THE DEPENDENCE OF CECROPIA YOLK FORMATION IN VITRO ON SPECIFIC BLOOD PROTEINS

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ABSTRACT

The capacity of cecropia vitellogenic follicles to form yolk during short-term in vitro incubation in female blood was analyzed by labeling with fluorescein-conjugated serum globulin, tritiated cecropia blood proteins, or tritiated amino acid. As judged by fluorescence microscopy or autoradiography, yolk formation during 3–8 hr in vitro was similar in rate and in protein uptake specificity to that observed in vivo. When follicles were incubated in cecropia male blood, 6% gamma globulin, or cecropia saline, the yolk produced was markedly inferior in quality and quantity to that generated in female blood. Purified preparations of vitellogenin, the primary female blood protein deposited in the yolk, were equivalent to whole female blood in supporting yolk formation; this protein seems, therefore, to have a specific stimulatory role. An enhancement of the rate of pinocytosis at the oocyte surface by vitellogenin is postulated.

INTRODUCTION

Protein transport mechanisms which are able to sort out specific molecules from heterogeneous extracellular mixtures have been demonstrated in several types of animal tissue. Particularly clear examples are provided by maternal antibody transport by the embryonic yolk sac in rabbits, and by the small intestine in suckling rodents (reviewed by Brambell, 1966), and vitellogenic blood protein sequestration by the ovarian follicles of amphibians (Wallace and Jared, 1969a) and of insects (Telfer, 1960). In the ovaries of the cecropia moth, the transport mechanism favors a sex-limited blood protein, vitellogenin, which becomes 20–30 times more concentrated in the oocyte than in the blood, while other proteins, including injected foreign proteins, such as bovine gamma globulin, are largely excluded (Telfer, 1960).

The process of vitellogenin transport includes permeation of the intercellular spaces of the follicle (Telfer, 1961), followed by pinocytosis at the oocyte surface entailing coated vesicles of the sort described in mosquito oocytes by Roth and Porter (1964). While the route of entry seems clear, the mechanism by which vitellogenin is sorted out from other extracellular proteins prior to enclosure within the pinocytic vesicles remains obscure. Selective adsorption to the oocyte surface (Telfer, 1961; Roth and Porter, 1964) and preliminary interactions with adsorbents in the intercellular spaces of the follicular epithelium (Anderson and Telfer, 1970, a and b) are both potentially involved. The system is sufficiently complex, however, so that the relative contributions of the two steps have not as yet been determined.

In order to make the vitellogenin transport
mechanism more accessible to experimental analysis, the capacity of ovarian follicles to continue protein uptake and yolk deposition in vitro has now been explored, using fluorescein-conjugated rabbit serum globulin (FSG) as a label. The follicle has proven sufficiently resilient to continue vitellogenesis for at least 8 hr after removal from the animal. The reliability of the incubation and labeling procedures is established here by showing that both the rate of yolk deposition and the selectivity of the transport mechanism in vitro conform to expectations based on the performance of the follicle in situ.

Using these techniques, we found that the amount of yolk deposited in vitro is strongly dependent upon the availability of vitellogenin—bovine gamma globulin and cecropia male blood proteins were not by themselves able to support significant amounts of yolk deposition. While there are several potential explanations of this finding, it is proposed that the follicular protein transport mechanism both selects and requires activation by vitellogenin.

METHODS

FSG Labeling and Incubation Media

The use of fluorescein-labeled rabbit serum globulin (FSG) as a label was suggested by unpublished observations on its behavior in vivo. Following injection into developing cecropia females, FSG was deposited in all newly formed yolk spheres, and even after 24 hr these remained in a stable fluorescent stratum, 80-100 µ thick, without mixing with the unlabeled yolk that had been laid down prior to the injection. Labeling of the yolk 3 hr after injection of 0.25 ml of the FSG solution described below is illustrated in Fig. 1. Since strata of comparable depths have been observed with other methods of labeling (Melius and Telfer, 1969), FSG has no detectable effect on the vitellogenic mechanism. FSG injections have also been used successfully to demonstrate yolk formation from blood proteins by Rhodnius prolincus (Coles, 1963).

A solution of FSG (Nutritional Biochemicals Co., Cleveland, Ohio, the globulin fraction of a rabbit antisem to ovalbumin, conjugated with fluorescein isothiocyanate and preserved with 1:10,000 merthiolate; total protein content about 6%) composed 10-30% by volume of the incubation media. The remaining 70-90% was made up of experimental mixtures whose components included various combinations of female or male cecropia blood, dialyzed blood proteins, a preparation of purified vitellogenin, or a 6% solution of gamma globulin (Cohn Fraction II) in cecropia saline (Anderson and Telfer, 1969).

Cecropia blood was collected in a test tube containing a few crystals of phenylthiourea to prevent melanin formation and cleared of cells by centrifugation at 10,000 g at 4°C for 10 min. The blood either could be used fresh, in which case it was often from the same animal as the follicles being incubated, or it could be stored at -20°C. The blood of either developing adults or diapausing pupae sufficed, though in the latter case it required dilution to 1/5 of its original volume with cecropia saline to reduce the osmotic pressure. The blood and tissues of diapausing pupae may contain as much as 0.6 M glycerol (Wilhelm et al., 1961), and we have found that developing tissues fare better in such blood after dilution.

Preparation of Purified Vitellogenin

Vitellogenin was purified from the blood of three females (14-15 day of adult development) on a diethylaminoethyl (DEAE)-cellulose column by the method of Pan and Wallace (personal communication), using a nonlinear Tris-citrate buffer gradient (starting buffer: 22.5 mM Tris, 1.5 mM citric acid, pH 8.7; limiting buffer: 225 mM Tris, 75 mM citric acid, pH 6.0). Vitellogenin was identified in the eluents by Oudin's antiserum-agar test (Telfer and Williams, 1953).

Approximately 1 ml of blood from each female was chromatographed separately and the fraction of the eluents containing the vitellogenin was concentrated by ultrafiltration to a final volume of about 1 ml. The three concentrates were then pooled and further concentrated to a volume of 0.5 ml, which was dialyzed against cecropia saline for 2 hr at 4°C. The final preparation was assayed by the Oudin test for vitellogenin and a carotenoid protein which also occurs in both blood and yolk (Telfer, 1960). The concentrations of these proteins, relative to their levels in a standard consisting of blood from a female on day 17 of adult development, were approximately 110% for vitellogenin, and 3% for carotenoid protein. To estimate the total concentration of protein in the mixture the absorption at 280 μM of a 1:50 dilution of the sample in cecropia saline was compared with that of a bovine gamma globulin standard. The protein concentration in the vitellogenin sample was approximately 3 mg/ml.

Incubation Procedures

Follicles to be used for incubation were removed from females on day 17-20 of adult development through a dorsal incision in the abdomen; the follicles selected were 1.4-1.6 mm long and still retained their cap of nurse cells. Thus, under normal in vivo conditions they would have continued to form yolk
for at least 24 hr (Telfer and Anderson, 1968). In some cases the follicles were rinsed for about 3 sec in cecropia saline and transferred directly to 0.1-0.25 ml of the incubation mixture in spot plate depression slides. The depression was covered with a glass coverslip and sealed with paraffin or placed in a humidified Petri dish. The plates were then agitated on a mechanical shaker (approximately 1 r.p.m.) at 26°C.

In other experiments follicles were first placed in a 6% solution of unlabeled gamma globulin in cecropia saline to extract extracellular blood proteins (Anderson and Telfer, 1970) and to permit removal of the ovariole sheath before incubation. When transferred to labeling medium, these follicles produced the same amounts of fluorescent yolk as follicles which had been transferred directly to the incubation medium from the animal, indicating that neither the ovariole sheath nor the extracellular blood proteins initially trapped in the ovariole significantly affected the results.

As a test of survival at the termination of incubation, a few follicles from some experiments were placed in a 1% solution of trypan blue in modified cecropia saline (Anderson and Telfer, 1970) for 15 min and then rinsed in cecropia saline. In healthy follicles, like those freshly dissected from the animal, the dye is excluded from the cytoplasm of the cells, but is bound intercellularly in the follicular epithelium (Telfer and Anderson, 1968). Dead cells and oocytes could be readily detected because they were deeply stained with dye. Such fatality occurred only rarely during the experiments described here, and could not be attributed to any particular feature of the incubation procedures.

**Histology and Microscopy**

At the conclusion of the incubation period the follicles were freeze-substituted (Melius and Telfer, 1969), embedded in paraffin, and cut into sections 10 µ thick. Sections were then mounted on glass slides. Although freeze-substitution may distort somewhat the intercellular spaces of the follicle (Anderson and Telfer, 1970), it preserves both the structure of the yolk and the fluorescence of the marker satisfactorily. For low-power fluorescence microscopy it was not necessary to remove the paraffin; in fact, the fluorescence was found to be more stable if the sections remained in paraffin.

Microscopy was performed with a Zeiss Universal Fluorescence Microscope with a high-pressure mercury burner as the light source, and with the filter combination II-50/47. Under these conditions, the fluorescence due to FSG was visible as a bright, greenish yellow against a background of pale green autofluorescence. The thickness of the band of fluorescent yolk spheres was measured with a filar micrometer and recorded photographically with either Tri-X Pan (3-5 min exposure time) or High Contrast Copy Film (10 min exposure time). When the thickness of the stratum varied in different regions of a given follicle, that stratum of greatest thickness was measured, since the objective was to determine the maximum vitellogenic capability under the given conditions, and some local injury was expected in handling. All results reported are representative of experiments repeated at least three times on follicles of different animals.

**Autoradiography**

To confirm the results of FSG labeling, the follicles in some experiments were incubated with tritiated histidine (l-histidine-2,5-3H, 14,300 mCi/mmmole, 1 mCi/ml; Nuclear-Chicago, Des Plaines, Ill.) or blood proteins. To obtain male or female blood containing tritiated proteins, three animals of each sex were injected on day 13 or 14 of adult development with 0.1 ml leucine-3H (l-leucine-4,5-3H in sterile water; New England Nuclear Corp., Boston, Mass., 5000 mCi/mmmole, 1 mCi/ml), and the blood was collected after 24 hr. The labeled male blood samples were centrifuged at 10,000 g, pooled, and stored at -20°C. From the labeled female blood, vitellogenin was isolated by DEAE-cellulose chromatography and concentrated by ultrafiltration as previously described. Oudin tests indicated that the preparation contained vitellogenin at 100% and carotenoid protein at 2.5% of their concentrations in day 17 female blood. The amounts perchloric acid-precipitable label in the male blood and in the vitellogenin preparation differed by only 3%, as determined by a glass fiber filter disc method (Mans and Novelli, 1961).

Following incubation in the radioactive medium, the follicles were fixed either by freeze-substitution or in Bouin's solution, embedded in paraffin, and cut into sections 10 µ thick. Sections were coated with NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y.), exposed for 3 wk, and developed with Kodak D-11 for 4 min at 20°C.

**RESULTS**

**Yolk Formation in Female Blood In Vitro**

Follicles incubated in female blood and FSG formed strata of fluorescent yolk spheres comparable to those observed after in vivo labelling (Figs. 2 and 3). The labeled stratum was approximately 20 µ deep after 3 hr (Fig. 2), and increased in depth with time to about 40 µ after 6 hr (Fig. 3). Comparable results were obtained when the FSG comprised either 10% or 30% of the incubation medium, and when the follicles were...
FIGURE 1  A cecropia female in the last week of adult development was injected with 0.25 ml of a solution of fluorescein-conjugated serum globulin (FSG) and dissected after 3 hr. Follicles were fixed by freeze-substitution and processed as described in the text. Following the in vivo exposure to FSG, fluorescence was most conspicuous in the basal lamina of the follicular epithelium on the left and in the yolk spheres lying at the periphery of the oocyte. Several layers of unlabeled yolk spheres are seen at right. × 260. Scale on fluorescence micrographs is 20 µ.

FIGURES 2 and 3 Fluorescence micrographs of vitellogenic follicles after incubation in vitro in female blood containing FSG for 3 hr (Fig. 2) and 6 hr (Fig. 3). × 260.

either transferred directly from the animal to the test solution or preincubated for 1 hr in 6% gamma globulin.

Inclusion of 0.1–1.0 mM dinitrophenol (DNP) in the medium during a 3–6 hr period of incubation completely inhibited the formation of the fluorescent layer. When follicles that had been treated with DNP for 3–6 hr were returned to fresh blood for 3 hr, they formed a 15–20 µ deep layer of fluorescent yolk spheres, indicating that the inhibition was reversible. Thus, fluorescein labeling of the cortical yolk spheres is apparently an energy-requiring process, and is interpreted here as being due to the incorporation of FSG in yolk spheres formed during the incubation period.

Autoradiographic techniques allowed us to confirm that the rate and distribution of yolk produced in vitro were the same as in vivo, and also to show that the preference of the follicle for vitellogenin was maintained. Selectivity was demonstrated by incubating follicles in mixtures of male blood and purified vitellogenin, in which a tritium label was in some cases borne by the male blood proteins, and in others by the vitellogenin. The incubation mixtures contained equal volumes of the vitellogenin preparation and of male blood; they were identical with respect to vitellogenin content, concentration of label in protein, and amount of whole male blood, and should therefore have provided identical conditions for the promotion of yolk deposition. Since male blood labeled under the conditions used contains acid-soluble as well as protein-bound label (Melius and Telfer, 1969), 0.01 M leucine was included in both cultures to reduce incorporation of the tritiated precursor into the cells of the follicle. The capacity of the two mixtures to support yolk formation was confirmed by adding 10% (by volume) FSG in some experiments. Fluorescence microscopy indicated that in most follicles tested a 15–20 µ deep stratum of FSG-labeled yolk spheres was produced during 3 hr of in vitro incubation.

In autoradiographs of follicles incubated for 3 hr in labeled vitellogenin and unlabeled male blood, the silver grains were substantially more concentrated over the peripheral stratum of yolk spheres (Fig. 4) than over any other component of the follicle. In the reverse situation, unlabeled vitellogenin and labeled male blood, the peripheral yolk spheres were only sparsely covered.
FIGURES 4 and 5 Autoradiographs of vitellogenic follicles incubated for 3 hr in tritiated mixtures of male blood and purified vitellogenin. The tritium label was borne either by the vitellogenin (Fig. 4) or by the male proteins (Fig. 5). Silver grains over the follicular epithelium at left are largely out of focus in the photographs. X 600. Scale on autoradiographs is 10 µ.

with grains (Fig. 5). A low level of epithelial labeling occurred in both cases, particularly in the intercellular spaces which are traversed by vitellogenic blood proteins (Meltus and Telfer, 1969; Anderson and Telfer, 1970 b), but also in the epithelial cells of the follicles incubated in labeled male blood, which contained tritiated leucine as well as labeled protein. Aside from this difference, it was clear that labeled male blood proteins were not incorporated by the oocyte as readily as vitellogenin. Thus neither the in vitro conditions nor the presence of FSG interfered in a detectable way with the selectivity of protein incorporation by the oocyte.

Dependence of Yolk Formation on Female Blood

In order to examine the dependence of yolk formation on female blood, follicles were incubated for 7-hr periods in amounts of whole female blood ranging from 0 to 90% of the volume of the incubation medium. The FSG solution comprised 30% (10% for blood concentrations greater than 70%); and the balance of the volume was made up with 6% bovine gamma globulin in cecropia saline. This concentration was selected because it approximates the total protein content of female blood on the 16th to the 20th days of adult development. The follicles were preincubated for 1 hr in unlabeled gamma globulin solution to remove blood proteins present initially in the intercellular spaces.

In the absence of female blood, vitellogenesis was dramatically reduced: fluorescence was detected only in droplets less than 1 µ in diameter in the cortex of the oocyte (Fig. 6). The presence of as little as 2% female blood resulted in the formation of a measurable stratum of yolk spheres of 2-4 µ size. The depth of the stratum increased with greater concentration to a maximum thickness of 45 µ, obtained with a blood concentration of 50% (Table I, Fig. 3).

To ascertain whether small molecular weight metabolites present in the blood contributed to the requirement for female blood, a sample of blood was dialyzed for 3 hr in the cold against two changes of cecropia saline prior to use in an incubation mixture. The nondialyzable fraction was found to be as effective as whole blood in promoting yolk formation during a 3 hr incubation (compare Fig. 7 with Fig. 2). Thus, the dialyzable components of the blood can be replaced by cecropia saline during the incubation time employed without obviously impairing yolk deposition.

Stimulation of Yolk Formation by Vitellogenin

The next question to be considered was the extent to which different blood proteins were
capable of stimulating the production of yolk spheres. Cecropia male blood is qualitatively similar to female blood with the exception of a much reduced concentration of vitellogenin (Telfer, 1954), and thus provided a medium for testing the effect of the absence of this protein. Follicles were incubated in dilutions of developing male blood ranging from 2 to 70%. The yolk which formed was confined to a shallower stratum than that observed following incubation in female blood, and within that stratum consisted of smaller and more scattered yolk spheres, so that measurement of depth had little meaning (Fig. 8). As in the case of female blood, greater concentrations of male blood seemed to yield greater amounts of yolk.

That male blood stimulated more FSG labeling of yolk than did bovine gamma globulin may be attributed to the fact that it contains several components normally involved in yolk deposition. An example of this is the carotenoid protein, which is present in yolk at a concentration comparable to that in the blood, and for which the follicles therefore have a greater avidity than for bovine gamma globulin (Telfer, 1960). By the same token, the small amount of yolk formed in male blood compared to female blood seemed likely to be due to the absence of vitellogenin. This possibility was first tested by supplementing male blood with dialyzed female blood proteins; these were found to restore yolk formation to a normal level (Fig. 9).

The proposal that vitellogenin is the main component of female blood responsible for the promotion of yolk formation was confirmed by observing the genesis of yolk spheres in male blood to which a purified fraction of vitellogenin had been added. After 3 hr in a mixture containing, by volume, 45% male blood, 45% purified vitellogenin solution (see Methods), and 10% FSG, the follicles formed a layer of fluorescent yolk spheres 15-20 µ deep, and therefore comparable to that formed within 3 hr in 50-90% female blood. The size and refractility of the newly formed yolk spheres were also comparable under the two conditions.

Finally, follicles were incubated in purified vitellogenin alone plus 10% FSG. As seen in Fig. 10, the labeled yolk formed was similar to that generated in the same period in whole female blood (Fig. 2), or dialyzed female blood proteins (Fig. 7). The purified vitellogenin fraction alone was therefore able to promote FSG uptake to a degree which neither male blood proteins nor gamma globulin could support.

In Vitro Yolk Formation Labeled with Histidine-3H

Incubation of follicles in vitro in female blood containing tritiated histidine results in the appearance of fixable label in the cells of the follicular epithelium and in the newly formed peripheral yolk spheres (Anderson and Telfer, 1969). While some of the labeled material in the yolk spheres may be synthesized in the oocyte cortex, it was shown that a more prominent fraction is derived, like the blood proteins, from the intercellular spaces, into which it had presumably been secreted by the follicle cells. In the absence of cecropia blood proteins in the medium, this material forms labeled bodies in the oocyte cortex very much like

| Concentration of female blood | Thickness of fluorescent yolk stratum after 7 hr incubation |
|-------------------------------|------------------------------------------------------------|
| % by volume of the incubation medium | µ |
| 2 | 11 ± 0.9$ (5)$ |
| 5 | 14 ± 1.3 (4) |
| 7 | 17 ± 2.4 (5) |
| 10 | 23 ± 1.5 (13) |
| 20 | 28 ± 4.0 (27) |
| 30 | 30 ± 2.9 (18) |
| 40 | 33 ± 4.1 (30) |
| 50 | 40 ± 3.4 (13) |
| 60 | 39 ± 5.4 (16) |
| 70 | 43 ± 4.4 (24) |
| 80 | 40 ± 5.5 (13) |
| 90 | 37 ± 5.3 (11) |
| 100| 36 ± 5.5 (7) |

* Follicles were incubated in a medium consisting of female blood at the concentration indicated, 30% FSG, and, when necessary, 6% gamma globulin in cecropia saline.
† Standard deviation.
§ No. of measurements.
|| The depth of yolk made in 100% female blood in vitro was determined by injecting a female with the label prior to incubation in unlabeled, 100% female blood; the displacement of the labeled layer then indicated the depth of the yolk made in vitro.

Table I

Effect of the Concentration of Female Blood on Yolk Deposition*
FIGURE 6  Fluorescence micrograph of follicles preincubated in 6% gamma globulin for 1 hr and then incubated for 6 hr in 6% gamma globulin containing 30% FSG. A few small spheres (arrow) in the oocyte cortex contained label. (The patchy luminescence of the mature yolk spheres deeper in the oocyte was due to refraction of incident light by irregularities in the section, rather than to fluorescence.) × 260.

FIGURE 7  Fluorescence micrograph of follicles incubated for 3 hr in a solution containing 90% dialyzed female blood and 10% FSG. The labeled yolk formed is comparable to that generated in whole female blood under similar conditions (Fig. 2). × 260.

FIGURE 8  Two preincubated follicles exposed for 5.5 hr to a medium consisting of 50% male blood, 30% FSG, and 20% gamma globulin (6% gamma globulin in cecropia saline). The follicles are separated by some of the labeled incubation medium fixed there by freeze-substitution. × 260.

FIGURE 9  A follicle incubated for 8 hr in a medium containing 30% male blood, 40% dialyzed female blood proteins, and 30% FSG. × 260.

FIGURE 10  Fluorescence micrograph of a follicle incubated for 3 hr in a solution containing purified vitellogenin and 10% FSG. A fragment of the ovariole sheath is present at left. × 260.

the small fluorescent bodies (Fig. 6) generated by follicles incubated in 6% gamma globulin and FSG. It seemed possible that the two procedures were detecting functionally equivalent structures, i.e., incipient yolk spheres produced by pinocytosis of extracellular materials lacking in vitellogenic blood proteins.

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sphere precursors provided an opportunity both to study the recovery of the oocyte from vitellogenin deprivation and to confirm autoradiographically the differences seen above in the capacity of various protein solutions to promote yolk deposition.

For this purpose follicles were washed for 1.5 hr in cecropia saline to elute the blood proteins in the intercellular spaces, and then incubated for 2 hr in saline containing 0.1 mCi/ml of histidine$^3$H for 2 hr. The follicle cells, the brush border at the oocyte surface, and scattered small bodies in the cortex of the oocyte became heavily labeled. $\times 600$.

**Figures 11-14** Autoradiographs of follicles soaked in saline for 1.5 hr and labeled with histidine$^3$H as in Fig. 11; they were then incubated for 3 hr in either female blood (Fig. 12), 6% gamma globulin in saline (Fig. 13), or male blood (Fig. 14). $\times 600$.

...
bation in histidine-\textsuperscript{3H}. The small prelabeled bodies were, in any case, no longer evident either in the cortex or deeper in the oocyte, and had therefore apparently retained their ability to combine with the yolk spheres formed in female blood.

In contrast, when prelabeled follicles were incubated in 6\% gamma globulin, the silver grains remained confined to the 2–5 \( \mu \) bodies in the cortex of the oocyte (Fig. 13). During incubation in male blood, yolk formation resumed to only a small extent, producing an 8–10 \( \mu \) stratum of 3–7 \( \mu \) diameter labeled yolk spheres (Fig. 14). In short, the capacity of each of the four mixtures to promote yolk formation in follicles prelabeled with histidine-\textsuperscript{3H} paralleled its ability, described above, to promote the genesis of yolk spheres in the presence of FSG. The two independent labeling techniques therefore agree in indicating that normal vitellogenesis is dependent on the presence of female blood proteins.

**DISCUSSION**

In order to reach the surface of the moth oocyte, yolk proteins must pass through the intercellular spaces of a porous epithelium, where they appear to be complexed with secretory products of the follicle itself (Anderson and Telfer, 1970\textsuperscript{b}). A variety of experiments, made possible by the methods of in vitro incubation which are described fully for the first time here, strongly suggest that vitellogenic blood proteins are deposited in the yolk spheres in combination with these endogenous secretory products (Anderson and Telfer, 1969; 1970\textsuperscript{a} and \textsuperscript{b}). The interactions between blood proteins and the vitellogenic follicle are thus potentially more complex than the events leading up to pinocytosis in physically independent cells (e.g. Marshall et al., 1959, for *Chaos chaos*; Chapman-Andresen, 1965, for *Amoeba proteus*; Ryser, 1968, for mammalian cells in culture). Nevertheless, the product of pinocytosis in the cecropia oocyte is a clear and measurable stratum of yolk spheres containing a well-defined selection of blood proteins (Telfer, 1960). Despite the complexity of the follicle, therefore, several aspects of its protein transport mechanism can now be analyzed in vitro.

The data obtained thus far indicate that the capacity of a blood protein to be accumulated in vivo is directly related to its ability to promote FSG-labeled yolk deposition. Bovine gamma globulin, which is accumulated in only trivial amounts by the protein uptake mechanism in vivo, was able to support the formation of no more than a thin stratum of small cortical bodies when it was the primary constituent of an in vitro incubation medium. The small amount of FSG uptake that did occur may not have required the 6\% gamma globulin in the medium, for autoradiographic experiments showed that similar cortical bodies (Fig. 11), probably containing secretory products of the epithelium (Anderson and Telfer, 1969; 1970\textsuperscript{a}), were produced upon incubation in cecropia saline without proteins. Finally, an intermediate level of yolk deposition was elicited by male blood proteins, which, though lacking vitellogenin, included molecules such as the carotenoid protein with a significantly greater capacity to enter the yolk in vivo than is shown by gamma globulin.

What then is the mechanism of vitellogenin's stimulatory action? Blood proteins are incorporated along with extracellular follicle cell products in coated pinocytotic vesicles which fuse to form the mature 10–20 \( \mu \) wide yolk spheres. The production of small fluorescent bodies in the cortex in the absence of vitellogenin indicates that pinocytosis had continued to some degree under these conditions, so that vitellogenin is not an absolute requirement for pinocytosis, although it grossly affects the volume of materials deposited in the yolk spheres.

Such an effect might arise in one of three ways. First, it might be that the rate of pinocytotic uptake by the oocyte is specifically stimulated by vitellogenin. By this hypothesis, the arrival of vitellogenin in combination with epithelial products at the oocyte surface would either accelerate the formation of pinocytotic vesicles or increase their size.

A second possibility would be that the oocyte incorporated extracellular materials at equal rates with and without vitellogenin, but that in the latter instance, the ingested materials were either resorbed or dispersed throughout the deeper strata of cytoplasm in a form which could not be seen by fluorescence microscopy. While present data do not disprove this interpretation, the fact that the small yolk precursor bodies are retained in the cortex through a 6 hr incubation period without being disseminated or released suggests that pinocyotosed material remains localized there.

A third consideration acknowledges the fact that, despite their density and refractility, yolk spheres contain 60–70\% water (Telfer and
Anderson, 1968), much of which could conceivably be added to preformed pinosomes by osmosis rather than incorporated during pinocytosis. If most of the water of the yolk spheres were obtained in this manner, the material entering by pinocytosis would have, prior to osmotic swelling, a volume equaling 30-40% of the mature yolk volume. If in the present experiments the effect of vitellogenin deprivation was to prevent osmotic swelling rather than to reduce the volume or number of the pinosomes produced, then the FSG-labeled bodies produced in 6% gamma globulin should have achieved 30-40% of the mature yolk sphere volume. The total volume of the FSG-labeled bodies in the cortex of the oocytes incubated in gamma globulin was probably not more than a few percent of that of the yolk produced in vitellogenin, however, (compare Figs. 3 and 6), so that it seems unlikely that a lack of hydration, without a simultaneous reduction in the volume of pinocytosed material, could account for this result.

The most likely possibility, therefore, is the first mentioned above, that vitellogenin determines the volume of yolk deposited, at least in part by controlling the rate of pinocytotic intake at the oocyte surface. The complexity of the system prevents us, on the basis of present evidence, from defining in detail the mechanism by which vitellogenin could promote pinocytosis, although several alternatives are evident. It could directly stimulate pinocytosis upon combining with the oocyte surface; on the other hand, it could stimulate the follicle cells to release an activator; or it could simply penetrate the follicular epithelium and associated membranes more readily than other proteins.

With the incubation and labeling system whose validity is established here, these uncertainties concerning the mechanism of protein transport in insect follicles appear for the first time to be analyzable in vitro. Wallace and Jared (1969) have recently succeeded in obtaining in vitro the selective uptake of proteins by the ovary of Xenopus laevis, so that the protein transport mechanisms in both vertebrate and insect ovaries now appear to be amenable to study by these methods.

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