough endoplasmic reticulum by the lysosomal system. Crinophagy and autophagy are the forms of the lysosomal degradation of cellular organelles (Raikhel, 1986b, with permission).

logenic development in order to gain this competence (Gwardz & Spielman, 1973). We described the developmental events leading to oocyte competence to internalize proteins, and hormonal control of these events in the mosquito (Raikhel & Lea, 1985). The oocytes of newly eclosed females had an undifferentiated cortex. During previtellogenic development, numerous endocytotic organelles, coated vesicles and uncoated endosomes, were formed in the oocyte cortex. Only the oocytes containing these endocytotic organelles were competent for protein uptake. The formation of endocytotic organelles was controlled by juvenile hormone from the corpora allata. This developmental event was blocked by ablation of the corpora allata at eclosion, but it was restored by either implantation of this gland into allatectomized females, or the aplication of juvenile hormone (Fig. 7).

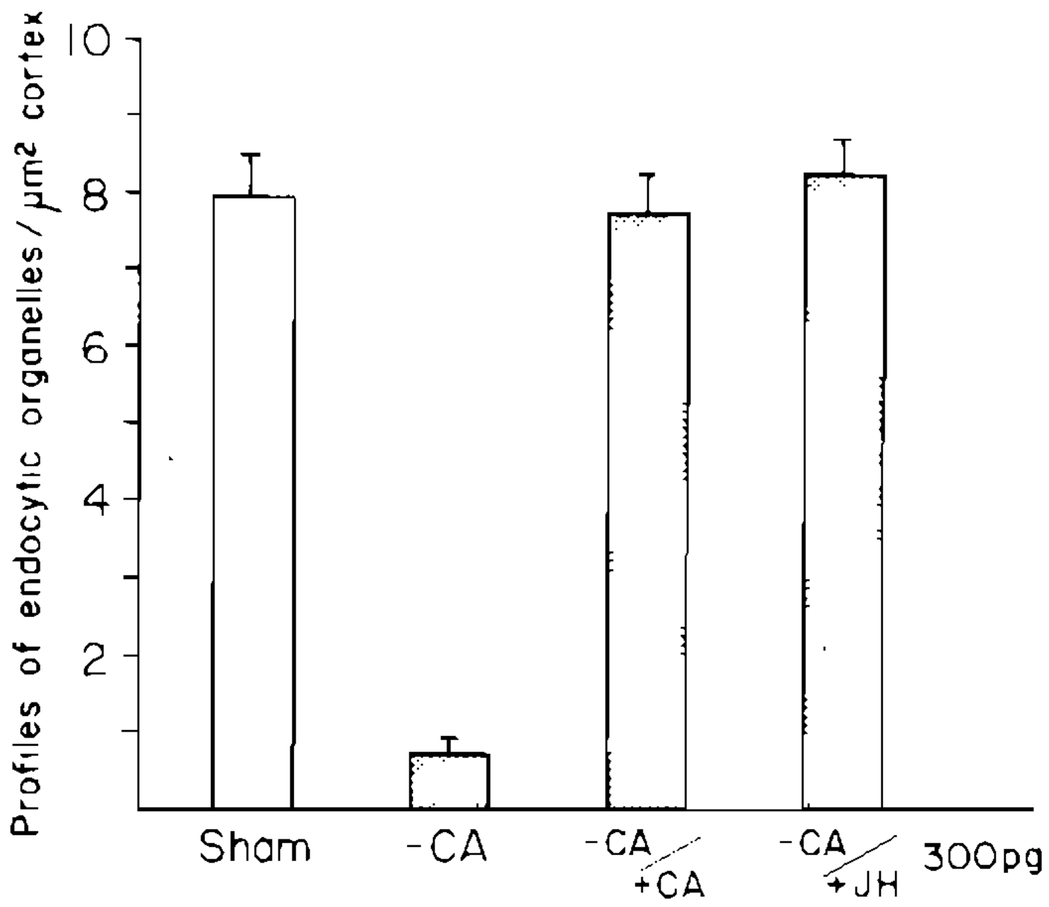

Fig. 7. Effect of the corpora allata and juvenile hormone III on the formation of endocytotic organelles in previtellogenic mosquito oocytes. SHAM and -CA – mosquitoes were sham-operated or allatectomized respectively within 1 hr after eclosion and examined 48 hr later; -CA/+CA – mosquitoes were allatectomized at eclosion, implanted with active corpora allata 48 hr later, and examined 48 hr after the implantation; -CA/+JH – allatectomized mosquitoes or abdomens isolated at eclosion were treated with 300 pg of JH III (three applications, 100 pg each). All ovaries were examined 48 hr after eclosion. Values expressed as means ± standard errors.

Selective endocytosis of yolk protein by mosquito oocytes: In order to determine the nature of the protein uptake in the mosquito oocyte, we perfomed *in vitro* uptake experiments utilizing iodinated YP, either VG from the fat body or VT from the ovary, and non-

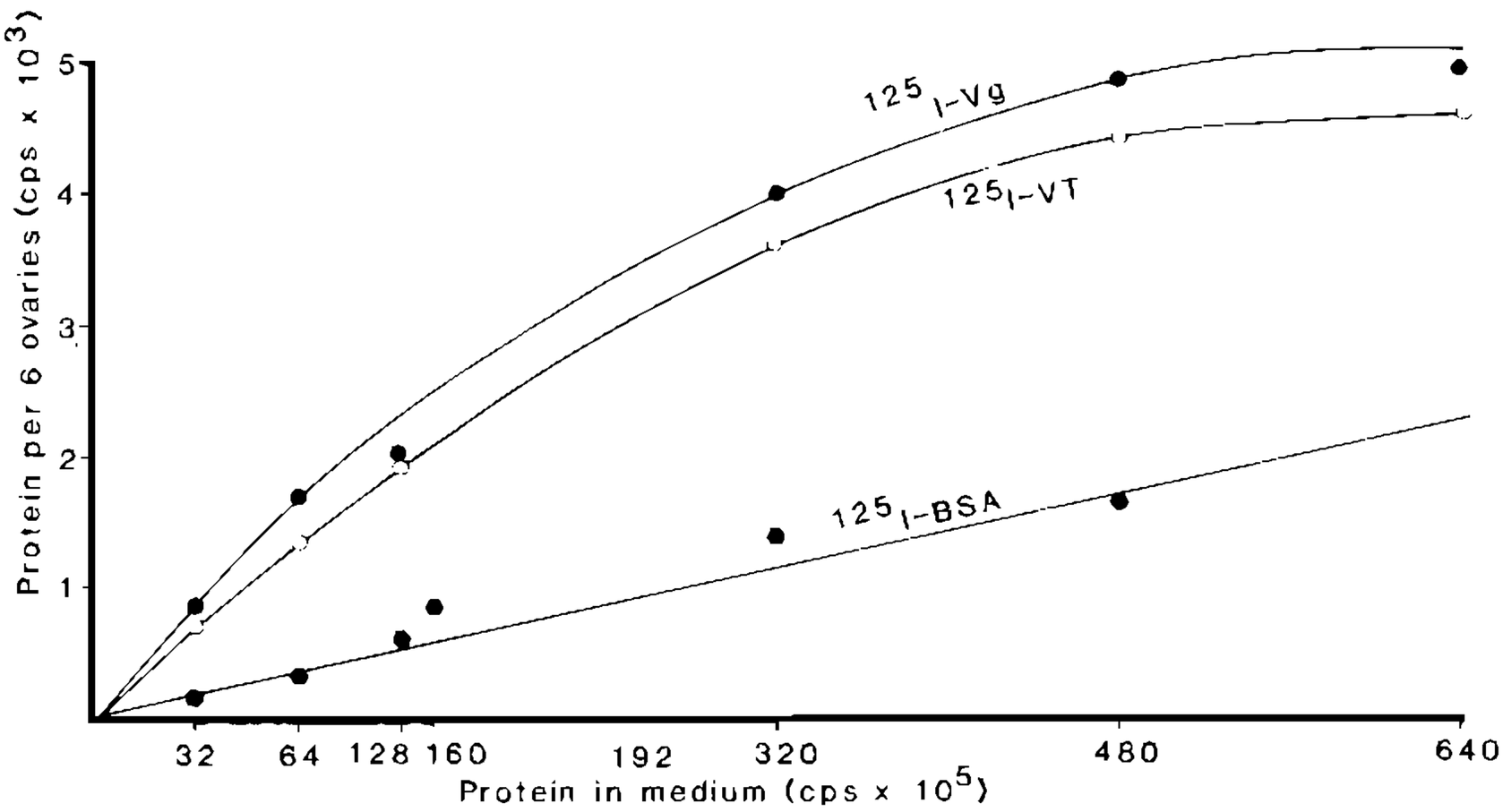

Fig. 8. Effect of increasing protein concentration in the medium on uptake rates. After incubation of ovaries in culture medium for 1 hr at 27°C, amount of accumulated protein was determined. Previtellogenic but competent, ovaries from 3-day-old mosquitoes were used for this experiment.

specific protein, bovine serum albumin (BSA). First, we used competent, but non-stimulated previtellogenic, oocytes, i.e. they had not been previously exposed to any of the factors, which could stimulate endocytosis. The uptake of yolk proteins, ^{125}I-VG and ^{125}I-VT, was saturable at high concentrations (Fig. 8). In contrast, uptake of ^{125}I-BSA was linear. This showed that both VG and VT were internalized by receptor-mediated endocytosis, while BSA by fluid-phase endocytosis. The uptake rates of VG and VT were not significantly different from each other. Therefore, recognition moieties of the YP molecules were not altered during their internalization and crystallization in the oocyte. Next, we used stimulated oocytes, 3 hr after blood feeding. Although the size of these oocytes increased insignificantly compared to competent previtellogenic oocytes, the uptake rate of iodinated VG and VT rose nearly three fold. This points out a stimulatory effect of factors, which are involved in initiation of vitellogenesis after a blood meal, on the rate of YP uptake.

Accumulative pathway of the yolk protein in the oocyte: Utilization of high-resolution immunocytochemistry gave important insight to the accumulative pathway of VG in the mosquito oocyte (Raikhel, 1984). The oocyte membrane is differentiated into microdomains; VG binds to receptors located at the base of and between the oocyte microvilli (Fig. 10). After binding to its receptors, VG is internalized by coated vesicles. The dissociation of VG from its receptors occurs in the next compartment, the endosome. Endosomes coalesced into a transitional body, a specific intermediate compartment, which plays an important role in VG processing in oocytes. In this compartment, VG undergoes condensation and begins to crystallize. The transitional yolk body transforms into the mature yolk body, which is a final compartment containing crystalline YP.

Factors regulating the routing of proteins in the oocyte: One of the central problems of the selective endocytosis is understanding the mechanism governing postendocytotic routing and sorting of macromolecules.

With a panel of monoclonal antibodies specific to different YP subunits, we investigated the routing of the YP subunits and their possible sorting in the oocyte. Double immunocytochemistry with subunit-specific mABs demonstrated that both YP subunits followed the same accumulative route in the oocyte where they were accumulated together in mature yolk bodies (Fig. 9).

We also studied factors determining the routing of different proteins in the mosquito oocyte (Raikhel & Lea, 1986). This study has shown that proteins internalized by the oocyte follow either a specific accumulative route or a lysosomal degradative route (Fig. 10). Via coated vesicles, all proteins enter the same compartment, the endosome, where they dissociate from membrane-binding sites. The route to

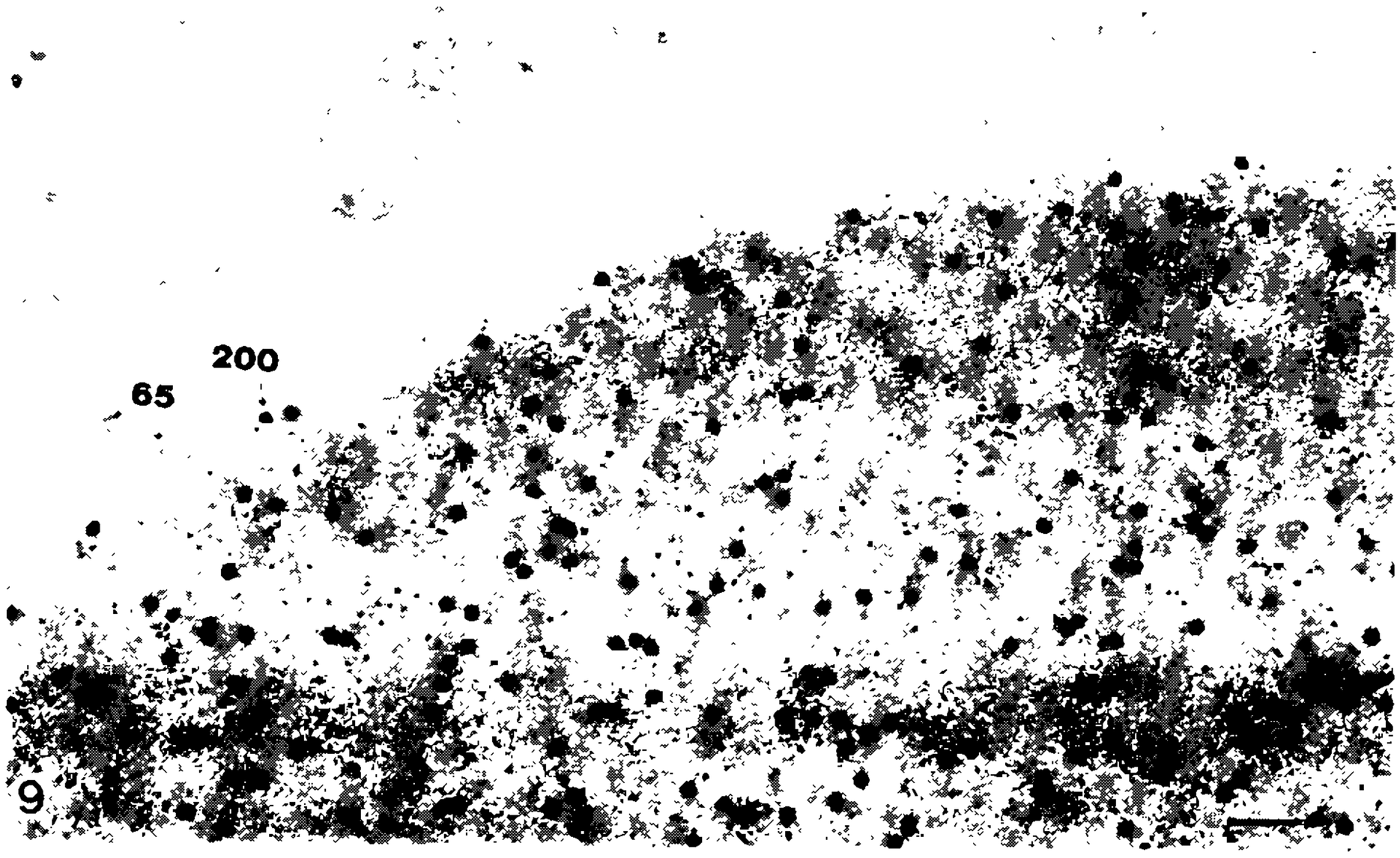

Fig. 9 Double immunocytochemistry with subunit-specific mABs (as in Fig. 4) and colloidal gold of different sizes. Colocalization of the YP subunits in the mature yolk body of the mosquito oocyte; 5 nm gold marks a small YP subunit (65), 15 nm gold a large YP subunit (200). 110,000 X, bar is 0.1 μm.

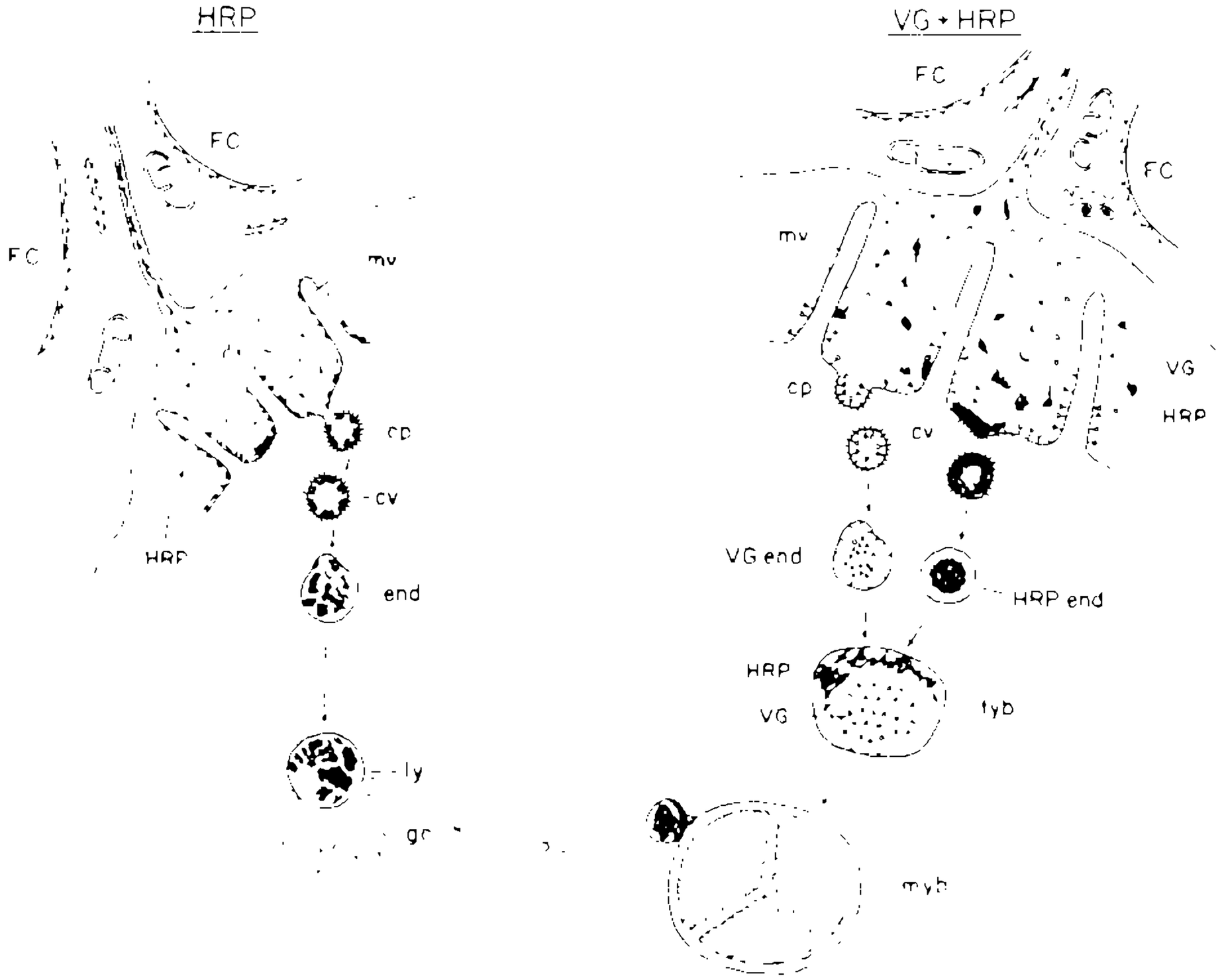

Fig. 10 A schematic representation of the internalization routes of non specific and specific proteins in the mosquito oocyte. cp, coated pit; cv, coated vesicle; end, endosome; gc, Golgi complex; FC, follicle cell; HRP, horseradish peroxidase; ly, lysosome; mv, microvillus; myb, mature yolk body; tyb, transitional yolk body; VG, vitellogenin (Raikhel & Lea, 1986, with permission).

their final destination depends on the presence of the specific ligand, the yolk protein. In its absence, the degradative route is followed and the endosome with non-specific protein fuses with lysosomes. In the presence of the specific ligand, the accumulative route is followed, and both proteins are first delivered into an accumulative compartment, the transitional yolk body. During the transformation of a transitional yolk body into the final storage compartment, a mature yolk body, YP undergoes crystallization, whereas non-specific protein is concentrated in small vesicular extentions of the compartment membrane. These vesicles are separated from the yolk bodies and, apparently, deliver the nonspecific protein into the lysosomal system. We concluded that any protein bound to the membrane would be internalized by the oocyte, but only binding of VG to its receptor serves as a transmembrane signal stimulating the formation of accumulative compartments.

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