THE ROUTE OF ENTRY AND LOCALIZATION OF BLOOD PROTEINS IN THE OOCYTES OF SATURNIID MOTHS

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ABSTRACT

The oocytes of saturniid moths take up proteins selectively from the blood. The distribution of blood proteins in the ovary during protein uptake was investigated by staining 2 μ sections of freeze-dried ovaries with fluorescein-labeled antibodies. The results indicate that blood proteins occur primarily in the intercellular spaces of the follicle cell layer, in association with a brush border at the surface of the oocyte, and within the oocyte in the yolk spheres. That proteins derived from the blood are associated with the yolk spheres was confirmed by isolating these bodies and showing that lysis, which can be induced by any of a number of mechanical means, causes them to release immunologically defined proteins known to be derived from the blood. That the level of blood proteins in the cytoplasm is low relatively to that in the yolk spheres was confirmed by the observation that the yellow pigments associated with several blood proteins, although conspicuous in the yolk spheres, are not visible in the translucent layer of centrifuged oocytes. From these and previous physiological observations, it is proposed that blood proteins reach the surface of the oocyte by an intercellular route, that they combine with some component of the brush border, and that they are transformed into yolk spheres by a process akin to pinocytosis.

The oocytes of saturniid moths remove copious amounts of proteins from the blood during their period of yolk formation. The evidence for this includes the observations that many proteins in the oocyte are antigenically indistinguishable from proteins occurring in the blood, and that foreign proteins injected into the blood subsequently appear in the oocyte (28, 30). As much as 10 per cent of the wet weight of the mature oocyte may consist of proteins derived from the blood; protein uptake is thus a major aspect of the growth of this cell.

A study of the distribution of blood proteins in the ovary during the period of yolk formation was undertaken. The objectives were to discover the route by which proteins penetrate the layers of cells which envelope the oocyte, to determine their final distribution within the oocyte, and thus to characterize further the mechanism of protein uptake.

Evidence is presented that the blood proteins reach the oocyte via an intercellular route, that they become associated with a brush border at the oocyte surface, and that they are finally deposited within discrete cytoplasmic particles, the protein yolk spheres. Since blood proteins were not detected in the cytoplasm surrounding the yolk spheres, the mechanism may entail a direct transfer of protein from the cell surface into the growing yolk spheres. Mechanisms are discussed which could account for these and other attributes of yolk formation and protein uptake.

OBSERVATIONS

The Distribution of Blood Proteins in the Ovaries as Revealed by Fluorescence Microscopy[1]

The ovaries of saturniid moths consist, as in the Lepidoptera generally, of eight tubular ovarioles, each containing a number of oocytes arranged in a linear sequence. Toward the end of metamorphosis, the oocytes enter a one week period of yolk formation during which they almost double their volume daily (32). Each oocyte is surrounded during this period by a layer of follicle cells and, in addition, is connected by a cytoplasmic bridge to a cluster of five nurse cells which lie at its anterior end (19, 26) (Fig. 1). Thus, in order to reach the oocyte a blood protein must pass from the hemocoel, through the wall of the ovariole, across the follicle cell layer, and into the oocyte, either directly or via the nurse cells. The localization of blood proteins with relation to these cells was sought by staining sections of fixed and embedded ovaries with fluorescein-labeled antibodies against blood proteins (9).

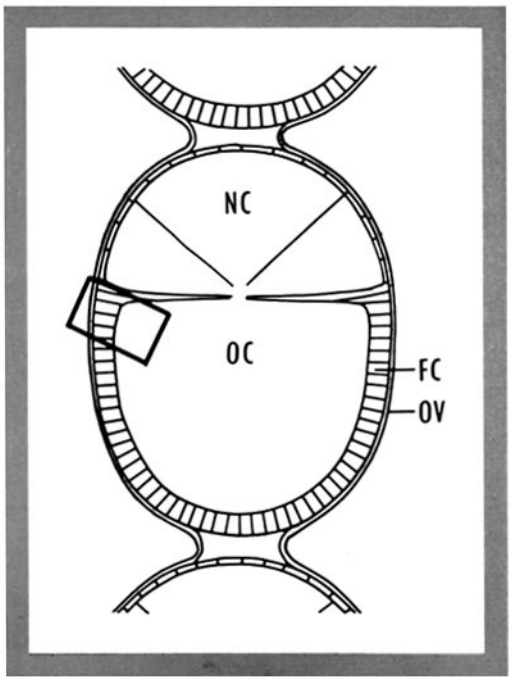


FIGURE 1

Diagram of an oocyte and associated cells as they appear in longitudinal sections of the ovary of a moth. The oocyte (*OC*) is surrounded by a layer of follicle cells (*FC*), and is associated with a cluster of five nurse cells (*NC*), three of which may appear in a section such as this. The entire complex of cells is enclosed in a tubular ovariole (*OV*) which, in the cecropia and polyphemus moths, contains up to sixty such groups arranged in linear order. The box indicates the region shown in Figs. 2 to 5.

An ovariole was removed from a female cecropia moth on the 15th day of her transformation from pupa to moth and placed transitorily in a drop of blood, its normal medium within the moth. The largest oocyte in the ovariole was approximately 1 mm. long, and was thus about one-quarter of the way through its period of yolk formation (32). Every fourth occyte, along with the associated follicle cells, nurse cells, and ovariole wall, was frozen in liquid propane at about −180°C., dried in vacuum, and embedded in diethylene glycol distearate. Longitudinal sections, 2 μ thick, were cut with a glass knife onto a flotation medium containing 1 per cent formaldehyde and 50 per cent dioxane in water. After the sections had remained on this medium for 5 to 10 minutes, they were mounted on slides and dried at 40°C. The embedding medium was removed by immersing the slides for 3 minutes in xylene; the sections were then put through a series of alcohol solutions of decreasing concentration, and finally washed in 0.15 M NaCl, buffered at pH 7.4.

The technique described has been found to fix soluble proteins in mammalian tissue sections sufficiently to prevent their extraction during the subsequent stages of antibody staining and washing, without seriously impairing their reactivity with specific antibodies (20). From the results described below, the method appears to be equally useful for the fixation of blood proteins in the insect ovary.

The sections were stained by an indirect technique in which the slide was first bathed with unlabeled rabbit antibodies against cecropia blood proteins for 2 hours. Rabbit serum proteins which had not precipitated on the section were removed by washing the slides with four changes of cold, buffered saline over a total period of 16 hours. Finally, the sections were stained by immersion in fluorescein-labeled chicken antibodies against rabbit gamma globulin. After four more washings in cold, buffered saline, the sections were mounted in 95 per cent glycerol (adjusted to pH 8–9).

The rabbit antiserum used was one whose reactions with cecropia blood proteins and oocyte extracts has been subjected to extensive analysis (adult antiserum

[1] The fluorescence microscopy was performed in the laboratory of Dr. J. M. Marshall, Jr., whose assistance with this aspect of the work is gratefully acknowledged.

d in 28, 30). In antiserum agar tests with either cecropia blood or oocyte preparations, it produced two major zones of precipitation, one with a carotenoid protein and one with the female protein—so named because it circulates in female blood at approximately one thousand times its level in the blood of males. Both of these proteins have been shown to be removed from the blood and deposited in the oocytes in copious amounts. Experiments involving heterospecific blood transfusions have shown that neither protein is synthesized within the ovary to a significant extent (30). The antiserum contained minor amounts of precipitating antibodies against several additional blood proteins, all of which, according to absorption tests, occur in the oocytes. Thus, the antibodies under consideration probably reacted exclusively with proteins which enter the occyte from the blood.

Control sections were treated by the procedures described above, except that the rabbit antiserum against cecropia blood protein was replaced by a rabbit antiserum against lobster hemocyanin, or the chicken antibodies against rabbit gamma globulin were replaced by fluorescein-labeled gamma globulin from unimmunized chickens.

After either type of control treatment, fluorescence appeared in the sections in the form of a thin, bright line at the point of juncture of the oocyte with the follicle cells (Fig. 2). Because of its apparent affinity for serum proteins, we were unable to determine whether this structure normally contains significant amounts of cecropia blood proteins. Otherwise, all components of the ovary, including any blood adhering to its surface, were either unstained or weakly fluorescent, in the control sections.

Sections stained so that the distribution of fluorescence should reflect the distribution of blood proteins appeared as shown in Figs. 3 and 4. The cellular structures which fluoresced included the large, spherical structures which lie in the cytoplasm of the oocyte and which are termed yolk spheres (34), a brushy structure at the interface between the follicle cells and the oocyte, and occasional small droplets in the cytoplasm of the follicle cells. As in the control sections, a line of fluorescence separated the oocyte from the follicle cells. This line of non-immunological fluorescence was located within the brushy zone so that two-thirds of the latter lay on the oocyte side and one-third lay on the follicle cell side. The nurse cells, which are not portrayed in Figs. 3 and 4, were not detectably fluorescent except, as in the case of the follicle cells, for an occasional droplet in their cytoplasm. The ovariole wall was too attenuated to allow observations on its cellular structure.

All the extracellular spaces in the ovary were intensely fluorescent. These included especially the spaces which lie between the follicle cells, and which are seen in freeze-dried preparations to be approximately one-fourth as wide as the follicle cells. In those cases in which blood adhered to the outer surface of the ovary (Fig. 4), this also was strongly fluorescent, as would be required if our interpretation of the fluorescence is correct.

These observations raise the possibility that blood proteins reach the surface of the oocyte by an intercellular route. If they are transmitted through the cytoplasm of the follicle cells, they are either kept at a low level of detectability or bound in some fashion that makes them unable to combine with antibody. The complementary fact that blood proteins are abundantly present in intercellular spaces which extend from the ovariole wall to the brush border of the oocyte makes it more probable that this is the primary route by which proteins penetrate the layer of follicle cells.

The presence of fluorescence in the protein yolk spheres indicates that these bodies are depositories of blood proteins, accumulated by the oocyte. This observation is confirmed by two other methods described in the subsequent sections. The undetectability of fluorescence in the cytoplasm surrounding the yolk spheres suggests, in addition, that blood proteins may pass directly from the surface of the oocyte to the growing yolk spheres.

Phase microscopy of sections prepared for staining with labeled antibody confirmed the existence of a brush border at the surface of the oocyte (Fig. 5). Small droplets, well under 0.5 μ in diameter, are closely associated with the inner side of the brush border. The gradual transition in the size and appearance of these droplets from the smallest ones adjacent to the brush border to the 20 to 25 μ yolk spheres deeper in the oocyte suggests that the yolk spheres originate and grow in the proximity of the brush border from which blood proteins and other substances are derived.

Isolation of the Yolk Spheres

If the results of fluorescent antibody staining are correct, blood proteins should be extractable from

yolk spheres. To test this assumption, a technique was developed for separating the yolk spheres from the other components of the oocyte.

Centrifugation of the mature, unfertilized egg of either the cecropia or the polyphemus moth yields two strata which are discernible through the dissecting microscope: a centripetal cap of colorless particles, and a centrifugal mass which is colored yellow and which occupies more than 95 per cent of the oocyte. Dissection of such an oocyte reveals that the centrifugal mass is composed primarily of yellow spheres whose size and abundance identify them as the protein yolk spheres. They vary in diameter up to 25 μ, and are numerous enough to be very nearly close-packed in the mature oocyte.

When an oocyte is dissected in one of the various media described in the next section, and its contents are dispersed by gentle agitation, most of the yellow bodies settle to the bottom within several minutes, while the colorless particles remain in suspension or rise to the surface. The separation can be completed in less than one minute by centrifugation at 500 *g*.

The lipid nature of the centripetally moving particles is suggested by the observation that they rise in an aqueous medium with a specific gravity of close to 1.0. Since they do not coalesce when compressed by centrifugation, their structure is more complex than that of an oil droplet and they may therefore be classified as lipochondria, such as are observed in animal oocytes generally (*cf.* 14). Various other particles, such as mitochondria, must also be present in the oocyte and these are presumably represented in the smaller bodies visible in the suspended oocyte contents. Many of these failed to sediment at the forces used, however, and those which did represented an insignificant proportion of the centrifugal pellet. The procedure thus yielded a preparation consisting almost exclusively of yolk spheres.

The Physical Properties of the Yolk Spheres[2]

The yolk spheres can assume any of several configurations, according to the nature of the medium in which they are suspended. In 0.3 M NaCl, most of the yolk spheres of the mature oocyte appeared at first bright yellow, spherical, and highly refractile (Fig. 6). After about 30 minutes they lysed, losing both their refractility and their yellow color. The residual "ghost" was a fragile structure which was readily deformed into elongated configurations by turbulence in the medium (Fig. 7); the surface of the ghost exhibited undulations due to Brownian motion. The pigment which was lost from the yolk spheres

[2] This aspect of the work was carried out with the assistance of Mr. William E. Koch.

FIGURES 2 to 5

Longitudinal sections of a cecropia oocyte with its adjacent follicle cells. A standardized corner of each section is shown as indicated in Fig. 1. × 900. The sections were treated and viewed as follows:

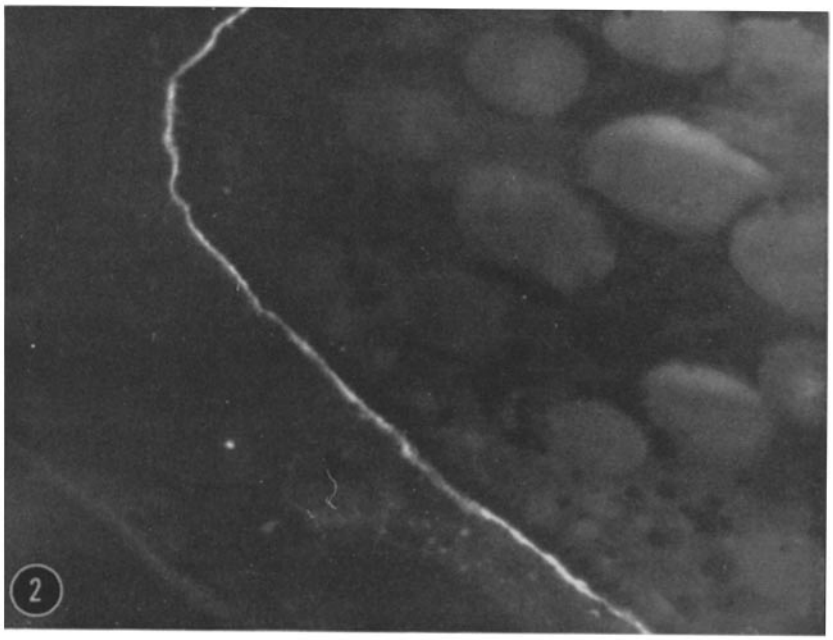
FIGURE 2

Fluorescence microscope view of a control section treated so that the distribution of fluorescence should reflect the adsorption of rabbit or chicken serum proteins on the section by non-immunological mechanisms. The section was treated with rabbit antiserum against lobster hemocyanin, followed by fluorescein-labeled chicken antibodies against rabbit gamma globulin. The bright line of fluorescence lies between the follicle cells (left) and the oocyte (right) and corresponds to a similar line appearing in Figs. 3, 4, and 5.

FIGURE 3

Fluorescence microscope view of a section treated so that the distribution of fluorescence should reflect the distribution of blood proteins. The section was treated with rabbit antiserum against cecropia blood proteins, followed by fluorescein-labeled chicken antibodies against rabbit gamma globulin.

The fluorescent spheres (right) are yolk spheres lying in the cytoplasm of the oocyte. The oocyte is separated from the follicle cells (left) by a brush border which is fluorescent and which includes the line of non-immunological adsorption seen in Fig. 2. The follicle cells are separated from one another by fluorescent intercellular spaces. The weakly fluorescent band (far left) is the attenuated wall of the ovariole.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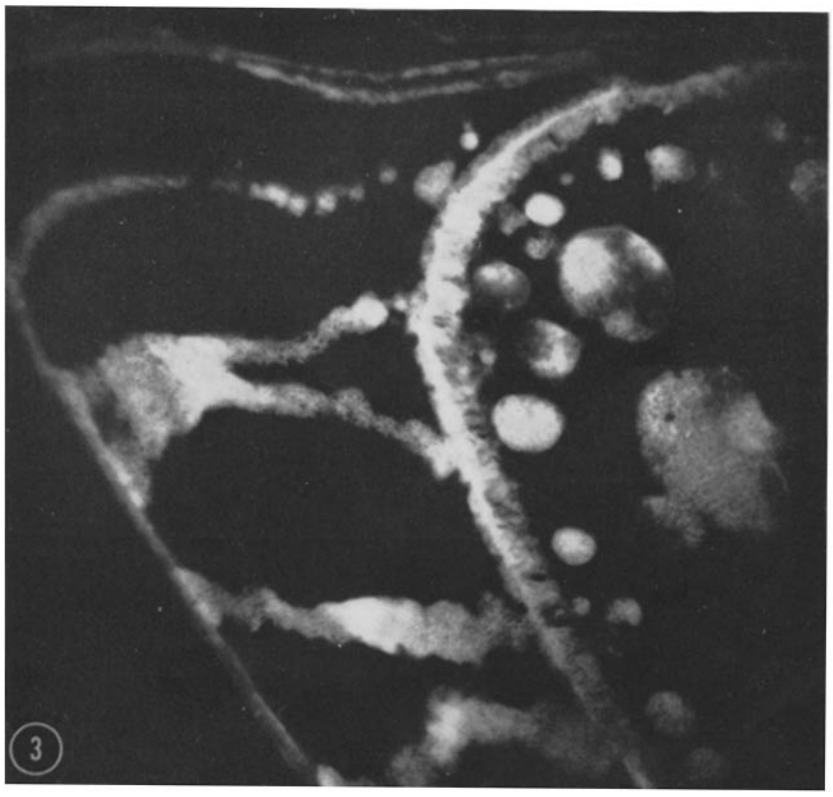
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during lysis appeared in the surrounding medium, from which it could no longer be sedimented by centrifugation, except by prolonged exposure to forces which suffice for the sedimentation of proteins in solution (for instance, 10 hours at 100,000 *g*).

Lysis also occurred when the yolk spheres were suspended in 0.15 M NaCl, although in this case a time lag was not apparent. In 0.6 M NaCl, the yolk spheres were shaped like ellipsoids with crinkled surfaces (Fig. 8). With time, individual yolk spheres in this medium reassumed their smooth, spherical appearance, and finally lysed in the fashion that occurred more promptly in less concentrated solutions.

That the behavior of the yolk spheres in NaCl solutions can be explained in terms of osmotic effects was confirmed by studying the effects of non-ionic solutes such as sucrose. Although variability in the behavior of different yolk spheres was too great to permit a precise measure of the equivalent concentrations of different solutes, it was apparent that the molar concentrations of sucrose which produced lysis and crinkling were approximately twice the molar concentrations of the NaCl solutions with corresponding effects.

Two differences were noted between the effects of sucrose and of NaCl. In hypertonic solutions of sucrose, the crinkled configuration was retained for periods as long as several days, rather than gradually giving way to lysis.

The second difference resided in the appearance of the yolk spheres that had lysed. When lysis occurred in 0.3 M sucrose, the contents of the yolk sphere became granular and one could directly observe the granules exuding through a point of rupture in an enveloping membrane (Fig. 9). Dissiptation of the granules by Brownian movement finally left the membrane in the form of an empty shell. There was little doubt that, under these conditions, a membrane was present at the surface of the yolk sphere. The explanation of the granular appearance of the contents resides in the fact that some of the proteins in the yolk sphere are insoluble in aqueous media of low ionic strength. This interpretation is based on the observation that a precipitation formed when a lysate of yolk spheres in 0.15 M NaCl was diluted with water, but not when the lysate was diluted with 0.15 M NaCl. The fact that intact yolk spheres suspended in 0.3 M sucrose appeared optically homogeneous rather than granular suggests that the enveloping membrane is impermeable not only to sucrose, but also to the substances which normally provide the ionic environment necessary for retaining these proteins in solution.

The yolk spheres can be mechanically disrupted by means other than exposure to hypotonic solutions. They were lysed, for instance, by freezing at −20°C. and subsequent thawing, either in isolation or in the intact, mature oocyte. As has already been described (30), crushing a mature oocyte with a mortar and pestel caused their lysis. Microscopic examination indicated that, in preparations obtained by carefully tearing the chorion with forceps and suspending the yolk spheres in a hypertonic medium, 10 per cent or more of the yolk spheres were lysed. Finally, two or three successive washings by centrifugation and resuspension resulted in lysing all the yolk spheres, even in a medium in which, in the absence of agitation, they remained in good condition for several days. Both in their normal environment within the oocyte and in isolation media, therefore, the yolk spheres are readily lysed by mechanical damage.

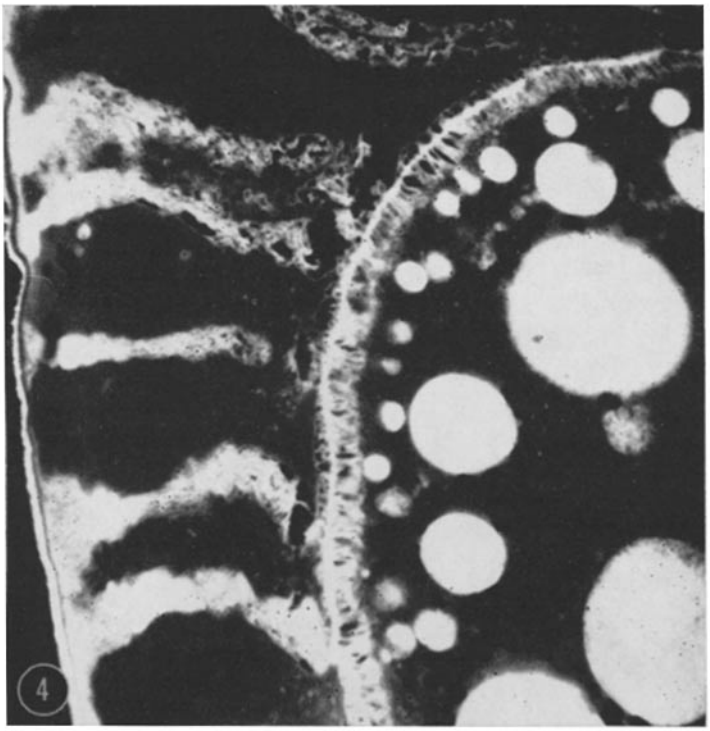
Whether lysis is due entirely to the breakdown of a membrane, or also entails the disruption of an optically homogeneous matrix enclosed by the

FIGURE 4

A section treated in the same manner as that shown in Fig. 3. Ice crystals had partially disrupted the brush border, revealing its structure in greater detail, and a film of blood which appears brightly fluorescent (left) adhered to the outer surface of the ovariole.

FIGURE 5

Phase microscope view of a section prepared for fluorescent antibody staining. Small droplets lie in the cytoplasm of the oocyte adjacent to the brush border. There is a gradation in the size of the droplets up to the 20 to 25 μ yolk spheres which lie at a greater distance from the surface of the oocyte.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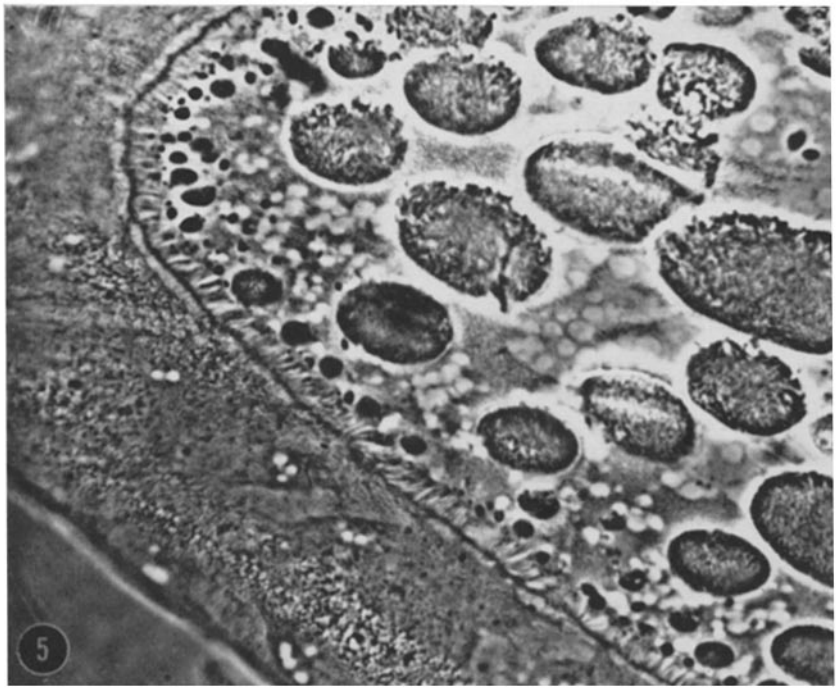
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membrane, is not indicated by the available data. In either case, it is clear that the yolk spheres can be isolated intact and that they can be largely solubilized by a number of relatively gentle procedures.

The Association of Blood Antigens with the Yolk Spheres

With the assistance of procedures for the isolation and lysis of the yolk spheres, it was possible to confirm the suggestion that these structures contain those proteins of the oocyte which are derived from the maternal blood.

The yolk spheres of mature polyphemus oocytes were separated from the lipochondria and soluble constituents of the oocyte in a medium containing 0.6 M sucrose and 0.15 M NaCl, a solution which is hypertonic to most of the yolk spheres. Each isolation was accomplished by tearing open the chorions of from four to ten oocytes in 0.2 ml. of the medium and suspending their contents by gentle agitation. When the yolk spheres had been sedimented by centrifugation (500 *g* for 1 minute), the supernatant was withdrawn. A volume of fresh medium, equal to that of the withdrawn supernatant, was added to the sedimented yolk spheres so that they could be resuspended at their initial concentration. The suspension was frozen at −20°C. and subsequently thawed in order to lyse the yolk spheres. The supernatant preparation was similarly frozen and thawed and the two solutions were then analyzed for their content of one of three oocyte proteins which are known to be derived from the blood.

An extract of yolk spheres obtained by this procedure thus contained the lysed spheres of from four to ten oocytes and, in addition, other cellular matter which had been trapped in the interstices between the yolk spheres when they were initially packed by centrifugation. Absent from the yolk extract were 10 per cent or more of the yolk spheres which had been mechanically lysed during the isolation procedure. The supernatant preparation thus contained lysed yolk spheres to addition to the constituents of the oocyte which are not sedimented at 500 *g*. If corrections could be made for the extent to which each fraction was contaminated by the other, a comparison of the concentrations of a protein in the two preparations would reveal the proportion of that protein in the oocyte which is associated with the yolk spheres.

The antigenic composition of the two preparations was analyzed with the antiserum-agar technique of Oudin. The details of our application of this technique and its potential sources of error have already been discussed in detail (24, 33). The composition of the extraction medium and of the antiserum-agar was planned so that the potential sources of error in the measurement of antigen concentrations were limited to possible structural differences between an antigen extracted from the yolk spheres and its counterpart in the supernatant.

Three proteins were studied: the female protein, a carotenoid protein, and bovine serum albumin which had been injected into the maternal blood before the period of yolk formation had commenced (30). In all three cases, four to five times as much protein was derived from the sedimented yolk spheres as from the supernatant (Table I). The observed ratios are necessarily lower than they should be. In particular, the fact that some

FIGURES 6 to 9

Yolk spheres suspended in various solutions of NaCl and sucrose and viewed by standard light microscopy. × 900.

FIGURE 6

Five minutes in 0.3 M NaCl.

FIGURE 7

Thirty minutes in 0.3 M NaCl.

FIGURE 8

Five minutes in 0.6 M NaCl.

FIGURE 9

Five minutes in 0.3 M sucrose.

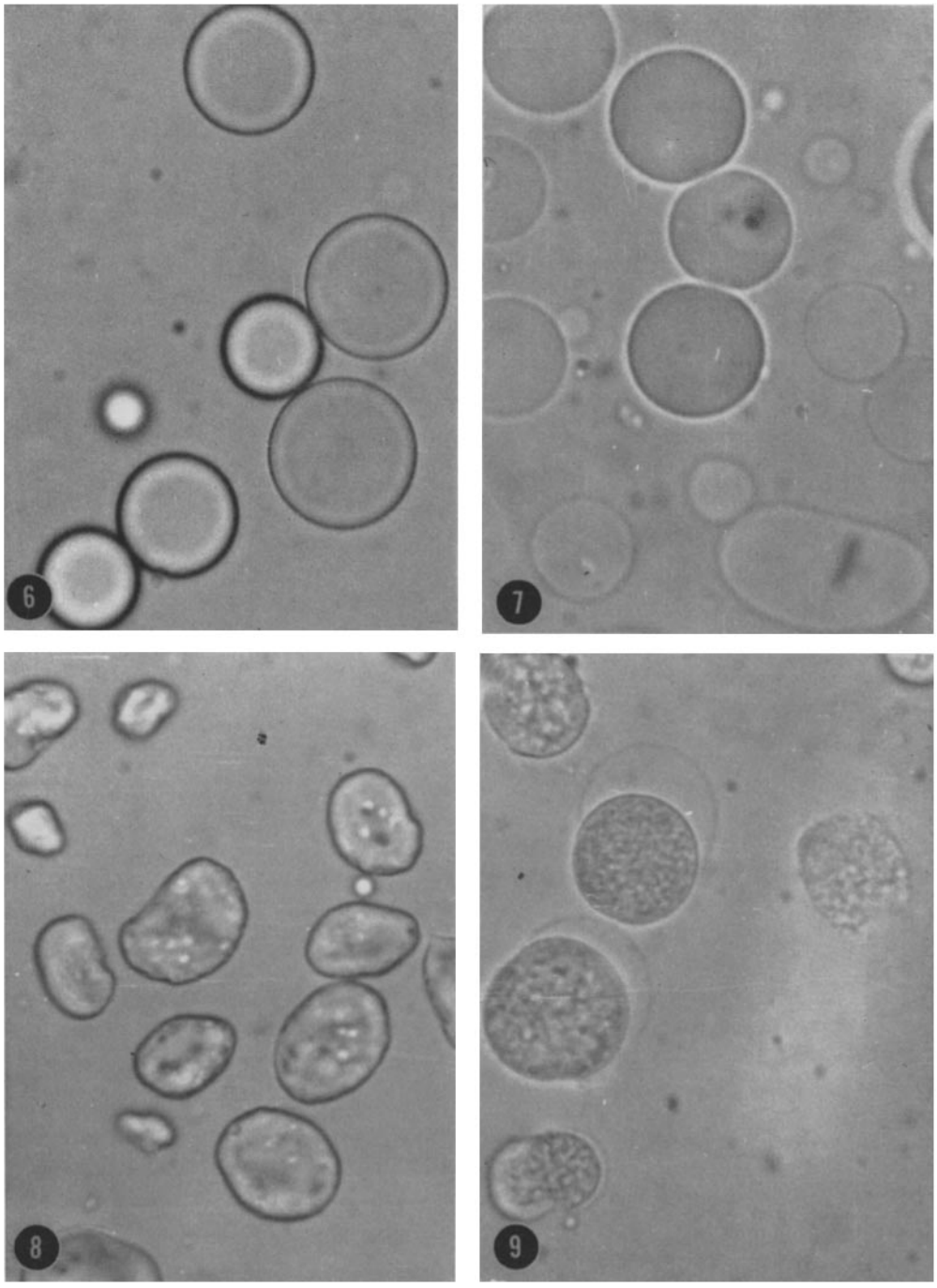
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TABLE I

The Distribution of Three Blood Proteins Between Two Centrifugal Fractions Obtained from Mature Polyphemus Oocytes

Blood protein	Amount of protein in the yolk spheres relative to the amount in the 500 *g* supernatant		
	No. of observations	Range	Mean
Polyphemus female protein	8	3.4–6.5	4.6
Polyphemus carotenoid protein	8	3.3–6.6	4.9
Bovine serum albumin	6	2.5–7.9	3.9

of the yolk spheres lysed during the separation could account for much or all of the blood-derived protein present in the supernatant. The results thus agree with fluorescent antibody staining in indicating that blood proteins are localized primarily in the yolk spheres.

The Distribution of Yellow Pigments in the Oocyte

The fact that the yolk spheres are colored yellow has already been noted; a variety of types of evidence suggest that this is due to pigments which are associated with the proteins obtained from the blood. The blood also is colored yellow and the pigments of both oocyte and blood are protein-bound: they are non-dialyzable and are reversibly precipitated by 60 per cent saturated ammonium sulfate solution. The carotenoid and female proteins which are present in both blood and yolk carry conspicuous quantities of yellow pigments (33 and unpublished observations). Thus, if these proteins are deposited exclusively in the yolk spheres after accumulation by the oocytes, the cytoplasm surrounding the yolk spheres should be colorless.

Oocytes in the early stages of yolk formation are not as yet close-packed with particulate matter, so that centrifugation for 5 minutes at 10,000 *g* yielded a stratum of translucent cytoplasm between the layers of yolk and lipochondria. In contrast to the bright yellow yolk spheres, the translucent layer was not distinguishably pigmented. Fluorescent antibody staining thus appears to have been accurate in indicating that blood proteins are present in substantially greater concentration in the yolk spheres than in the cytoplasm which surrounds them. Indeed, blood proteins are not detectable in the cytoplasm by either of the techniques utilized.

DISCUSSION

The three types of observations described indicate that oocyte proteins which are known to be derived from the blood are localized primarily, and perhaps exclusively, in the protein yolk spheres. The consequences of this conclusion, and of a number of the observations which led to it, are discussed here with relation to the mechanisms of protein accumulation and of yolk formation.

The Site of Selectivity of the Protein-Accumulating Mechanism

Blood protein accumulation by the saturniid oocyte is non-selective to the extent that most, and possibly all, proteins in the blood enter the oocyte to some extent. The mechanism is adapted for the preferential uptake of particular proteins, however (30). The female protein is twenty to thirty times more concentrated in the oocyte as a whole than in the blood at the end of yolk formation, while other proteins appear in the oocyte in concentrations equal to or well under their concentrations in the blood. As will be demonstrated in a later paper, there is no apparent relationship between the diffusion coefficient of a protein and its capacity to enter the oocyte. Selectivity must in this case be explained by a differential adsorption on, or solubility in, some component of the ovary.

The possible locations of the mechanism of selectivity include the ovariole wall, the nurse and follicle cells, the surface of the oocyte, and the yolk spheres. Two observations are reported here which should aid in identifying the site of selectivity.

The results of fluorescence microscopy indicate that blood proteins are abundantly present between the follicle cells. Indeed, the unusually large size of these intercellular spaces might be considered an adaptation facilitating the diffusion of proteins from the blood to the surface of the oocyte. Though there is no evidence that intercellular blood proteins of the ovary are, in fact, *en route* to the oocyte, the sparsity of blood proteins within the follicle and nurse cells makes this interpretation plausible. It is thus unlikely that

these cells constitute a barrier which could admit proteins to the oocyte selectively.

If selective accumulation were due to a preferential avidity of the yolk spheres for particular blood proteins which have already reached the cytoplasm of the oocyte, one would expect to find corresponding differences in the distributions of different proteins between the cytoplasm and the yolk spheres. A molecule of female protein is accumulated twenty times more readily than a molecule of the carotenoid protein, and up to three hundred times more readily than a molecule of bovine serum albumin (30). Yet these three proteins were found to be similarly distributed between isolated yolk spheres and the remainder of the oocyte.

The analysis suggests that neither the yolk spheres nor the follicle and nurse cells determine the extent to which a blood protein will accumulate in the oocyte. By a process of elimination, the ovariole wall and the surface of the oocyte remain the most likely candidates, and a combined action of the two structures is a possibility.

The Nature of the Protein-Accumulating Mechanism

The disposition of the accumulated blood proteins within a cytoplasmic particle is a feature which the oocyte has in common with cells that obtain proteins by pinocytosis. One is thus led to question whether the oocyte also accumulates proteins by pinocytosis, and whether the yolk sphere is thus produced from pinocytic vacuoles. An alternative proposal is that blood proteins traverse the oocyte surface as individual molecules by diffusion or transport, and, having entered the cytoplasm, are incorporated secondarily by the growing yolk spheres.

One might propose that selectivity of protein accumulation is an argument against yolk formation by pinocytosis, since such a process would appear to be inherently non-selective. This consideration holds, however, only for the lumen of the pinocytic vacuole. Adsorption of protein on the cell surface, and consequently on the lining of the vacuole, has been demonstrated in amebae undergoing pinocytosis (7, 27), and what may well be a protein-carrier complex has been isolated (21). An adsorption of this sort which affected proteins differentially could readily lead to selective accumulation (1); a high degree of selectivity could be achieved by making the volume of the vacuole sufficiently small, and the proportion of its surface which is covered by the protein sufficiently large.

At the present time, blood proteins which have entered the saturniid oocyte have been unequivocally demonstrated only in the yolk spheres. There is no evidence that they occur in the cytoplasm of either mature or growing oocytes. There are two possible interpretations of this result. One is that blood proteins are in fact absent from the cytoplasm—that they pass directly from the surface of the oocyte to the yolk spheres. Pinocytosis could obviously accomplish this result if the droplets thus produced were precursors of the yolk spheres.

A second possibility is that blood proteins destined for the yolk spheres actually pass through the cytoplasm, but never accumulate there in amounts sufficient to be detected by the methods used here. The distinction between an individual molecule destined to be incorporated into a yolk sphere and a group of such molecules bound within a pinocytic droplet may be theoretically possible, but is difficult to achieve. At this point we need postulate only that a device exists for assuring that those blood proteins which enter the oocyte will be incorporated into the yolk spheres, rather than being released in quantity to the cytoplasm in a free state. It is important to note that this postulate calls for a synthetic contribution of the ovary to the yolk. The ovary does not play a passive role in the formation of yolk, but, by the present proposal, must synthesize those components of the oocyte surface which account for the selectivity of protein uptake and for the incorporation of such proteins into the yolk spheres.

A final point concerns the cytological structure of the oocyte surface. The brushy surface of the cecropia oocyte resembles to some extent the zona radiata of the growing oocytes of chickens (6) and of fish (8) and presumably has a fine structure similar to that of the microvillar surface of the frog's oocyte (17). Such a cellular structure occurs generally where rapid absorption is under way and particularly where pinocytosis is occurring (13); its conspicuous association with blood proteins (Figs. 3 and 4) confirms this interpretation in the present case.

On the basis of these considerations, the following scheme for protein uptake by the moth oocyte is proposed: *A* blood protein which has penetrated the ovariole wall crosses the follicle cell layer by

an intercellular route; on reaching the surface of the oocyte, it combines with a hypothetical carrier substance, the avidity of the combination depending on the structure of the protein; the protein then enters the oocyte, possibly in the form of a small pinocytic droplet, which is then transformed into a microscopically visible yolk sphere. Growth of the yolk sphere could then be due either to fusion with other yolk spheres (13), to the continued passage of blood proteins through a temporary connection with the oocyte surface, or to the uptake of substances derived from the cytoplasm of the oocyte. Though this scheme is in part speculative, it suffices to explain in reasonably simple terms the information at hand concerning the mechanism of blood protein accumulation in the saturniid oocyte.

The Site of Formation of the Protein Yolk Spheres of Insects

The preceding physiological considerations have implicated the oocyte surface in the formation of the yolk spheres. To what extent does this view conform with the cytological literature on the origin of yolk? Though there is a dismaying diversity of theories concerning the site of origin of the yolk (4), recent studies of a number of insects producing very yolky oocytes indicate that in these cases the protein yolk spheres grow primarily in the cytoplasm at the periphery of the cell (2, 3, 5, 22, 23, 25). At the outset of yolk formation in insects representing the Hemiptera, Isoptera, Orthoptera, and Coleoptera, the smallest identifiable precursors of the yolk spheres are localized at the periphery of the oocyte. During the subsequent period of yolk deposition, the periphery is the only region in which yolk spheres of graded size are observed. A similar growth zone is suggested here for the yolk spheres of moths.

Since the region of yolk sphere growth is adjacent to the layer of follicle cells, these cells have been implicated as possible sites of synthesis of the proteins and PAS-positive substances whose presence in the yolk spheres has been demonstrated histochemically (3, 22). This concept is reinforced by the fact that the major groups of animals which produce very yolky oocytes—Cephalopoda, Arthropoda, and Chordata—are also the groups in which the differentiating oocyte is invested by follicle cells. Unfortunately, experimental evidence for a synthetic contribution of the follicle cells to the yolk is still wanting.

Though it is clear that blood protein accumulation is involved in the formation of the yolk spheres, an additional contribution from the follicle cells is not as a consequence precluded. A more generalized proposal would be that the yolk sphere is formed at the oocyte surface from substances synthesized primarily outside the oocyte. This would leave open the interesting possibility that the follicle cells secrete substances into the intercellular spaces of the ovary and that these are incorporated by the oocyte along with and by the same mechanism as the proteins derived from the blood.

Participation of the nurse cells in yolk formation has been proposed for some insects (15, 16). Unless it develops that different insects have different mechanisms for producing the protein yolk spheres, however, the nurse cells cannot be directly involved. The oocytes of the Orthoptera and Homoptera, though surrounded by follicle cells, are not associated with nurse cells; yet they form protein yolk spheres with facility. Synthetic contributions of the nurse cells, in those insects which possess them, presumably reach the oocyte via a unique route: the cytoplasmic connectives retained from the last oogonial divisions. If one is correct in proposing that the formation of yolk spheres is a phenomenon of the oocyte surface, the nurse cells must be concerned with the synthesis of reserves possessing a different cytological form.

Yolk Formation in Vertebrates

Many of the attributes of yolk formation noted in the insect have been observed in the oocytes of vertebrates. Such features include a peripheral location of the yolk-forming mechanism in the oocytes of chickens (6) and of fish (8) and to some degree in frogs (35), the existence of a brush border at the surface of the oocyte (6, 8, 17), and the accumulation of serum proteins (11, 18) and their association with yolk bodies (10, 12).

The results of fluorescent antibody staining (12) and of differential centrifugation studies (10) have led to the proposal that the serum-like antigens of the frog oocyte, unlike those of the moth, are not exclusively associated with the yolk bodies, but are also detectable in the surrounding cyto-

plasm. Further analysis will be required to determine whether this difference results from technical discrepancies or from physiological differences in the mechanism of protein uptake. Otherwise, the resemblances are sufficient to warrant the general proposal that the protein yolk bodies in animal oocytes are primarily depositories of proteins accumulated by the oocyte from its environment.

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