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THE CORPUS ALLATUM AND OOGENESIS IN *RHODNIUS PROLIXUS* (STÅL.)

I. THE EFFECTS OF ALLATECTOMY

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INTRODUCTION

A mated female of *Rhodnius prolixus* produces its eggs in cyclical fashion: each feed results in a cycle of egg production and oviposition, the number of eggs produced depending upon the size of the meal ingested (Buxton, 1930; Friend, Choy & Cartwright, 1965). The corpus allatum has long been known to be essential for the full expression of egg production (Wigglesworth, 1936). More recently, quantitative studies demonstrated that allatectomized mated females lay eggs at about one-third the rate of normal females (Davey, 1967). It is also known that virgin females lay fewer eggs than mated females and that the effects of mating upon oviposition are mediated through the neurosecretory cells of the pars intercerebralis (Buxton, 1930; Davey, 1965, 1967). In the present paper some of the effects of allatectomy will be analysed. Subsequent papers will deal with the relationship of the gonadotropin of the corpus allatum to mating and starvation.

MATERIALS AND METHODS

Experimental animals

The insects used in these experiments were part of a large culture maintained in a humid environment at 28 °C. Females were separated from males at moulting and exposed, usually overnight, to two males in individual glass jars. The subsequent appearance of a spermatophere in the jar was taken as evidence of mating. The animals were fed 7–10 days after emergence, and measurements were made upon the first cycle of egg production.

Allatectomy was carried out as described earlier (Davey, 1967) during the post-emergence period before the first feed. Survival of the operated animals was better than 80%, although many of the allatectomized animals did not gorge fully and were eliminated from the experiments. Allatectomy as performed in this laboratory depresses slightly the level of protease activity in the intestine, but does not affect the cyclical nature of this activity (Persaud & Davey, 1971).

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Preparation of ovarioles for measurement

Ovaries were removed on successive days after feeding and fixed in 4% glutaraldehyde in Sørensen's buffer at pH 7·2. The ovarioles were dissected from the ovary and stained overnight in 1% Ponceau S in 5% aqueous trichloracetic acid, washed in 5% trichloracetic acid until no further stain was eluted, dehydrated in ethanol, cleared in xylol and mounted in Canada balsam. Ovarioles prepared in this way are suitable for measuring the length of the three most terminal oocytes with negligible distortion. The patency of the follicular epithelium is also discernible, and the refractile bodies in the tropharium are also easily observed.

In order to count the mitoses in the tropharium, ovaries were fixed for 30 min in Carnoy's fluid, washed and rehydrated. The ovaries were stained in Hansen's trioxyhaematin for 90 sec, dehydrated in an alcohol series and cleared in xylol. The cleared ovaries were dissected and the ovarioles were mounted in Permount. Mitotic figures are readily identified and counts were made of all the mitoses in all of the ovarioles from two animals on each of the first 5 days after feeding, in normal and in allatectomized animals.

The patency of the follicular epithelium was most readily tested by immersing freshly dissected ovarioles in a 1% solution of Evans blue in saline. The ovarioles were transferred to a slide without rinsing and viewed by transmitted light without a coverslip. Measurements of the length of the oocyte can be made at the same time as the observation of the penetration of the dye into the lateral intercellular spaces of the follicular epithelium.

Yolk protein in the haemolymph

Yolk-protein concentrations were determined in freshly collected and diluted haemolymph by minor modifications of the radial immunodiffusion technique of Fahey & McKelvey (1965). Cross-reacting yolk protein was prepared from homogenates of ovaries by centrifugation and repeated chromatography on columns of Sephadex G-200. Antiserum was prepared in rabbits by three injections, administered at weekly intervals, of 10 mg quantities in Freund's complete adjuvent. Details of the preparation and properties of the purified yolk protein and its antiserum will appear elsewhere (Pratt, in preparation). Radial immunodiffusion was carried out with 5 μl samples of haemolymph serially diluted in the saline solution. Antiserum was employed at a dilution of 1/15 in 0·75% agarose in 0·02 M sodium barbitone buffer at pH 8·3. Each radial diffusion was separated from interference from neighbouring samples by pouring measured quantities of the antiserum-agarose solution into individual circular wells 1·6 cm in diameter and 2 mm deep. The test antigen was added to a hole punched in the centre of the cooled gel and left to diffuse for 18–22 h at 20 °C in high humidity. Standards of purified yolk protein were run with each analysis and a standard curve was drawn. The relationship was highly reproducible, but differed from that reported by Fahey & McKelvey (1965) in not being strictly logarithmic. In practice, the titre of yolk proteins in any haemolymph sample was determined by the response given by at least two dilutions. The sensitivity of the method is inversely related to the precipitation titre of the antiserum employed and reaches its limit when the ring of precipitation is no longer visible to the eye under dark-ground illumination. In the present series of observations the test was run at mean maximal sensitivity and

The detection limit was 0·5 μg of yolk protein per sample well. In most cases, suitable dilutions of haemolymph were in the range 1/5 to 1/20, so that there was negligible influence of sample viscosity on the diffusion of antigen into the gel loaded with antiserum.

Fluorescein-labelled yolk protein

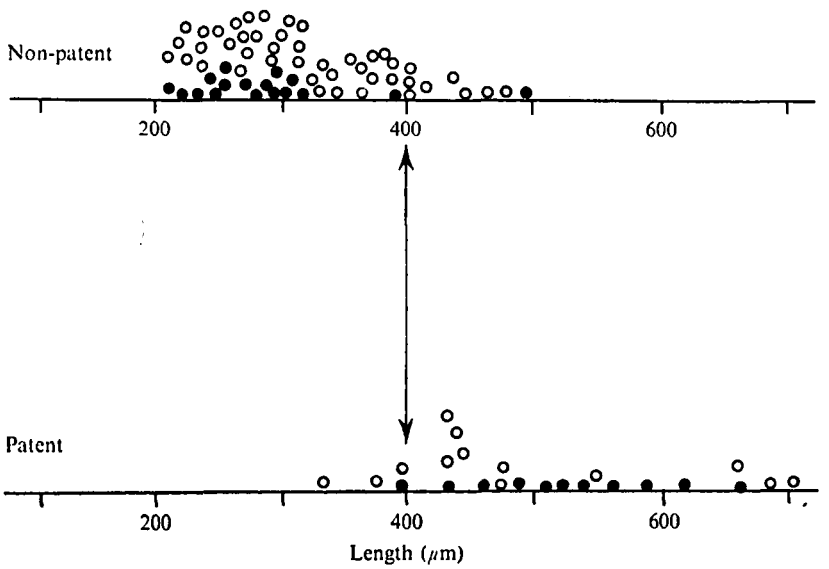
Preparations containing fluorescein-labelled yolk protein were obtained from homogenates of washed whole ovaries in 0·01 M tris-citrate buffer at pH 7·8. The homogenate was centrifuged at 3000*g* for 20 min and passed through a 0·22 μm Millipore filter. The clear solution was filtered through small columns of G-25 Sephadex into the carbonate-bicarbonate buffer of Rinderknecht (1962). After labelling with fluorescein isothiocyanate on Celite according to Rinderknecht (1962) for 2 min at 0 °C, the material was rapidly filtered through G-25 Sephadex into physiological saline solution. Protein concentrations were adjusted to 10 mg/ml by concentration with dry Biogel P-2 in centrifuge-basket adapters fitted with nylon mesh of 10 μm pore size.

RESULTS

Oocyte size, follicular patency and vitellogenesis

Histochemical studies on *Rhodnius* have suggested that protein from the haemolymph passes between the cells of the follicular epithelium to the surface of the oocyte (Patchin & Davey, 1968), and it has been established for other species that the yolk protein enters the growing oocyte through the follicle layer (Telfer, 1965).

In an oocyte which has not yet entered vitellogenesis sections reveal no obvious extracellular spaces in the follicular layer (Pl. 1, fig. 1), while a follicle in active



Text-fig. 1. Oocyte size at which spaces appear in the follicular epithelium. Open circles: allatectomized mated females. Solid circles: normal mated females. The arrow indicates the critical size of activation: above 400 μm the follicles are patent, while below this value most of the follicles do not have marked intercellular spaces.

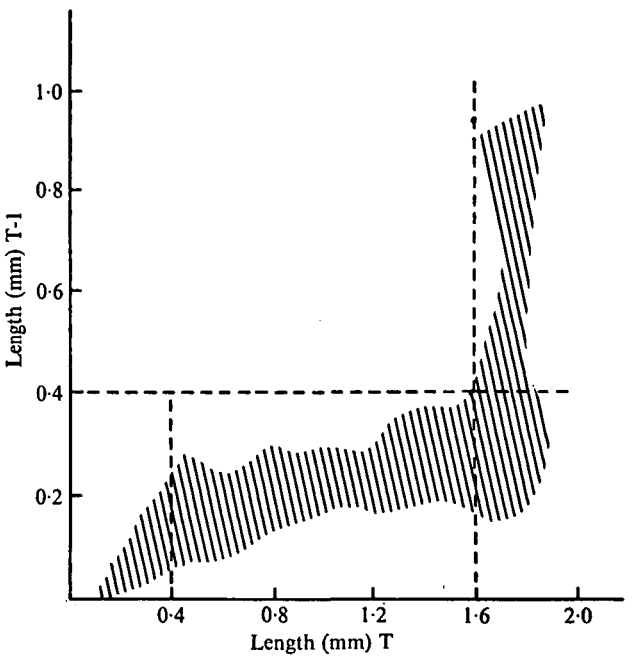
vitellogenesis (Pl. 1, fig. 2) shows abundant extracellular spaces. These spaces can be readily demonstrated by immersing a freshly dissected ovariole in Evans blue. In an actively vitellogenic follicle, the dye penetrates within seconds into the spaces, thus clearly demarcating the cells of the follicular epithelium (Pl. 1, fig. 3), while in a smaller, non-vitellogenic follicle (Pl. 1, fig. 4) the follicle cells adhere closely to one another and the dye does not penetrate.

The spaces can also be observed in whole mounts of fixed material stained with Ponceau S, where the relationship between size of oocyte and onset of patency can be readily quantified (Text-fig. 1). Thus, the measurement of oocyte length can substitute for a test for patency in that all oocytes greater than 400 μm in length can be regarded as being surrounded by a follicular epithelium which is to some degree patent. Furthermore, the onset of patency occurs at the same critical size whether the animal be normal or allatectomized, although the degree of patency (Pl. 1, fig. 6) is less in an allatectomized female.

Final confirmation of the functional validity of this criterion was obtained using fluorescein-labelled yolk protein injected into mated females 4 days after feeding. Upon dissection 12 or 18 h after injection, only those oocytes greater than 400 μm in length contained fluorescent yolk spheres.

Co-ordination of growth within the ovariole

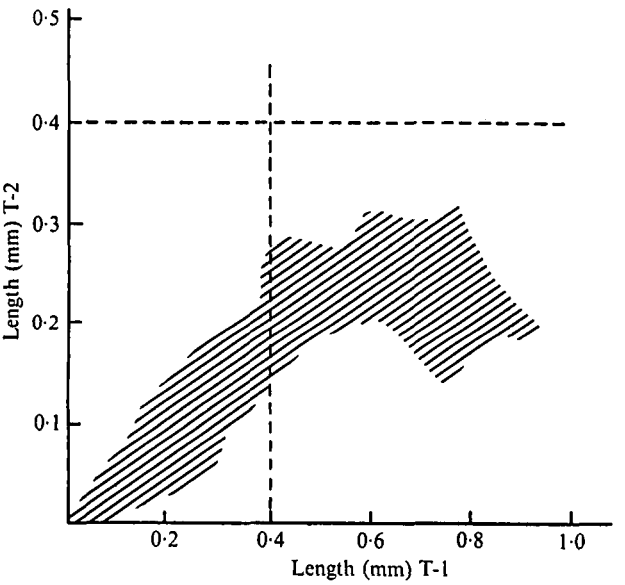
The larger oocytes in any ovariole represent a longitudinally arranged series in which the terminal oocyte (T) nearest the base of the ovariole is the oldest and largest oocyte



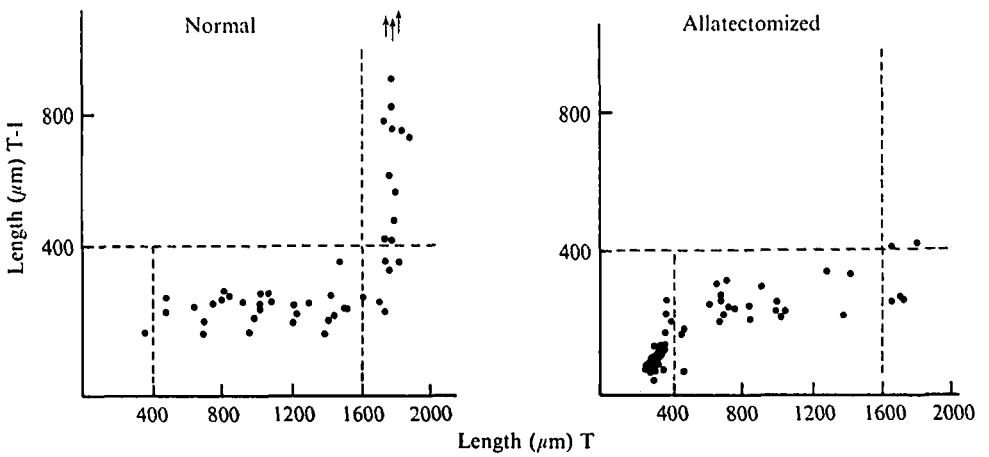
Text-fig. 2. The length of the T oocyte plotted against that of the T–1 oocyte from 658 ovarioles. The points fall within the shaded area in the graph and the broken lines indicate the size at which patency of the follicular epithelium (0·4 mm) occurs or deposition of the chorion (1·6 mm) begins.

and its younger neighbours, T-1, T-2, etc., represent successively earlier stages in oocyte growth. Some evidence of co-ordination of growth within the ovariole is obtained from the observation that in the many hundreds of ovarioles which we have examined there is never more than one oocyte in active vitellogenesis at any time. It is as if the entry into vitellogenesis of the T-1 oocyte is delayed until the T follicle has begun to produce a chorion.

Text-fig. 2 plots the size of the T oocytes against the size of the T-1 oocyte for 658 ovarioles. It is clear that the random scatter of points which would be expected



Text-fig. 3. As in Text-fig. 2, but for T-1 and T-2 oocytes.



Text-fig. 4. The length of T oocytes plotted against that of T-1 oocytes from ovarioles removed from normal or allatectomized females on the fourth day after feeding. The broken lines indicate the size at which patency of the follicular epithelium occurs (400 μm) and the chorion is deposited (1600 μm).

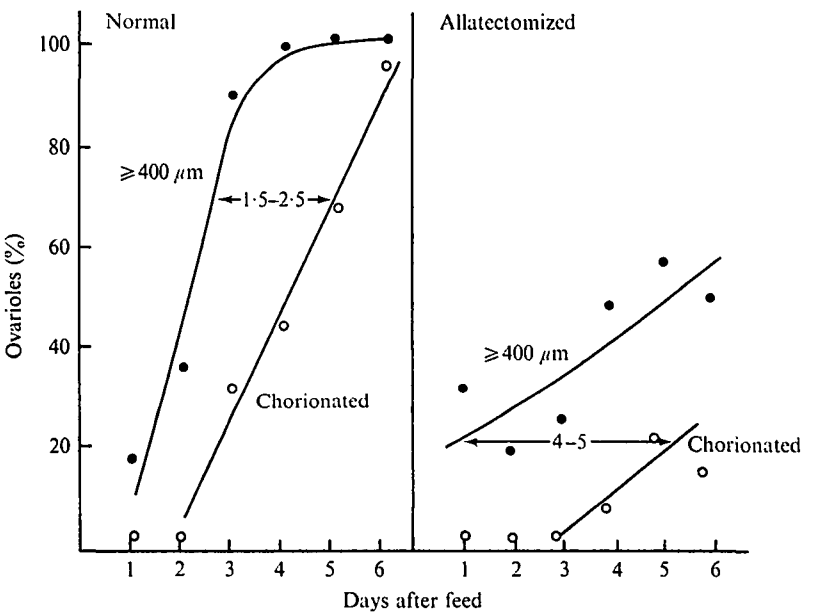
if the growth of these oocytes were independent is not in fact obtained. A similar relationship was obtained between the T-1 and T-2 oocytes (Text-fig. 3).

Furthermore, Text-fig. 4 shows the relationship obtained between T and T-1 measured 4 days after feeding in both normal and allatectomized animals. It is clear that allatectomy does not abolish this co-ordination. Similar data have been obtained for each of the first 6 days after feeding. The broken line at 1600 μm in the graphs in Text-fig. 4 indicates the smallest size at which the chorion has been observed. The average size at which the chorion appeared was approximately 1800 μm.

Thus, within any one ovariole, the larger oocytes do not grow independently of one another but are markedly co-ordinated in both normal and allatectomized animals. It follows therefore that if a deficiency in allatum hormone inhibits the growth of an oocyte when it is of a particular size or within a particular size range, this deficiency must also manifest itself as an inhibition in the growth of the neighbours of the affected oocyte. This conclusion, which derives directly from our observations, complicates any interpretation in terms of the site of action of the allatum hormone.

Allatectomy and vitellogenesis

The percentage of ovarioles containing T oocytes which had exceeded 400 μm and entered vitellogenesis was compared with the percentage of ovarioles containing a mature oocyte complete with chorion ('chorionated') for each of the first six days

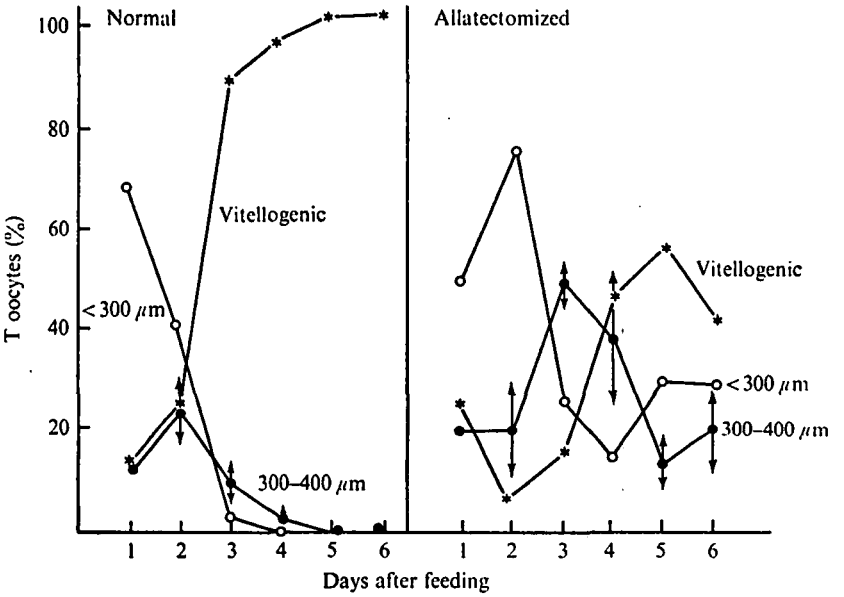


Text-fig. 5. The percentage of ovarioles from normal and allatectomized females whose T oocytes have become activated (≥ 400 μm) and have deposited chorion, on each of the first 6 days after feeding.

after feeding – in both normal and allatectomized animals. The data are displayed in the pair of graphs in Text-fig. 5. A delay in entry into vitellogenesis among allatectomized animals is evident: 90% of the normal ovarioles have initiated vitellogenesis

by day 3 and 100% by day 5, while the figures for the allatectomized animals are 30% and 50% respectively.

Accompanying this delay in entry into vitellogenesis is a reduced rate of vitellogenesis. For the normal ovarioles, a comparison of the percentage vitellogenic with the percentage chorionated indicates an elapsed time of 1·5–2·5 days, while the allatectomized ovarioles appear to require 4–5 days to complete the process.



Text-fig. 6. The percentage of T oocytes in each of three size-classes in ovarioles from normal and allatectomized animals. Solid circles: 300–400 μm. Open circles: less than 300 μm. Asterisks: vitellogenesis (larger than 400 μm). The arrows indicate standard errors.

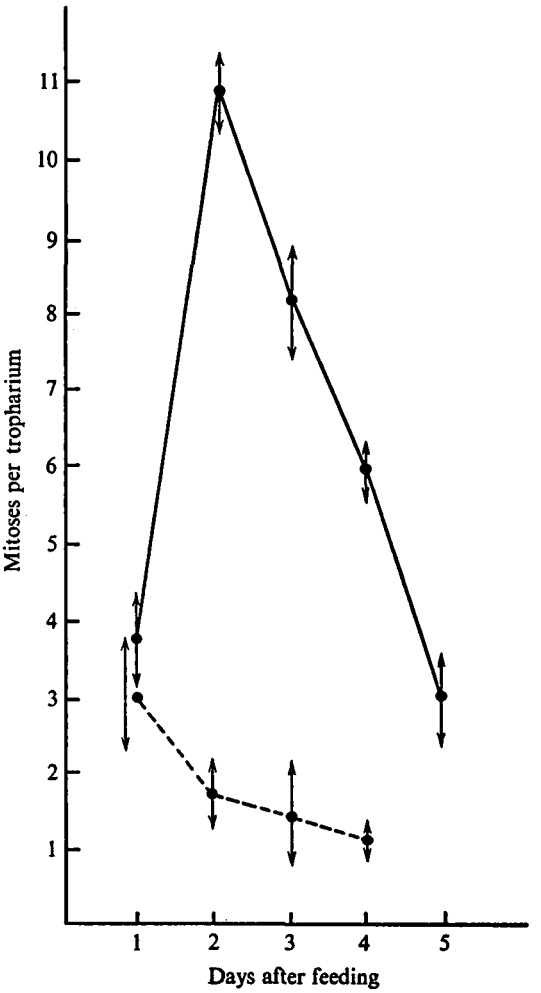
At the same time there is a temporary accumulation of oocytes in the size range immediately preceding vitellogenesis. Thus Text-fig. 6 shows the percent of ovarioles in which the T oocytes fall into the size ranges smaller than 300 μm, 300–400 μm, and greater than 400 μm (vitellogenic) for normal and allatectomized females. It is clear that there is a transient synchrony in the T oocytes within the critical 300–400 μm size range at about 3 days after feeding in the allatectomized animal. Apparently the oocytes in allatectomized animals readily grow up to the size of activation, but many exhibit a reluctance to become activated.

Allatectomy and pre-vitellogenic growth

One of the first events in the process of pre-vitellogenic growth is that of making available new nurse cells to nourish the continuing waves of oocytes. This is achieved by mitoses in the tropharial primordium. Text-fig. 7 shows the number of mitotic figures per tropharium in normal and allatectomized ovarioles. It is obvious that a cycle of mitoses with a pronounced peak at 2 days after feeding exists in the normal ovariole and that this cycle is entirely absent from the allatectomized animals.

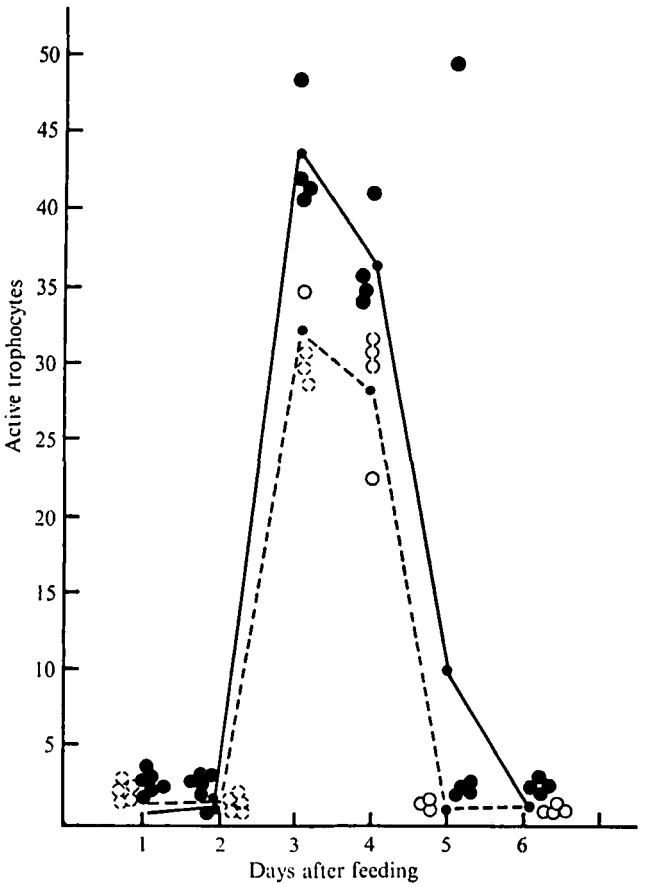
The products of these mitoses eventually enlarge and contribute their contents, or

part of them, to the developing oocytes via the trophic core and the trophic cords. As the enlarged trophocytes become active, numerous spherical bodies of high refractive index can be visualized in fresh or fixed whole mounts of ovarioles (Pl. 1, fig. 5). These bodies can be vitally stained with acridine orange and exhibit the intense red fluorescence typical of aggregates of ribonucleoprotein.



Text-fig. 7. The number of mitoses per tropharium in ovarioles removed from normal (solid line) or allatectomized females (dotted line) at various times after feeding. The lines are drawn through the means and the arrows indicate the standard errors.

We have counted the number of such enlarged cells containing these spherical bodies in a total of 500 tropharia in whole mounts of ovarioles from normal and allatectomized animals over the first 6 days after feeding. The results appear in Text-fig. 8, and reveal a slight increase in the peak numbers in the allatectomized animals.



Text-fig. 8. The number of active trophocytes per tropharium in ovarioles removed from normal (open circles, dotted line) or allatectomized (closed circles, solid line) on each of the first 6 days after feeding. The lines have been drawn through the means of the points, each of which represents one animal.

Allatectomy and yolk protein in the haemolymph

Using the radial immunodiffusion assay of Fahey & McKelvey (1965) with an essentially monospecific antiserum prepared as described elsewhere (Pratt, in preparation), the apparent concentration of yolk protein in the haemolymph of normal and allatectomized animals was compared. The standard employed was yolk protein purified from ovarian homogenates and the titres were expressed in terms of equivalent protein concentration using bovine serum albumin as standard for biuret determinations. Eight normal animals yielded a mean of $4{\cdot}79 \pm 0{\cdot}38$ S.E. mg/ml while 11 allatectomized animals yielded a mean of $2{\cdot}71 \pm 0{\cdot}64$ S.E. mg/ml. Clearly allatectomy results in a significant depression in the level of the yolk protein in the haemolymph.

DISCUSSION

In considering the site of action of the gonadotropin from the corpus allatum there are several levels at which a deficiency in hormone caused by allatectomy might intervene to produce a depression in egg production. Those levels which operate

outside the ovariole culminate in the production of the yolk protein which is transferred from the haemolymph to the oocyte. It is clear that the level of yolk protein in the blood is depressed by allatectomy, and this might be regarded as evidence favouring the hypothesis that the corpus allatum controls the rate of egg production by controlling the synthesis of this protein. Such hypotheses have been proposed for *Rhodnius* (Vanderberg, 1963; Coles, 1965). Engelmann (1969) has demonstrated that juvenile hormone or implanted corpora allata will bring about *de novo* synthesis of a female-specific protein in allatectomized females of *Leucophaea maderae*. De Loof & de Wilde (1970) have found that synthesis of yolk protein in the potato beetle requires juvenile hormone, and Bell (1969) and Bell & Barth (1970) likewise recognize that the level of yolk protein in the haemolymph of the cockroach *Byrsotria* is affected by the juvenile hormone. In *Rhodnius*, however, it is not yet clear that this potential control of oogenesis via a control of the level of yolk protein is in fact realized in the intact animal. Is the decrease in egg production following allatectomy a consequence of a decreased level of yolk protein in the haemolymph? This question will be examined again in a later paper.

The observation that events within the ovariole are subject to internal co-ordination is an important one, for it complicates any interpretation concerning the site of action of the allatum hormone. In an attempt to understand how this co-ordination might be achieved, it is important to recognize that at least two sorts of phenomena may be involved.

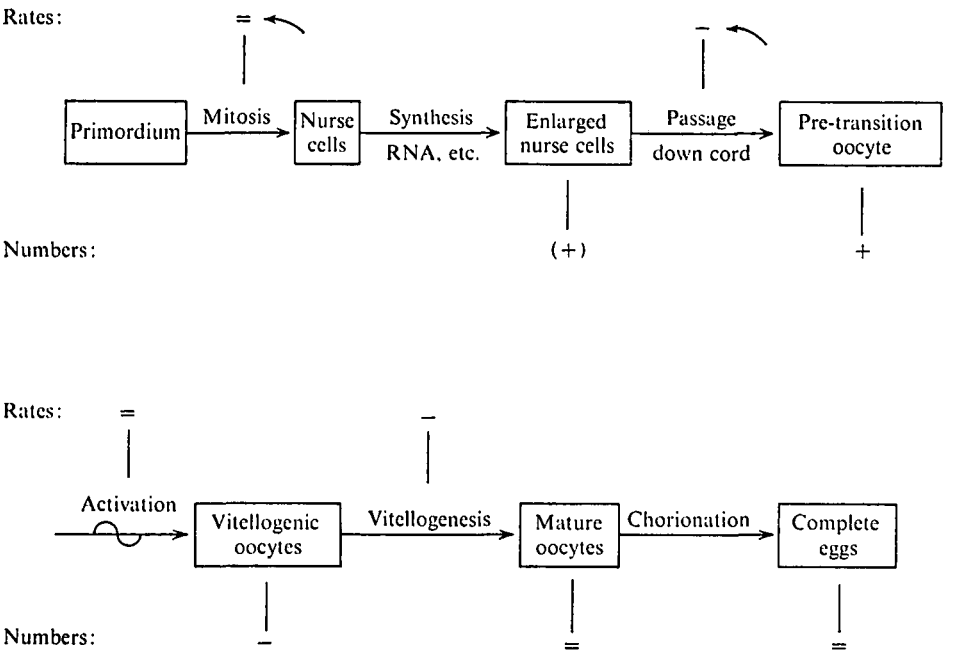
In the telotrophic ovariole of *Rhodnius* the pre-vitellogenic oocytes and trophocytes are all connected without the intervention of cell membranes through the trophic core (Huebner, 1970). This connexion exists for the flow of nutrient material from the trophocytes to the oocytes, but it may function additionally to co-ordinate the growth of the pre-vitellogenic oocytes, not only among themselves, but also with elements in the tropharium.

However, this explanation may be inadequate to account for the observation that an ovariole never contains more than one vitellogenic oocyte. First, it is not clear how long the trophic cord remains functionally intact in a vitellogenic oocyte. We have observed trophic cords attached to vitellogenic oocytes, an observation which does not agree with that of Wigglesworth (1936). Secondly, a similar relationship whereby the presence of a vitellogenic oocyte inhibits vitellogenesis in younger oocytes in the same ovariole has been observed in other insects with non-telotrophic ovarioles. Thus Roth (1968) described the patterns of ovarial development in a number of species of cockroaches which formed a series from the more primitive where up to three oocytes may be simultaneously vitellogenic to the specialized, notably these which carry the ootheca; where only the terminal oocytes are vitellogenic. Adams, Hintz & Pomonis (1968) have shown that the presence of maturing eggs in the housefly inhibits the development of the earlier oocytes beyond the stage at which they are activated by the corpus allatum. This co-ordination appears to be mediated by the release from maturing oocytes of an oostatic hormone which inhibits the release of the gonadotropin from the corpus allatum (Adams, 1970).

An important difference between *Rhodnius* and *Musca* lies in the fact that whereas in *Musca* the development of all of the ovarioles within one animal is closely synchronized, there is little or no such synchrony in an unoperated unstarved *Rhodnius*. Thus

Adjacent ovarioles are simultaneously in different developmental stages and the circulating titre of gonadotropic hormone cannot be held responsible for ensuring that within any one ovariole only one oocyte will be in vitellogenesis. The signal which imposes this restriction must originate and remain within each ovariole.

Let us now consider the effects of allatectomy upon the various stages of this co-ordinated growth, in an attempt to discover at which precise stages external influences such as the gonadotropin interact and have their primary effect. One possibility is that controls occur at a very early stage in oocyte development, namely oocyte proliferation.



Text-fig. 9. A diagram representing the processes within the ovariole. For explanation, see text.

However, work in this laboratory (Case, 1970) has shown that the oocytes of *Rhodnius* become differentiated and undergo their first reduction division in the larva. This means of control is therefore not open in the adult.

The subsequent development of the oocyte revolves around the transfer of materials from the mature nurse cells down the trophic cord and into the oocyte. Our observations have shown that while the production of new nurse cells (as evidenced by mitoses in the trophic primordium) is very much decreased in allatectomized females, the transient increase in number of ribonucleoprotein-like aggregates is no less than in the normal animals. Furthermore, the transient accumulation of oocytes in the 300–400 μm range indicates that pre-vitellogenic growth has occurred at the normal rate. We therefore conclude that allatectomy has no direct effect on pre-vitellogenic growth, a conclusion which agrees with that of Wigglesworth (1936).

In a large follicle (up to 400 μm) which has not yet begun vitellogenesis the follicle cells are mutually adherent, but spaces open between them just before vitellogenesis begins. We have shown that the patency of the follicular epithelium as revealed both

by the penetration of dye into the intercellular spaces and by the incorporation *in vivo* of fluorescent yolk protein into oocytes occurs at this same critical size. This complex process, whereby the pre-vitellogenic processes wane in importance, the follicular layer becomes patent and the machinery of adsorption and sequestration of yolk protein begins, we term 'activation'.

In allatectomized animals activation is markedly delayed, as evidenced both by the transient synchrony in the number of oocytes in the 300–400 μm range and by a delay of entry into vitellogenesis. An attractive hypothesis can explain these observations by focusing on the follicular layer as a possible site of action for the hormone. The gonadotropin, according to this hypothesis, initiates vitellogenesis by bringing about the development of intercellular spaces in the follicular epithelium, thus providing free access to the oocyte surface for the haemolymph proteins. The possibility that the rate of vitellogenesis, once initiated, can be controlled in the unoperated animal by the degree of patency of the follicular epithelium will be explained in another paper in this series.

These results may be expressed in another way which is represented by Text-fig. 9, in which each element which we have examined in the ovariole is represented by a box. Each element contributes to or is transformed into the next element in the series by a process. The negative effects of allatectomy upon the rates of processes are represented by minus signs. Thus allatectomy severely depresses mitoses in the trophic primordium, inhibits the transfer of nutrients down the trophic cord, markedly inhibits activation and inhibits vitellogenesis. A consideration of the numbers of cells found at various stages reveals a slight increase in enlarged nurse cells, an increase in the number of pre-transition oocytes, a decrease in vitellogenic oocytes, and a marked decrease in the numbers of oocytes and complete eggs. Our attention is focused on the process of activation because it is the only process which is inhibited by allatectomy and which results in an increase in numbers of elements immediately upstream from the process accompanied by a decrease in the numbers downstream from the process. Since we believe vitellogenesis to be governed in the same way as activation, it is appropriate that its rate should be less. On the other hand, the decrease in rate of mitosis and of passage of materials down the trophic cords are accompanied by increases in elements downstream from these processes and are probably feedback phenomena as a result of coordination of events within the ovariole.

SUMMARY

1. In the ovarioles of both normal and allatectomized females the spaces open between the cells of the follicle layer and vitellogenesis begins as the terminal oocyte grows through the 350–400 μm size range.

2. In both normal and allatectomized ovarioles the growth of the larger oocytes is markedly co-ordinated so that, for example, each ovariole contains only one oocyte in active vitellogenesis.

3. A consideration of the size distribution of the terminal oocytes in normal and allatectomized ovarioles demonstrates that vitellogenesis begins later and proceeds more slowly in allatectomized animals.

4. Allatectomy markedly depresses the number of mitoses in the trophic primor-

ium, but this is held to be a feedback phenomenon resulting from a decreased demand for the products of mature trophocytes.

5. There is a decreased level of yolk protein in the haemolymph of allatectomized animals.

6. This evidence favours the hypothesis that the primary site of action in *Rhodnius* of the gonadotropin from the corpus allatum is situated at the level of activation, whereby spaces open between the follicle cells and proteins from the haemolymph are allowed free access to the oocyte surface.

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EXPLANATION OF PLATE

Fig. 1. Section of the follicular epithelium before yolk has begun to form. The follicle cells (arrow) are closely adherent. Osmium-ethyl gallate. ×650.

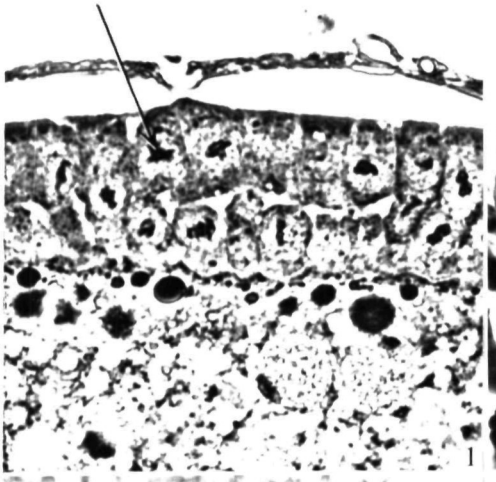
Fig. 2. As fig. 1, but taken from a follicle which is actively taking up yolk. The follicle cells adhere only by narrow projections, and large spaces have opened between them. Osmium-ethyl gallate. ×650.

Fig. 3. Surface view of an actively vitellogenic follicle which has been immersed in Evans blue. The dye has penetrated into the extracellular spaces. ×400.

Fig. 4. As in fig. 3, but from a follicle which has not yet begun to take up yolk. ×400.

Fig. 5. A freshly dissected tropharium from a mated female fed 4 days previously. The spherical bodies (arrow) indicating active trophocytes are clearly visible. ×120.

Fig. 6. Surface view of a large follicle from an allatectomized female after the ovariole had been immersed in Evans blue. The spaces between the follicle cells are small and relatively few in number. Compare with fig. 3. ×300.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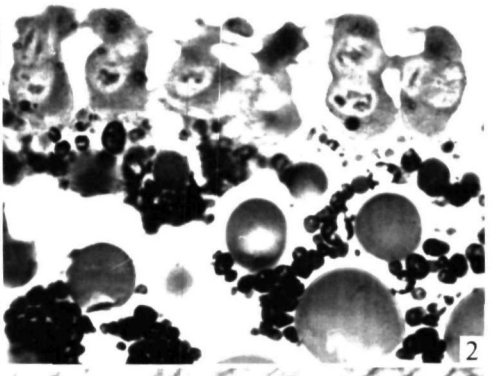
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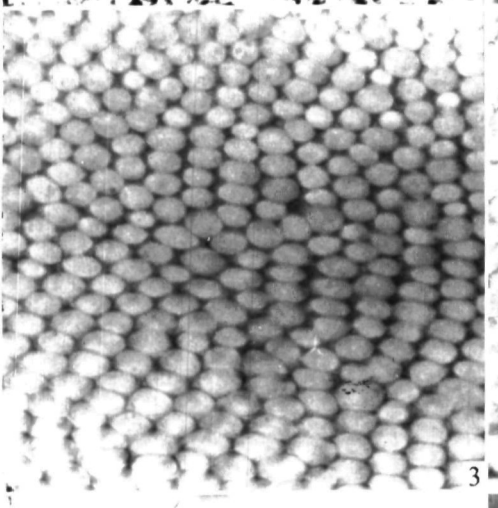
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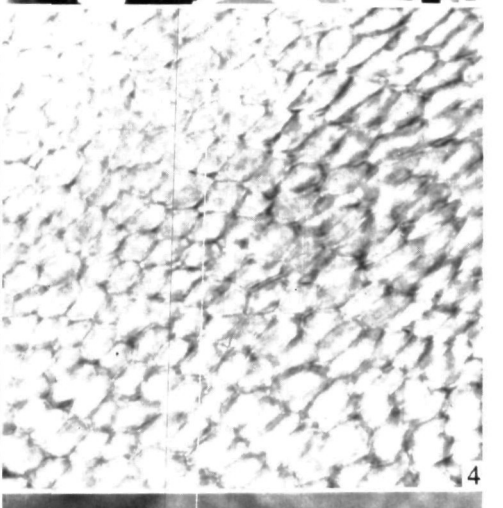
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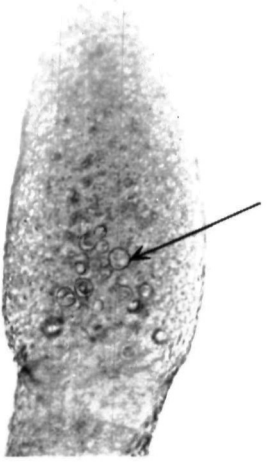
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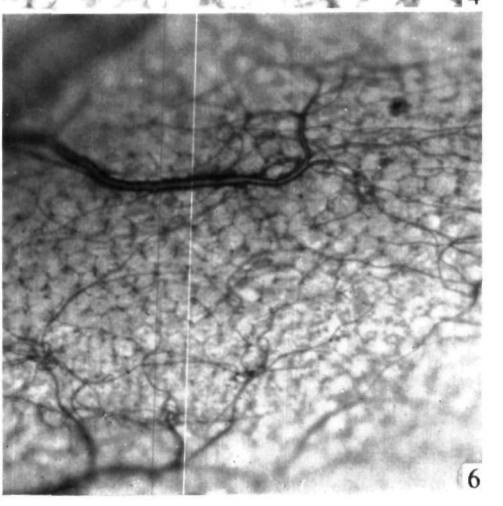
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