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PROTEIN TRANSPORT: A SELECTIVE MEMBRANE MECHANISM

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Proteins are selectively sequestered by a number of cell types. However, only in oocytes is the process sufficiently aggravated and specific to be readily studied. In these cells certain serum proteins are taken up in proportions different from those found in the serum.

In vitro incubations of hormonally stimulated and synchronous mosquito oocytes show that the only protein capable of initiating the transport process is the female specific yolk protein. Heterologous proteins such as IgG, bovine serum albumin, cytochrome C, and ferritin are inactive. The female specific protein is a phosphoglycolipoprotein. It is synthesized in the fat body, a liver analog in the insect, and passed into the serum before being transported into the oocytes. Preliminary kinetic analysis shows the uptake process to be specific with an apparent K_m of about 10^{-7} M. Glycolytic inhibitors stop protein uptake.

The receptor-mediated binding steps in the transport process are most easily studied in the chicken because of the enormous amount of oocyte membrane available from a given oocyte and because up to 1 gm of protein is normally transported per day per oocyte. IgG and the hen specific phosvitin lipovitellin are two of the physiologically important proteins that are transported intact into the chicken oocytes. The uptake appears selective as shown by studies with iodinated proteins. Ferritin conjugated to IgG is shown by electron microscopy to bind to isolated plasma membranes only where coated pits have formed, whereas ferritin alone is not seen localized on any membrane surface. These very specialized regions of the membrane are similar to micropinocytotic pits but, in addition, possess on their cytoplasmic side dense ridges that form the coat. Transport involves binding to the coated pits, the pinching off of the pits, and the subsequent movement of the coated vesicles in the cytoplasm.

INTRODUCTION

Selective protein transport is associated with specialized differentiations of the plasma membrane. These invaginations or coated pits appear to have a diffuse layer on the extracellular side of the plasma membrane and a short bristle-like coat on the cytoplasmic face. They have been described in a number of different cell types that are postulated to be actively involved in protein transport: e.g., erythroblasts taking up ferritin for hemoglobin synthesis (1), Kupffer cells lining the hepatic sinusoids of the

liver (2), the developing mosquito oocyte which is taking up yolk protein (3, 4), nerve endings (5, 6), rabbit oocytes (7), and rat vas deferens (8). The development of coated pits appears to be stimulated by the presence of the particular protein transported.

The transport of maternal protein to the developing oocyte and embryo has a profound impact on the young; without it death is certain. There are at least six different cases where protein transport may play a critical role in the development and maintenance of the mammalian oocyte, embryo, and neonate: transport of protein (1) into the developing oocyte within the ovary; (2) into the fertilized egg from the oviductual epithelium before and during early stages of cleavage; (3) across the yolk sac immediately after implantation; (4) transplacentally; (5) across the mammary epithelium; (6) and across the neonatal gut.

Oocytes of higher animals provide stores of protein in the form of proteinaceous yolk as a later source of amino acids for embryonic development. This is most obviously true in insects (9), fish (10), amphibia (11), and birds (12), all of which sequester large amounts of proteinaceous yolk of maternal origin in their oocytes. Mammals, perhaps because of the embryo's intimate connection with maternal circulation, store less maternally derived protein in their oocytes. The oocytes of mouse, rat, deer mouse, and hamster contain fibrous material that is thought to be protein yolk (13). This material may not be contained within membrane-bounded vesicles. However, several species, including rabbit, pig tail monkey, ferret, and sheep, accumulate large bounded vesicles filled with flocculent materal thought to be protein within their oocytes.

By the use of horseradish peroxidase, Anderson (14) has demonstrated that rabbit oocytes, which are generously supplied with coated pits, do transplant protein. Glass has found that mouse oocytes will transfer protein from serum, the rate determined by the development stage of the oocyte (15), the source of the protein (16), and the particular type of protein (17). Although both bovine globulin and albumin reached the zona pellucida, only the albumin was transported into the developing oocyte, demonstrating selectivity of the system.

Heterologous antigens injected into the serum prior to ovulation can be demonstrated in the oocyte at the time of ovulation, but disappear within 2–3 hr after ovulation (18). Glass has suggested that this disappearance may be due to metabolic breakdown of the protein stores within the oocyte.

In many mammalian systems the yolk sac represents the primary route of protein transport immediately after implantation. Only during the second half of in utero development is the transplacental route important in transport of macromolecules (19). The yolk sac splanchnopleure appears to be specialized for transmission of macromolecules by pinocytosis (20). The endodermal cells are covered with microvilli, with many coated pits and pinocytosing coated vesicles at the base of the projections (21). Studies of protein transport with radiolabeled serum proteins in the rabbit have shown that human serum proteins are adsorbed better than guinea pig proteins and that albumin is adsorbed better than globulin (19, 22). The site of selection for protein transport across the yolk sac is unclear. IgG is transported from maternal to fetal circulation while ferritin is not. Localization of ^{125}I-IgG and ferritin in the rabbit yolk sac endoderm, by use of autoradiography and electron microscopy, demonstrated that both proteins are found within the same micropinocytotic vesicles (23). Brambell (19) has suggested that the site of selection is intracellular. He has proposed that specific binding sites for transported protein protect that protein from intracellular lysosomal digestion, while protein which is not protected by binding to specific sites on the membrane is digested and therefore not transported.

In primates the transport of protein into the developing embryo is primarily via the placenta (24). Electron microscopic examination of the syncytial trophoblast reveals adaptations for pinocytotic transport very similar to the rabbit yolk sac endothelial cells (19). In humans and other primates the placental route is the most important one for the acquisition of passive immunity by the young. Interestingly, antibody titers to measles, rubella, and poliomyelitis are the same in premature as in full-term newborn and serum (25). The placenta, like the yolk sac splanchnopleure appears to be capable of selectively transporting protein intact from maternal to fetal circulation (26, 29).

Another route, through the mammary and the neonatal gut, appears to be primary among the ungulates (19). In the mouse, the mammary has been shown to transport proteins with the following selectivity: human IgA > human IgG > mouse IgG; mammary epithelium did not transport human albumin or IgG light chains (27, 28). The selectivity of the mammary is quite different from that of the placenta. In those animals where transfer of antibody takes place across the neonatal gut, the gut is in some ways morphologically similar to the rabbit yolk sac splanchnopleure and the human syncytial trophoblast, and appears active in pinocytotic transport. Neonatal gut is physiologically quite different from the adult (29), and is capable of selectively transporting proteins from the lumen (30, 31).

Vitellogenesis

It seems probable that the mechanism of selective protein transport has many common elements in all the cases discussed above. Furthermore, it seems probable that the mechanism of selective protein transport by the developing oocyte is similar throughout the animal kingdom. This process is exaggerated in certain groups that store large amounts of yolk protein, but uptake of protein by developing oocytes has been found in all animals which have been examined so far.

Uptake of yolk protein by the developing oocyte is termed vitellogenesis. Studies of this process in chickens has shown that estrogen specifically induces the liver to synthesize three plasma proteins, phosvitin, lipovitellin, and a riboflavin-carrying protein (12, 32). These proteins, as well as other normal plasma proteins, appear to be sequestered by the developing oocyte whose surface is adapted for selective protein uptake (33). Sequestration appears to be dependent upon the size of the oocyte as well as the particular type of protein. Cutting and Roth have found that when the oocyte reaches 200 mg, there is a sharp rise in its ability to specifically sequester IgG and PvLv and that uptake is selective (34, 35).

In amphibians estrogen induces the production of the yolk protein precursor, a lipophosphoprotein. The vitellogenin is very quickly cleared from the plasma in females, but has a half-life of 40 days in the male. Comparison of the uptake of vitellogenin with serum proteins labeled with ^{3}H demonstrates that the ovary selectively sequesters vitellogenin (36, 37). It was found that oocytes from vitellogenic females take up vitellogenin from the culture medium 15 times more rapidly than oocytes from non-vitellogenic animals (38).

Insects deposit large amounts of yolk in their developing oocytes. Telfer has shown that female specific proteins are removed quite specifically from the blood by the developing oocyte (39). Electron microscope examination shows that the oocytes are covered with micropinocytotic coated pits and vesicles (40, 41, 42, 43). The site of synthesis of the female specific or vitellogenic protein appears to be the fat body of the insect (44, 45). The production of vitellogenic protein is under hormonal control, as it is in both chicken and amphibian as discussed above.

The Vitellogenic Mosquito and Chicken

Selective protein transport via micropinocytic coated pits and coated vesicles appears to be similar through the animal kingdom, from mammals down to insects. Among those animals that store large amounts of proteinaceous yolk, protein transport in the developing oocyte is exaggerated, and therefore offers a convenient system in which to study the mechanism of selective protein sequestration.

The vitellogenic mosquito and chicken are particularly well suited for study of the mechanism of selective protein transport. Adult female mosquitoes contain two ovaries, each with 50 ovarioles. Each ovariole consists of a germarium, which produces oocytes and nurse cells, and a vitellarium in which the oocyte develops. The vitellarium contains one very immature follicle and one follicle in the process of maturation. Within each follicle one oocyte and seven nurse cells are surrounded by a single layer of follicle cells. Maturing follicles undergo two distinct periods of development. At the end of the first stage, which is initiated by juvenile hormone (46), the follicle enters a resting state. At this time the oocyte contains little or no proteinaceous yolk, there are no microvilli at the oocyte-follicle cell interface and very few coated pits are evident on the oolemma. The second stage of development, vitellogenesis, is initiated when the insect takes a blood meal. This developmental sequence is under complex hormonal control. The stimulus of the distended abdomen causes the release of a hormone from the brain, and with 4–8 hr a second hormone initiates the synthesis of yolk protein by the fat body of the mosquito (47). Yolk protein in the hemolymph stimulates an increase in the number of coated pits and all the oocytes in maturing follicles begin to sequester yolk protein in micropinocytic vesicles (40, 41). Thus, vitellogenesis is simultaneous for the 100 oocytes within each mosquito and can be so for a whole population of mosquitoes if they are blood fed within a short space of time. It is also possible to initiate vitellogenesis at will by giving the mosquitoes a meal of blood. The most active period of protein sequestration is 18–22 hr after the blood meal. Ovaries excised from mosquitoes 18 hr post-blood meal and incubated in vitro with ^{125}I-labeled yolk protein will continue to incorporate that protein for several hours. In vitro culture of the mosquito ovary has demonstrated that the transport of yolk protein from the fluid surrounding the oocytes is selective and is not accompanied by degradation of the yolk protein (4, 48, 49).

Hen oocytes share a morphological similarity with the mosquito oocyte but differ in being very much larger. It is this difference which makes them an ideal source of oocyte plasma membranes for binding studies and a possible source of receptors for the proteins being transported. Also, oocytes exist in a developmentally different continuum and thus permit a full spectrum of oocytes to be examined in a single ovary for their ability to sequester protein (34, 35).

By using the mosquito ovary to examine in vitro specific protein transport, reproducibility is enhanced by having the 50 synchronous oocytes in each ovary. On the other hand, hen oocytes provide the needed amounts of membrane for in vitro binding studies. The ubiquitous nature of the mechanism permits the results from both to have general significance.

METHODS

Mosquito Rearing and Organ Culture

Culex fatigans was reared by standard methods at 22–25°C (50). Adult females 1–2 days postemergence provided ovaries for culture that were previtellogenic; those 5

days old had developmentally mature oocytes, but unless they had received a meal of blood, they, too, were previtellogenic. Culture of the ovaries was in Grace's insect tissue culture medium (Gibco) or Drosophila Ringer's (51) at 25°C. Usually 5–10 ovaries were incubated simultaneously in a 50–110 μl drop in a siliconzied microscope depression slide placed in a high humidity chamber. Proteins were added as required by the particular experiment.

Chicken Rearing and Preparation of the Oocyte Plasma Membrane

Hens were obtained live from the local slaughter house and maintained for 1–15 weeks to monitor egg production. Only laying hens were sacrificed and the developmentally maturing follicles removed. Oocytes were drained of yolk and the oocyte plasma membranes dissected free from the overlying connective tissue and follicular epithelium under a dissecting microscope. The membranes were dissected, washed to remove adhering yolk, and minced in either Earle's Balanced Salts (Gibco) or Dulbecco's Minimal Salts (Gibco).

Isolation of Mosquito Yolk Protein

Rafts of 50–100 eggs were collected and frozen within 2 hr of being laid. 100–500 rafts were briefly homogenized in 0.3 M sucrose, 0.01 M Tris, pH 8, at 0°C. Low speed centrifugation sedimented the chorion, vitelline membranes, and yolk granules; other cytoplasmic material remained suspended. Successive extractions in 0.2 M and 0.1 M sucrose successfully removed contaminating organelles. Extraction of the pellet with 0.5 M NaCl, 0.01 M Tris, pH 8, at 0°C for 2–12 hr gave a preparation of yolk protein used in many of these experiments. Left in the pellet were the chorion and the vitelline membranes. A further purification by column chromatography and characterization on a variety of acrylamide gels was completed (52).

Isolation of Chicken IgG

Serum IgG was purified by two 33% saturated ammonium sulfate precipitations followed by chromatography on Biogel A 1.5 or P-300. Egg IgG was prepared by the method of Mok and Common (53) with subsequent sizing on Biogel A 1.5.

Iodination of Proteins

Iodination was either by the chloramine T method, essentially that described by Hunter and Greenwood (54), or by the lactoperoxidase method (55). Unbound radioactive iodine (New England Nuclear, ^{125}I or ^{131}I, carrier free) was separated from the protein by column chromatography and/or dialysis.

Electron Microscopy

Samples to be examined with the aid of the electron microscope were fixed in 0.5–2% glutaraldehyde, 0.1 M PO_4, pH 6.8, at 0°C for 1–8 hr, washed in buffer, and postfixed in 2% O_sO_4 for 1–3 hr. Excess O_sO_4 was washed free in four changes of tap water. After dehydration in a graded series of alcohols, the tissue was passed through propylene oxide and into EPON/NMA (1:1), followed by polymerization at 60°C. Sections were stained with uranyl Mg acetate and lead citrate.

Binding Studies

Hen oocyte plasma membranes from three to six oocytes (0.4–5 gm) were incubated with IgG or PvLv in 1 ml at 37°C at the concentrations indicated in the figures. In IgG-binding experiments, the diced membranes were separated from the reaction mixture by

centrifugation. Membranes were then washed by resuspension in one or more 10-vol excesses of buffer and then counted in a deep-well gamma counter after centrifugation and removal of the supernatant. Most recently, filtration has been used to recover and wash the membranes (56). 1 mM KI was present in all media to compete for free ^{125}I binding.

Time dependence of binding was assayed by incubating oocyte membranes as indicated above in the presence of ^{125}I-IgG. At 30 min intervals the membranes were chilled to 0°C, spun free from the incubation mixture, washed, counted, and then resuspended in the original reaction mixture at 37°C.

Competition for binding was done by adding to separate test tubes increasing amounts of unlabeled IgG in the presence of a constant amount of labeled IgG and membranes. Incubation was in 1 ml for 6 hr at 37°C in the presence of 0.5% bovine serum albumin (BSA) to reduce nonspecific binding of labeled IgG. Membranes were briefly washed and counted as before.

PvLv competition for iodinated IgG was done as above at final concentrations between 40 and 200 μg/ml in reaction volumes of 1 ml at 37°C. No competition by PvLv for iodinated IgG binding was found.

Ferritin-IgG Conjugation Procedure

Conjugates were prepared with recrystallized Pentex ferritin according to the procedures of Shick and Singer (57) with toluene 2-4 di-isocyanate. Purification of the conjugate was by chromatography on Biogel A 1.5 in 0.01 M phosphate-buffered normal saline, pH 7.4.

Inhibition of Uptake

Metabolic inhibitors at a variety of concentrations were incubated with ovaries in the presence of iodinated yolk protein. At the end of 3.5 hr, the ovaries were washed in Drosophila isotonic Ringer's (51) to remove loosely bound labeled protein. In the experiment reported here, the lowest effective concentration that produced inhibition of uptake was used. The methodology as well as the effect of these inhibitors on the metabolism of ^{14}C-glucose is reported elsewhere (58).

Kinetics

A concentration dependent uptake in the mosquito was shown by incubating sets of 10 ovaries in the presence of various concentrations of ^{125}I-labeled yolk protein. At the end of the experiment the ovaries were washed in four successive I-ml vol of isotonic Ringer's, each for 15 min, followed by centrifugation. Sequestered labeled protein was assayed by gammaspectrometry.

RESULTS

Morphological Correlates

Oocytes manifest the most aggravated instances of protein sequestration known. However, the process is under strict developmental and hormonal control. In order to determine what morphological alterations might be correlated with protein transport, oocytes before and during the periods of active yolk sequestration were examined in the electron microscope.

In Fig. 1, the ovary of a mosquito is illustrated in which there is an obvious lack of yolk granules which characterize the vitellogenic ovary. When viewed at higher magnification, the overlying follicular epithelia in both the quiescent and vitellogenically active

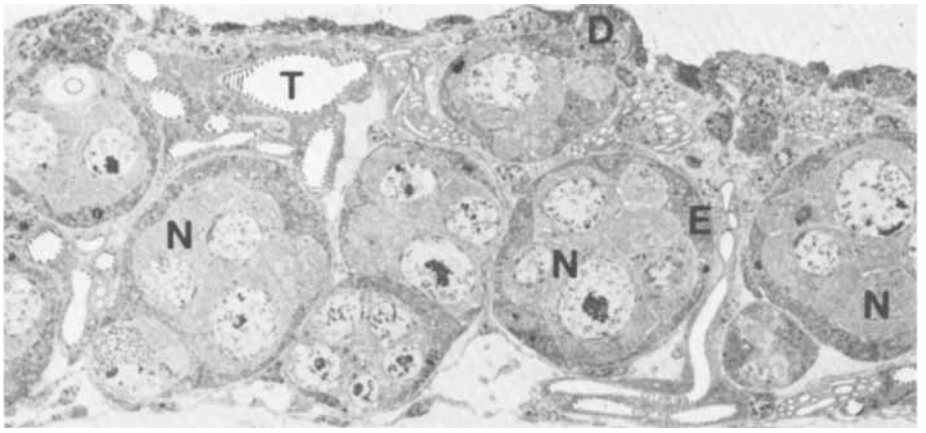

Fig. 1. A portion of a late pupal mosquito ovary. Formation of the morphologically identifiable sites associated with specific protein transport is developmentally controlled. The ovary is comprised of up to 50 ovarioles, each seen here as roughly round groups of cells. The conspicuous follicle of each ovariole contains one prominent oocyte and seven equally large nurse cells (N) encircled by a single squamous follicular epithelium of much smaller cells (E). Tracheae (T) and a portion of the oviduct (D) are visible. × 800.

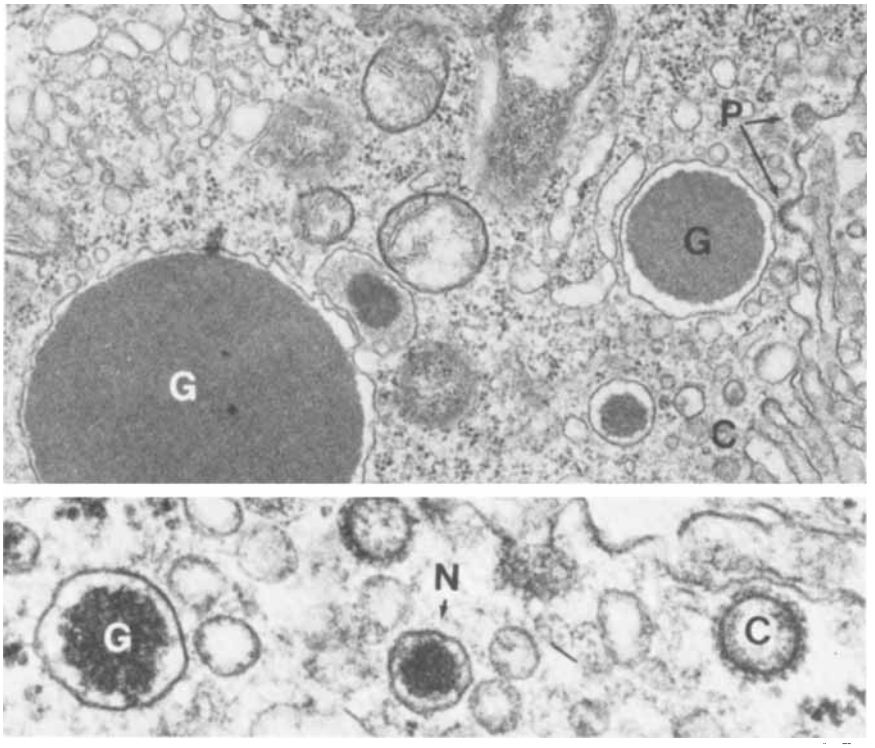

Fig. 2. Vitellogenic mosquito oocyte. The oocyte plasma membrane (M) is highly convoluted. Coated pits (P) at the oocyte surface give rise, in turn, to the coated vesicles (C), naked vesicles (N), and nascent yolk granules (G). × 29,700. In the inset, the structure of the coated vesicles with the adherent ridges on the cytoplasmic side and the included transported protein adherent to the vesicle membrane contrasts with the naked vesicles where the cytoplasmic coat is lost and the adherent yolk is freed from the membrane to the center of the vesicle. × 99,000.

ovaries are essentially identical. It is the surface of the oocytes that differs so dramatically one from the other in these stages. Note in particular the abundant yolk granules in varying stages of maturation which characterize the oocyte that is rapidly sequestering protein (Fig. 2).

When the plasma membrane of this oocyte is compared with that of the quiescent, the prevalent micropinocytotic coated pits are immediately apparent. It is this one difference that consistently correlates with the process of active protein uptake. Note, in the high magnification inset of Fig. 2, the particular structure of the coated vesicle.

This derivative of the coated pit lies in the cortex and, like the coated pit, it has on the cytoplasmic side of its enveloping membrane the array of characteristic ridges. In negative-stained preparations, such coated vesicles are arrayed with pentagonal arranged ridges interspersed with hexagons much like the pattern seen on a soccer ball (59). Note also the adherent layer of material on the inner side of the coated vesicle. This is thought to be the yolk protein.

With the loss of the cytoplasmic coat from the coated vesicles, the layer of adherent protein on the inner side soon becomes loosened from the membrane and freed to the center of the now naked vesicle (Fig. 2, inset). The fate of these naked vesicles is that of continued fusion with yet larger protein-containing vesicles and eventual coalescence into the paracrystalline arrays of protein that characterize the mosquito yolk.

A similar but even more dramatic comparison is evident when the surface of the chicken oocyte is compared before and during the period of active protein sequestration (Figs. 3 and 4). In both periods the oocyte is enveloped successively by an extracellular vitelline layer, the follicular epithelium, the basement layer, and then the thick and mechanically strong envelope of connective tissue in which the blood sinuses lie. As was the case with the mosquito, the chicken oocyte plasma membrane is relatively free of coated pits before yolk deposition but highly studded after uptake has commenced. As an adaptation to move the vast quantities of protein being sequestered, the cortex also is thrown into deep folds which increase the transport surface several hundredfold. Of particular note is the almost continuous array of coated pit-like material along these deep convolutions covering the cytoplasmic side of the oocyte plasma membrane (Fig. 3). The presence of this material in areas other than that just associated with the coated pits suggests that the formation of the pits is proceeding at a high rate with the nascent pits forming everywhere along the plasma membrane. Such a postulate is supported by the fact that more than 1 gm of protein is being moved from the serum into each oocyte per day. Subsequent steps in the process of protein uptake are essentially similar to those noted for the mosquito in that they involve a pinching off of the coated pits to form coated vesicles which, in turn, lose the coat, thus producing naked vesicles which fuse to produce large membrane-bound yolk vesicles. A diagram of our present understanding of the process is shown in Fig. 5.

In Vitro Uptake

To determine whether or not the uptake of protein yolk can be carried out in culture, mosquito ovaries were incubated in vitro with iodinated yolk protein. Both developmentally immature and transport-stimulated ovaries were used. Immature ovaries were obtained from 1–2-day old mosquitoes which had not received a meal of blood, whereas the transport-competent ovaries were removed from older animals which had received a meal of blood 18 hr earlier. Mosquitoes, after about 3 days, become developmentally competent to take a meal of blood which in turn triggers the synthesis of protein yolk by the fat body. Eighteen hr post-blood meal ovaries have already sequestered about one-third of the final complement of protein.

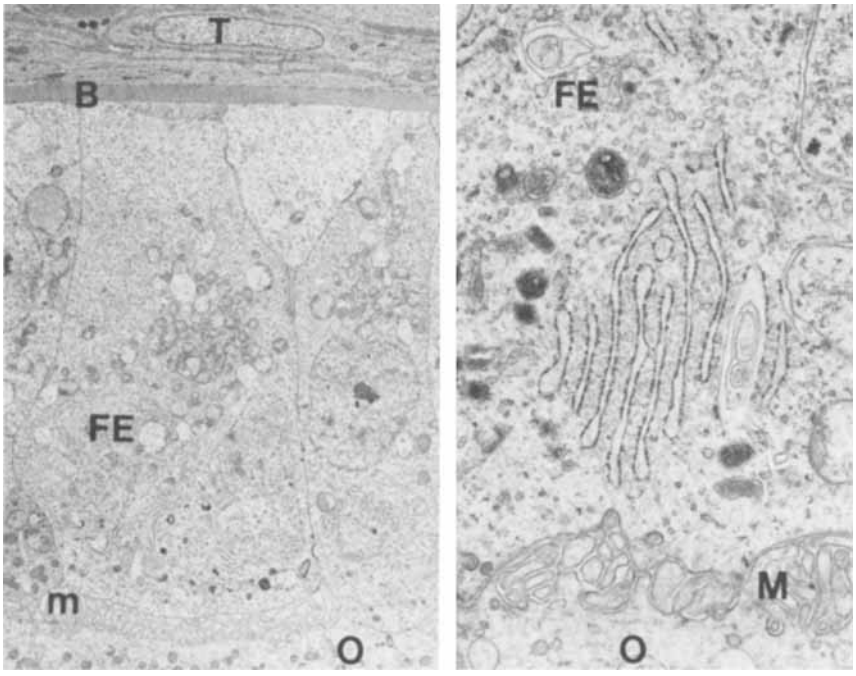

Fig. 3. Chicken oocytes weighing less than 0.2 gm are not highly differentiated for rapid protein uptake from the serum. In such an oocyte, as shown here, as well as in those heavier, a thick sheath of connective tissue (T) containing blood sinuses invests the oocyte (O) and the overlying cuboidal follicular epithelium (FE). A dense fibrous basement layer (B) separates the connective tissue from the FE. × 4,000. Chicken oocytes prior to the period of yolk protein transport, such as in the 1 mm oocyte imaged in this inset, exhibit few if any coated pits or vesicles on the oocyte plasma membrane (M) or in the cortex (C). × 14,600.

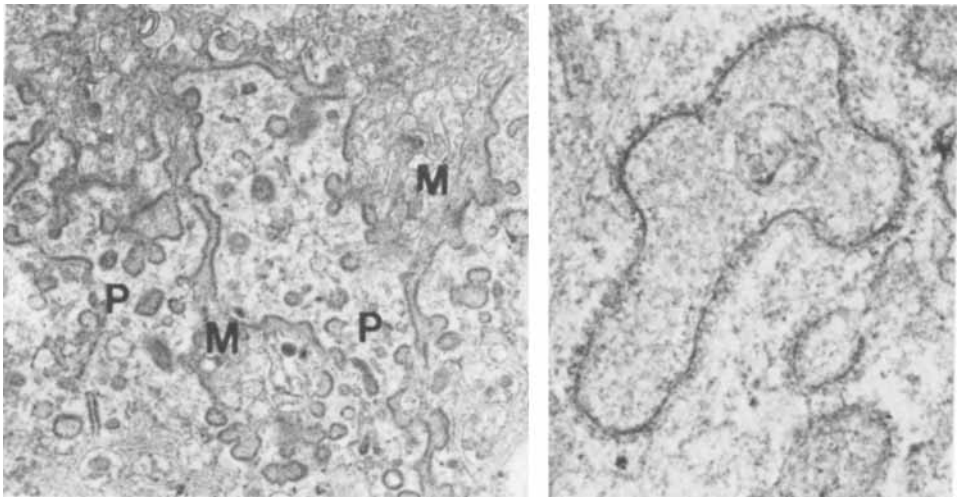

Fig. 4. Once yolk transport has become aggravated (> 0.2 gm), the correlated morphological image is remarkably different from that in Fig. 3. Note the deep folding of the oocyte plasma membrane (M). Especially evident are the numerous coated pits (P) budding into the cytoplasm (C). In fact, the whole of the plasma membrane is coated. × 14,800. Inset. At higher magnification this coating is indistinguishable from that on the pits. × 102,600.

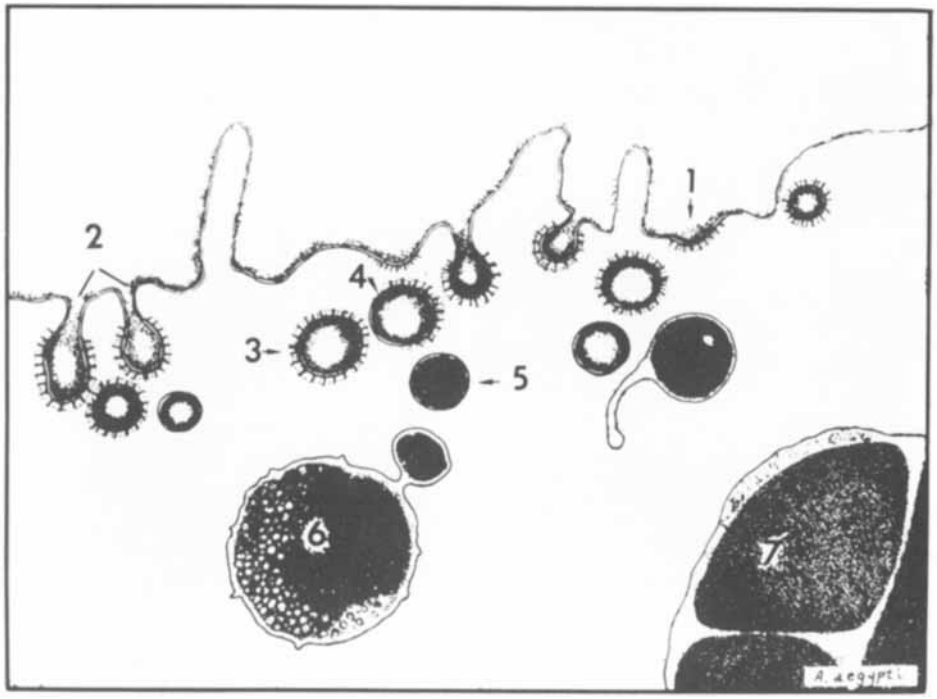

Fig. 5. This drawing depicts the formation of the coated pits as depressions in the plasma membrane (1) marked by the cytoplasmic coat, followed by the invagination and pinching off of the pits (2) as coated vesicles (3). Release of the cytoplasmic coat gives rise to transition vesicles (4) which in turn become naked (5) with attendant release of the membrane bound yolk within the vesicles. Subsequent fusion of naked vesicles give rise to nascent yolk granules (6) and in turn the mature yolk (7).

When cultured in the presence of the yolk protein isolated from mature eggs, uptake is approximately linear for at least 4 hr (Fig. 8). In the mosquito the protein that constitutes more than 90% of the yolk is a phosphoglycolipoprotein with a molecular weight of about 420,000 daltons on the basis of observations by agarose gel chromatography. In SDS it dissociates into two subunits. One is a 200,000 mw glycoprotein and the other a 100,000 mw phosphoglycoprotein (52). Note in Fig. 8 that the control or nonvitellogenic oocytes sequester or entrap little protein compared with the uptake manifested by the stimulated oocytes.

Specificity

It having been determined that transport of yolk protein was sustained for at least 4 hr in vitro, it was then possible to determine whether or not the process exhibited any specificity. To this end, stimulated ovaries were incubated in separate cultures for 4 hr with a constant amount of iodinated yolk protein and increasing concentrations of bovine serum albumin. As can be seen in Fig. 6, little effect upon the rate of yolk protein transport is seen. If BSA competed for the specific binding or uptake of yolk protein, a marked concentration-dependent decrease in iodinated protein would have been expected. Thus, under the conditions of our in vitro culture, BSA does not effectively compete for transport. Preliminary experiments with gamma globulin, cytochrome C, phosvitin, and transferin also show little if any competition. However, unlabeled yolk protein and trypan blue, a known inhibitor of uptake (60), both compete on an equimolar basis.

Kinetics

Since competition data indicated the process in vitro involved specific transport, it seemed reasonable to determine if the uptake was concentration dependent and if so, what kinetic parameters obtained. With increasing concentrations of yolk protein, the amount of iodinated yolk sequestered over 4 hr at each concentration was graphed as a double reciprocal plot (Fig. 7). The results of this study indicate that uptake of crude yolk protein has an apparent K_m of 10^{-7} M. It is important to remember that these particular experiments were not done with highly purified yolk protein. Due to contaminating molecules, the protein concentration may be overestimated by a factor of 2–5. Assuming equal efficiency in iodination, this would mean the concentration should be reduced and the K_m lowered to about 5×10^{-8} M. A more careful kinetic analysis using the highly purified phosphoglycolipoprotein is in progress.

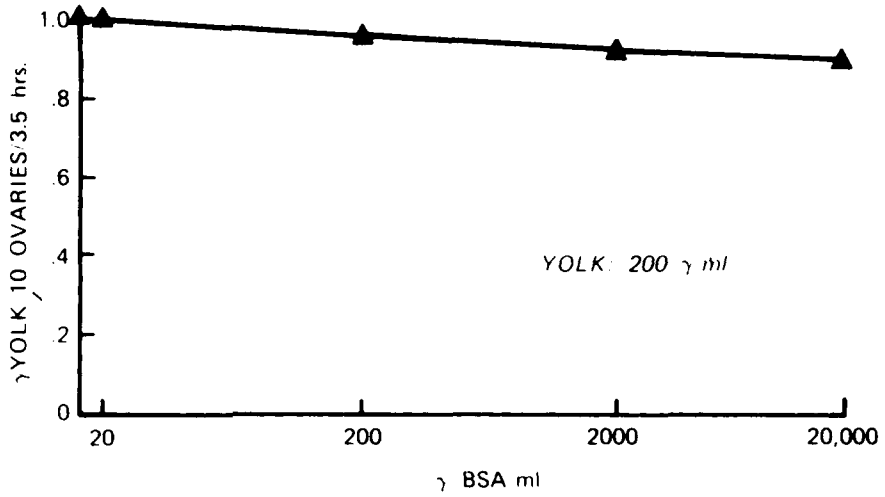

Fig. 6. Competition for uptake. To determine if the process of uptake was specific, transport competent mosquito ovaries were incubated with increasing concentrations of bovine serum albumin (BSA) in the presence of a constant amount (200 μg) of yolk protein. Little change in the rate of uptake of iodinated yolk protein was noted during the 4-hr time course of this incubation.

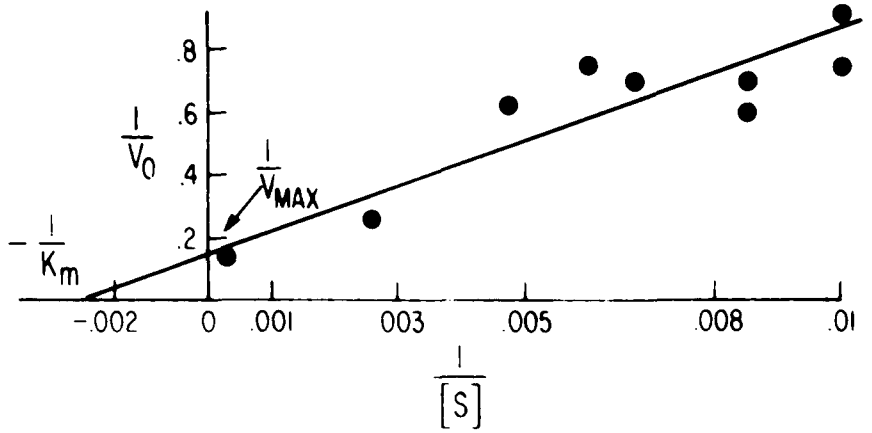

Fig. 7. A double reciprocal plot of the uptake of yolk protein by in vitro cultured mosquito ovaries. Transport during a series of 4-hr incubations gives a K_m of less than 10^{-7} M. (S = μg/ml).

Inhibition of Transport

To be better able to differentiate from one another the various steps in the transport process, a way was looked for to divest uptake from binding. Among the several methods attempted, inhibition of metabolic energy sources proved to be most reproducible (61). A variety of inhibitors were used, including indoacetic acid, fluoride, 2-deoxyglucose, cyanide, azide, antimycin A, and fluorocitrate. As summarized in Fig. 8, only inhibitors of glycolysis were effective in blocking protein sequestration. A more comprehensive examination of the inhibition of protein transport by metabolic inhibitors is reported elsewhere (58).

Binding Data

By incubating ovaries in the presence of either NaF (10^{-2} M), or 2-deoxyglucose (20 mg/ml) transport is stopped. Under such inhibition, it was possible to assay for binding of the iodinated yolk protein to the ovaries during a 4-hr period. Ovaries that were 18 hr post-blood meal and already laying down yolk, ovaries which were developmentally competent to deposit yolk but had not yet received a meal of blood to initiate the process, and developmentally immature ovaries were assayed in parallel cultures for binding of yolk protein. A clear difference exists between the two developmentally competent and the immature ovaries in their ability to bind iodinated yolk protein (Fig. 9).

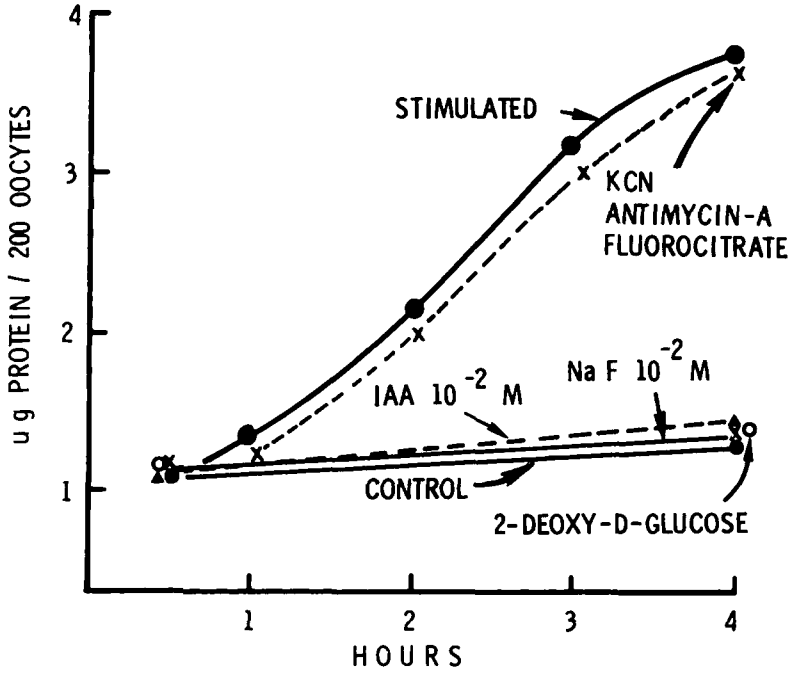

Fig. 8. Transport is prevented by inhibitors of glycolysis but not by inhibitors of respiration or oxidative phosphorylation. Control ovaries were taken from adults not yet stimulated by a blood meal to initiate sequestration of yolk from the hemolymph. Stimulated ovaries were from 18-hr post blood meal females.

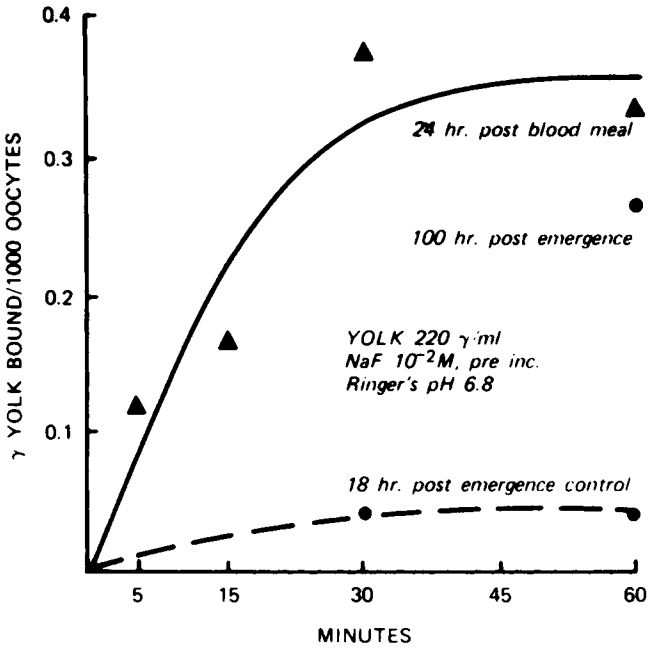

Fig. 9. Binding to inhibited ovaries. Uptake can be separated from binding by incubating the ovaries for 30 min with NaF and then with ^{125}I-mosquito yolk protein. A clear difference in binding is evident between the 18-hr post emergent adult (●) and either the nonblood-fed 100-hr postemergent adult or the blood-fed adult ovaries. This difference can be correlated with the relative numbers of coated pits on the oocyte plasma membrane.

When such ovaries are examined with the aid of the electron microscope, the immature oocytes are seen to lack the large number of coated pits found on the plasma membrane of both the mature and transporting oocytes. A quantitative correlation between the number of coated pits and the amount bound has not been done.

Specific Protein Regulation of Transport

Since protein transport in the mosquito appears to be a highly specific process involving a high affinity receptor, it is reasonable to ask if the overall process, once set in motion, continues to require the presence of the female specific protein. To answer this question, ovaries that were actively transporting protein were incubated in the usual manner but, in addition, an electron-dense marker, ferritin, was added in the presence or the absence of the female specific phosphoglycolipoprotein.

In those ovaries incubated for 30 min in the presence of female specific protein and ferritin, the ferritin was found localized in the nascent yolk granules (Fig. 10). Note its distribution at the periphery of the granule but centripetal to the enveloping membrane. In no case was ferritin found included in the paracrystalline array. Also, no ferritin was found elsewhere in the cytoplasm, but it was present in the extraoocyte space as well as between the follicular epithelial cells.

Ovaries incubated with ferritin alone or in the presence of BSA but without the female specific protein were also prepared for electron microscopy. In all cases the ferritin remains in the extraoocyte space; no ferritin can be found in the oocyte cytoplasm either free or in any vesicle (Fig. 10). In some few images a few particles of ferritin were noted

in large irregularly shaped vesicles, but only those very near the plasma membrane. It may well be that such "vesicles" are, in fact, invaginations of the membrane in which the content is continuous with the extraoocyte space out of the plane of section. In both experiments the oocyte still had coated pits along the surface, but, as was the case in all in vitro incubations, the number of pits was reduced to at least one-half the number noted on ovaries taken from the animal. The reason for this reduction is not yet clear.

From this rather simple experiment it is evident that yolk protein must bathe the oocytes if transport, once set in motion, is to be maintained. Whether other yolk proteins from other species will stimulate the transport process in the mosquito is not known.

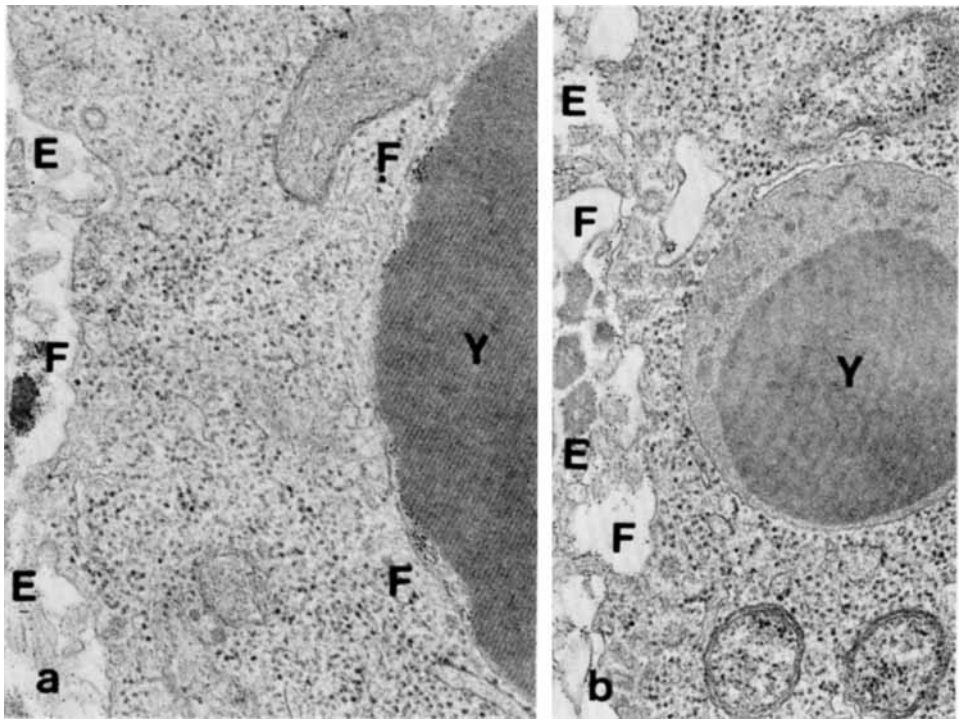

Fig. 10. (A) To determine if coated pits continued to carry in protein in the absence of exogenous yolk, mosquito ovaries already taking up yolk were then incubated with ferritin (F) in the absence of yolk. Note the absence of ferritin in the nascent yolk granules (Y) but its presence outside the oocyte (E) after 30 min of incubation. × 43,300.

(B). Similar mosquito ovaries incubated with ferritin and yolk protein show the ferritin now to be included within the yolk granule membrane at the periphery of the yolk (Y) as well as in the extracellular space (E) outside the oocyte. × 43,300.

Binding to Isolated Plasma Membranes

It soon became obvious that further characterization of the transport process would require quantities of membranes much greater than that afforded by the mosquito. The oocytes from the chicken seemed ideal as a source of this membrane, but it was first necessary to determine if yolk deposition in the chicken exhibited specific transport or merely gross pinocytotic engulfment. Of the six or seven serum proteins that constitute the chicken yolk, only immunogammaglobulin (IgG) and the estrogen-stimulated female specific phosvitin lipovitellin (PvLv) complex were used in these studies.

To determine if there was any indication of specific uptake, IgG and PvLv in the yolk and serum were quantitatively extracted by standard procedures, as outlined in Methods, and corrected for losses by isotopic dilution. In Table I it is apparent that the ratio of PvLv to IgG in the serum is approximately 10-fold different from the ratio of the two found in the yolk (35). Clearly, no simple engulfment can account for this difference in ratios. Of equal interest is the half-life of the two proteins in the serum as determined by isotopically labeled proteins and their removal from the serum. A strong correlation exists between the amount of the two proteins sequestered by the oocytes and the half-life of the two in the serum. Importantly, less than 1% of the label can be detected as low molecular weight material, nor is there appreciable accumulation of labeled material (< 10%) in the tissues of the reticuloendothelial system such as the liver, spleen, or lungs.

TABLE I. Comparison of Serum to Yolk

Substance	Phosvitin	Gamma Globulin
Serum concentration (g per 100 ml)	0.3	2.7
Yolk concentration (g per 100 ml)	1.75	1.5
Concentrating factor	6 X	0.6 X
Half life of ^{125}I protein in serum	1–2 hr	8–12 hr

Once there was evidence to suggest the process had some element of specificity associated with the yolk protein transport, it was necessary to determine which oocytes were actively transporting proteins. By injecting simultaneously ^{131}I-PvLv and ^{125}I-IgG into the circulation of hens, and then removing an ovary after 1, 2, 4, or 5 hr, a reproducible pattern of labeling of the various oocytes emerged, as reported in greater detail elsewhere (35) and as illustrated in Fig. 11. In every ovary there is a clear distinction between the oocytes larger than 0.2 gm and those smaller. The heavier oocytes are clearly vitellogenic and orange in color. These oocytes transport IgG and PvLv in a constant ratio to each other. Oocytes smaller than 0.2 gm, however, transport IgG and PvLv at rates that vary developmentally until 0.2 gm. For the isolation of the oocyte plasma membranes, only those greater than 0.2 gm were used in the experiments that follow.

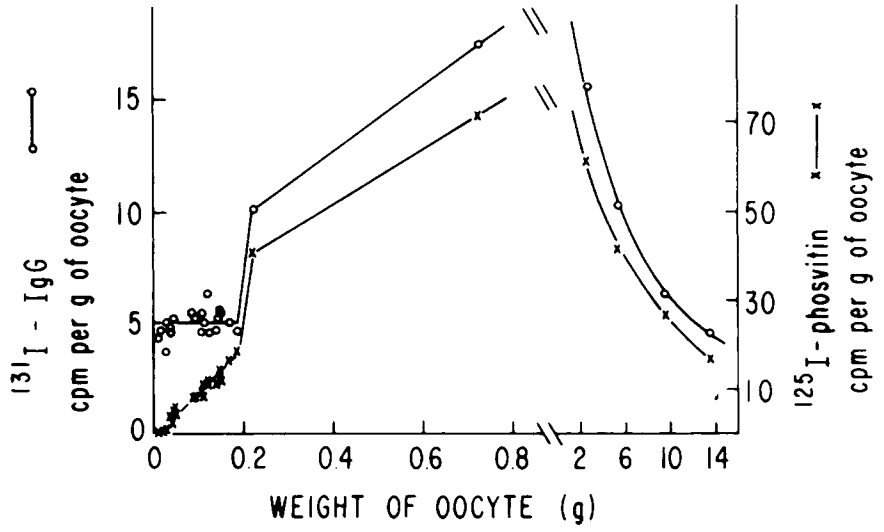

Fig. 11. Relative transport of phosvitin and IgG by chicken oocytes from the blood. Radioactive phosvitin and IgG were simultaneously injected into the wing vein. Two hrs later the radioactivity in each oocyte greater than 50 mg was determined.

Binding of IgG to Isolated Membranes

Since the data from the in vivo studies strongly suggest that there is specificity operating at some step in the uptake of serum proteins by the oocyte, selective binding of IgG to isolated plasma membranes was looked for. In those experiments 0.4 gm or larger oocytes were freed from the connective tissue sheath and the overlying epithelium by carefully dissecting free these layers. The oocyte plasma membrane was freed from adherent yolk and cytoplasm by gentle washing in Earle's Balanced Salts (EBS) solution. After a brief homogenization or dicing of the remaining membrane, the fragments were then incubated in EBS in the presence of ^{125}I-labeled IgG. Binding is progessive but approaching saturation by 2 hr with more than half the sites occupied by 30 min (Fig. 12).

Competition for Binding

If the process of binding involves a receptor and if the binding of labeled IgG is similar to that of the uniodinated IgG, increasing amounts of IgG should compete with labeled IgG for binding. Such competition is evident in Fig. 13, where for each doubling of IgG there is an approximate halving of bound ^{125}I-IgG. PvLv does not compete for IgG binding even when in 10-fold molar excess.

Binding of PvLv to Isolated Membranes

The hen female specific estrogen-stimulated protein is the phosvitin lipovitelline complex (PvLv). Binding of PvLv to isolated oocyte plasma membranes is highly specific, as reported elsewhere in this journal (56).

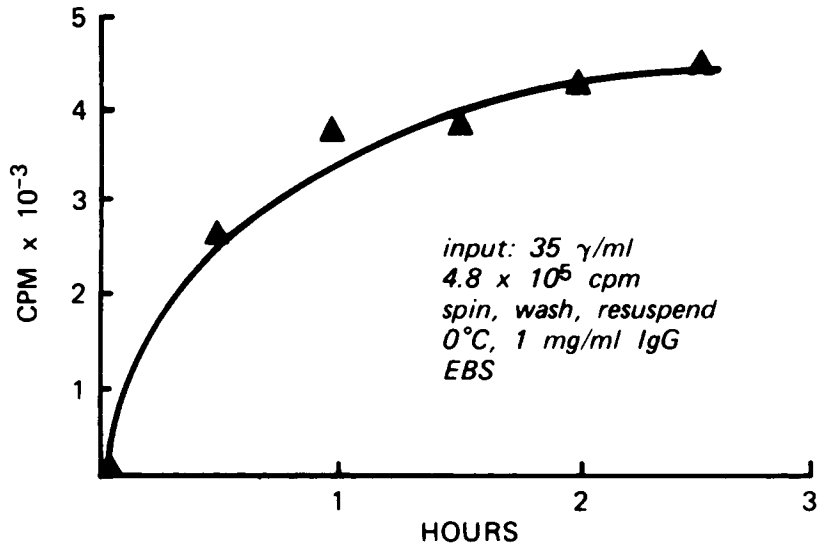

Fig. 12. Binding of IgG to chicken oocyte plasma membrane fragments. Binding was at 37°C, washes were at 0°C. Membranes were resuspended at each time point after counting in the original incubation mixture.

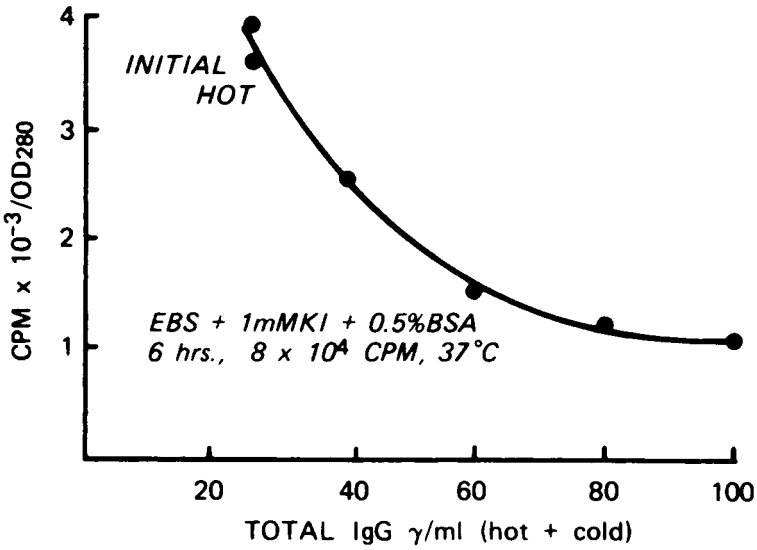

Fig. 13. Competition for b..ding by unlabeled IgG. Binding of labeled IgG to hen oocyte plasma membrane fragments was assayed in the presence of constant amounts of labeled IgG and increasing amounts of unlabeled IgG. Each data point is from an individual tube incubated for 6 hrs.

Localization of Bound IgG

From the foregoing data it appears that IgG binds specifically to isolated chicken oocyte membranes. Once this was established, it became reasonable to attempt to localize, with the aid of the electron microscope, the sites of binding by using ferritin conjugated to IgG. Freshly dissected oocyte plasma membranes were incubated in a drop of the conjugate (1–4 mg/ml) for 15–25 min, washed three times in EBS (5–10 min each), and then fixed and prepared for visualization in the electron microscope. At low magnification the overall morphology of the convoluted plasma membrane is evident, suggesting that the incubation did little except leach material from the cytoplasmic spaces between the plasma membrane folds. At higher magnification (Fig. 14) the distribution of the electron-dense ferritin conjugate is more readily visualized. Note that the pattern of ferritin placement can be separated into at least three classes. (1) Most of the ferritin appears to be localized to the extracellular spaces between membrane folds. In this space there is also seen a somewhat amorphous material not seen on the cytoplasmic side. Note that although the ferritin is visualized in this space, no pattern of association with the membrane is discernible. Also, no ferritin is found adherent to material on the cytoplasmic side of the plasma membrane. (2) Ferritin conjugates are visualized in some but not all of the coated pits. In most of the pits where ferritin is present, it is heterodispersed and again shows no pattern of association with the membrane. (3) A clear association with the membrane can be found in a small but consistent population of the coated pits. In such instances the ferritin lies centripetally located some 200–300 Å from the membrane, a distance consistent with the molecular size of the ferritin-IgG conjugate. Images which show coated and naked vesicles also have a distinct population which exhibits this centripetal localization. Although a thorough statistical analysis has not yet been completed, the population of coated pits which exhibits such a localization at the membrane appears to constitute less than 20% of the total.

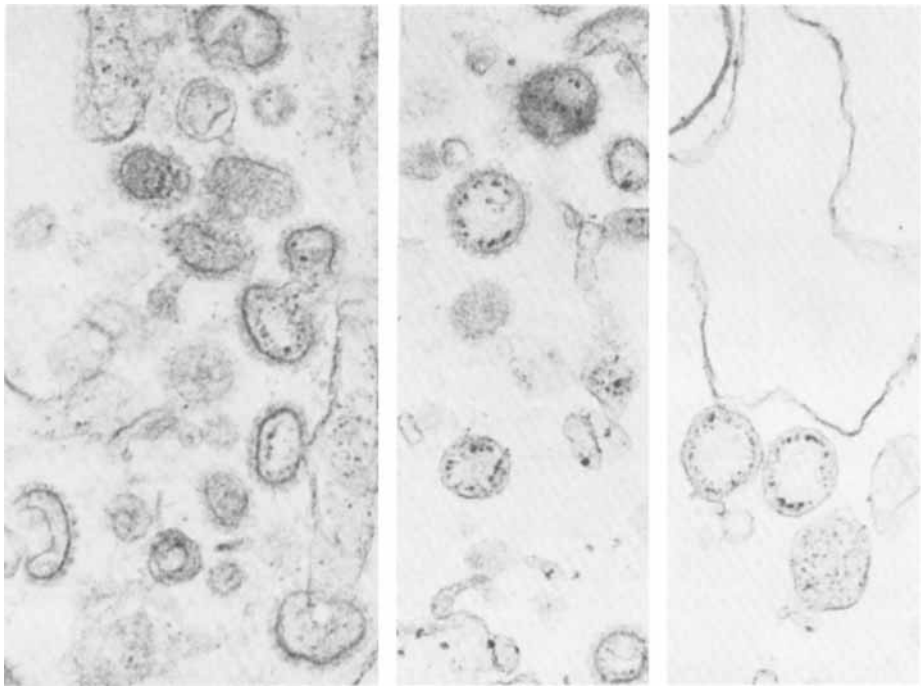

Fig. 14. Localization of IgG binding by ferritin-IgG conjugate. Chicken oocyte plasma membrane pieces were incubated in the presence of the conjugate, washed, and prepared for electron microscopy. Note the pattern of localization in the various coated pits and vesicles. Some contain no ferritin, in others the ferritin is heterodispersed while in a small but consistent population the ferritin is applied to the membrane at a distance consistent with the dimensions of the conjugate. × 77,400.

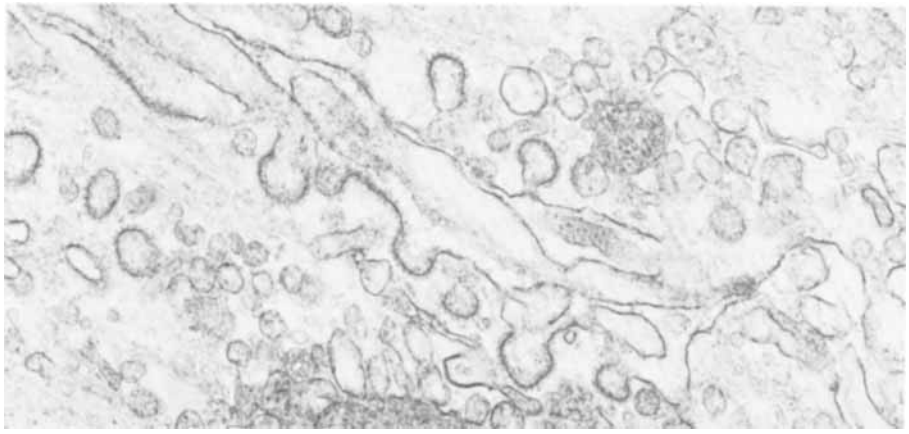

Fig. 15. Control chicken oocyte plasma membrane pieces were incubated with ferritin alone to determine if nonspecific binding occurs. After washing the membranes, little or no ferritin can be discerned in the cortical folds, coated pits, or vesicles. × 40,500.

Control images of similar membrane preparation incubated with free ferritin and then washed show no ferritin between the membrane folds (Fig. 15). The only ferritin noted in such controls is that bound generously to the vitelline filaments that lie along but outside the surface of the oocyte plasma membrane. Unwashed ferritin controls do show ferritin deep within the membrane.

DISCUSSION

Coated pits and pit-derived coated vesicles are found in all oocytes during the period of active sequestration of exogenous yolk protein. Oocytes must be both developmentally and hormonally competent before transport can occur. In both the chicken and mosquito, it is clear that until a particular stage in the developmental maturation of the oocyte is reached, relatively few coated pits are present along the oocyte plasma membrane. Once having reached the requisite stage, the mosquito system further awaits the stimulus of the blood meal to trigger the synthesis of the female specific phosphogylcolipoprotein. In certain autogenous mosquitoes, such as Culex pipiens pipiens, a blood meal is not required since the fat bodies synthesize the female specific protein without such an external trigger.

In the hen the hormonal environment is essential for maturation and yolk deposition, but here, too, the oocytes must first be developmentally competent or no deposition occurs. As in the mosquito, the hen will not continue to sequester yolk protein unless the female specific protein (PvLv) is present. Clearly there is a marked interdependence between the hormonal stimulus, the developmental competence, and the absolute requirement for the female specific protein to initiate and maintain the transport process. The ability to turn off the transport by withdrawing the mosquito yolk protein was absolute, as shown in Fig. 10. Others had implicated the presence of yolk as an absolute requirement in maintenance of the uptake (62, 63, 64).

Sequestration of the mosquito yolk protein appears to be receptor mediated. A K_m of less than 10^{-7} M and the lack of competition by a variety of other proteins give credence to this view. Further support for this mechanism comes from the inhibition studies which indicate that binding can occur in the absence of sequestration. Of particular note is the ability of both the inhibited previously sequestering oocytes and the developmentally mature but nonsequestering oocytes to bind nearly equal amounts of the mosquito female protein, whereas the developmentally immature oocytes bind considerably less. Such data prove a quantifiable assay of binding that closely parallels the distribution of morphologically identifiable coated pits along the oocyte surface.

Uptake of tagged protein by the mosquito oocyte was studied by Anderson and Spielman (65) and was critical in frimly establishing the route as via the coated pits and vesicle mechanism. Earlier studies had not used tagged proteins and relied upon correlative data. The tagged uptake studies also demonstrated the other aspect of the coated pit-mediated transport, that is, the nonspecific component of this otherwise specific transport process. In their experiments they used horseradish peroxidase as a cytochemical marker. However, the marker was not covalently attached. Thus, it entered the vesicles passively. If we examine the morphology of the coated pit, it is obvious from the dimensions that some small central volume exists which can be nonspecifically filled by any sufficiently small substance (Fig. 16). If the inside diameter of the coated pit is approximately 760 Å and protein is specifically bound to a depth of 150 Å, then the diameter of the void space is 460 Å which would give an approximate void volume equal to 22% of the total pit volume. This is not large but it is significant in a variety of oocyte

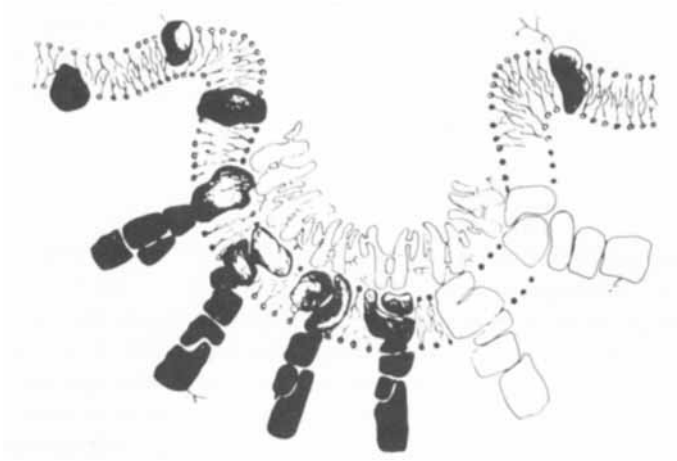

Fig. 16. Diagramatic presentation of a postulated molecular organization of the coated pit. Embedded in the lipid bilayer are the receptors to which the stylized IgG molecules are attached on the concave extracellular side. Dense blocks of protein which make up the ridgelike coat of the coated vesicle are tightly applied to the cytoplasmic side of the membrane.

systems. In the hen (17, 18), in particular, it has been demonstrated that heterologous molecules are transported into the egg in small quantities.

Chicken oocytes sequester several proteins in addition to PvLv and IgG. Although both IgG and PvLv appear to have mutally exclusive receptor sites by kinetic data, it is not established whether the receptors are segregated or integrated in the coated pits. Localization studies with ferritin-tagged IgG did not address this question but did provide some information that is applicable. It is evident that all coated pits do not bind Ft-IgG, whereas some few bind it very well. This suggests that the IgG receptors may be segregated into some coated pits and not others. However, these experiments should be interpreted with caution due to the large amount of conjugate found in the folds and not in the coated vesicles. It may be that the intermediate binding of the Ft-IgG to an amorphous material in this extra oocyte space may reflect the presence of some intermediary carrier or extracellular binding stuff, as suggested in the work of Anderson and Telfer (66). If such were the case, then the Ft-IgG would first be expected to bind to that material and thence the complex would in turn bind to the membrane receptor for uptake. This point can be more fully investigated once the receptors are isolated.

In oocytes the work of many scientists, such as that by E. Anderson, R. G. Kessels, W. H. Telfer, and R. A. Wallace, has contributed much to an understanding of yolk deposition. The process as thus far elucidated in oocytes has, as a morphological correlate, the coated pit as a membrane marker for specific protein uptake (2, 3). The plasma membrane of every cell of every higher animal carefully imaged in the electron microscope has coated pits that vary in number with the cell type and developmental stage. In such cells, little is known about their ability to bind and sequester large proteins. Thus far, in no case other than the oocyte has the coated pit-vesicle mechanism been shown to be specific. In all probability this is due to the lack of information as to what protein(s) is being specifically sequestered. Further, although many instances are known where a variety of exogenous large protein "'effectors" can regulate cellular function, what these proteins are is frequently unknown. In some cells the effector protein is known but the number of

molecules needed to regulate the cells is so low as to preclude at present any reasonable chance of specifically visualizing the uptake or binding process.

Although much is known concerning yolk deposition, there is little known about the nature of the receptors, the chemical composition of the coated pits and vesicles, and of their morphogenesis. At present, these ubiquitous and intriguing entities, the coated pit and coated vesicle, are best experimentally pursued in the oocytes.

ACKNOWLEDGMENTS

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