HAEMOLYMPH PROTEINS AND YOLK FORMATION IN RHODNIUS PROLIXUS STÅL.

By G. C. COLES

Department of Zoology, Cambridge University

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INTRODUCTION

Little work has been published on the changes in blood proteins of insects before and during egg production. In the adult female Schistocerca gregaria the concentration of blood protein rises after an initial lag period after emergence, and falls at the end of the oocyte development cycle (Hill, 1962). A similar rise and fall has been reported in Phormia regina fed on ‘medium diet’ during the first 6 days after commencement of feeding (Orr, 1964).

It is not certain how far these changes represent protein entering the oocytes from the blood, but that the natural proteins in the haemolymph can enter the oocyte was first suggested by Wigglesworth (1943) and clearly demonstrated in Hyalophora cecropia (Telfer, 1954). In this insect the protein is synthesized in the fat body (Laufer, 1960) and released into the blood where the amount is reduced in the course of its deposition in the ripening eggs (Telfer, 1954). Other studies suggest that a similar process occurs in Schistocerca gregaria (Hill from Wigglesworth, 1964), Calliphora erythrocephala (Bier, 1962) and Panorpa communis (Ramamurty, 1964), and electron microscope studies of the oocyte and associated tissues in Aedes aegypti (Roth & Porter, 1964), Lygaeus kalmii (Kessel & Beams, 1963), Periplaneta americana and Rhodnius prolixus (Anderson, 1964) have shown an oocyte surface organized for pinocytosis.

The aim of the present study was to follow the changes in the haemolymph of Rhodnius in order to demonstrate the importance of the blood proteins in yolk formation.

MATERIALS AND METHODS

The culture of Rhodnius was kept in a humid incubator at 28°C. and fed on rabbits by the standard laboratory technique derived from Buxton (1930). Experimental insects were kept at 26-5°C. and 80% relative humidity, using the caustic-soda method of Madge (1961).

Haemolymph was collected from insects either by cutting a leg and applying gentle pressure to the abdomen, or by cutting open the abdomen, and the haemolymph so collected was used directly for the determinations, all measurements being on whole blood. Blood volume was determined by exsanguination and, as an approximation, the density of the haemolymph was assumed to be 1 g./ml.

The total amount of protein in the haemolymph was estimated by the ultramicro method of Shaw & Beadle (1949). For separation of proteins two methods were used.

* Present address: Zoophysiological Laboratory B, Copenhagen University, Denmark
For qualitative work starch-gel electrophoresis was used as described by Smithies (1955) with the modified discontinuous buffer system of Poulik (1957) which, in the present investigation, was found to be superior to the buffer system of Smithies (1955). The gel was made in trays, 8 x 18 cm., and after electrophoresis the gels were sliced and stained with Amidoschwartz 10B. For quantitative work 3·3 μl. of haemolymph were placed at the centre of a strip of cellulose-acetate membrane 5 x 12 cm., and after separation for 1½ hr. using the buffer of Aronsson & Grönwall (1957) the bands were stained by the standard procedure of Scherr (1961) and eluted and read as described by Kohn (1960). Using this method for six determinations on a pooled sample of Rhodnius haemolymph all the percentages of any one fraction fell within a range of 1%. Correlations between the pattern on cellulose acetate and that on starch gel were obtained by separating the proteins on cellulose acetate, cutting out the bands and running these on starch gel. Immuno-electrophoresis was performed by the method of Poulik (1959). Antiserum was prepared by injecting three batches of 0·5 ml. of centrifuged haemolymph mixed with an equal volume of Freund's adjuvant at 2-weekly intervals into a rabbit.

In order to find where haemolymph proteins were going the whole haemolymph was labelled with fluorescein isothiocyanate following the method of Marshall, Eveland & Smith (1958). Proteins labelled in this way gave fluorescent precipitin lines with antiserum on cellulose acetate (using the method described by Kohn (1960)), and the large protein bands of the haemolymph were clearly visible, fluorescing strongly, after separation on starch gel and inspection under ultraviolet light. Ten microlitres of the labelled protein solution were injected into insects and the insects were left overnight. Tissues were then dissected out, washed carefully in three changes of Ringer's solution, mounted in Ringer's solution and inspected under the fluorescence microscope.

RESULTS

Qualitative studies

Comparison between haemolymphs from adult males and females and 5th-stage nymphs of Rhodnius by means of starch-gel electrophoresis revealed two additional slow migrating bands in the adults, but no qualitative sex difference. Electrophoresis of carefully washed eggs showed two large protein bands corresponding to the two proteins that are specific to the adult and six other small bands, but amongst these only a trace of one of the two major protein bands of the 5th-stage haemolymph (Text-fig 1). As judged by immuno-electrophoresis the two main egg bands were identical with the proteins specific to the adults. By contrast, there was no clear resemblance between the adult haemolymph proteins and the soluble proteins of the testis and the accessory glands of the male Rhodnius.

To demonstrate whether the adult-specific proteins found in the eggs are absorbed from the blood, female Rhodnius haemolymph was labelled with fluorescein and injected into female insects 4 days after feeding. Strongly fluorescing eggs were formed (Pl. 1). Some fluorescence was also observed when the ovaries had been incubated in vitro. If 5th-stage labelled haemolymph or rabbit serum was used, the eggs became fluorescent, but the intensity was small compared with that of eggs treated with female haemolymph. There was no uptake of free fluorescein.
The adult-specific proteins in the eggs seem to derive from the fat body, although they could not be extracted from the normal female fat body. However, they were extracted from female fat bodies incubated in vitro in 5th-stage haemolymph deproteinized by heating, and from fat bodies of previously starved females six days after they had been fed.

Text-fig. 1. The electrophoretic pattern of the haemolymph proteins and of the soluble proteins of the eggs of adult female *Rhodnius*, as separated on starch gel.

Quantitative studies

Since it has been indicated that protein formed in the fat body is transported by the haemolymph to the eggs, the quantitative changes of the proteins in the blood were followed to see how far these reflect the process of egg formation. All insects were starved for two weeks after moulting and were then allowed to feed. Text-fig. 2 shows the changes in protein-nitrogen concentration of male and female *Rhodnius* after feeding. Rapid egg-laying commenced at 6 days with a peak at 11 days, ceasing by 17 days. The blood volume increased rapidly but decreased gradually from one day after feeding. The quantitative changes of the four protein bands, as separated on cellulose acetate and which are not related to those on the starch gel, are shown on Text-fig. 3. Although the changes in band 3 apparently coincided with egg production, the adult specific proteins were found in band 2 and not in band 3. A utilization of band 3 occurred during starvation in the 5th-stage *Rhodnius*, and at the time when band 3 decreased in the female the crop was empty and the insect must have been entering upon starvation. During a similar period in the male there were, by contrast, no significant variations in any of the four protein fractions.
DISCUSSION

It was first established by Telfer (1954) that some haemolymph proteins enter the oocytes in insects. This is also indicated in a number of other studies of various insects, reviewed in the introduction, and it was therefore expected in Rhodnius.

It has been clearly demonstrated in the present work that specific blood proteins enter the oocyte of Rhodnius. The same proteins were found in the fat body and in the ovaries and, subsequently, it has been shown that they accumulate after ovariectomy (Coles, 1964). That this represents protein being transferred to the oocyte is clearly
confirmed by the use of direct labelling of haemolymph proteins and the formation of fluorescent droplets of yolk. Although it is a well-known technique, the use of directly labelled protein, in contrast to that of labelled antibodies, has largely been ignored in studies of insects and could well be used further.

Telfer (1960) demonstrated that, although the mechanism of protein uptake is strongly selective in *Hyalophora cecropia* and results in the great accumulation of the female-specific protein, bovine serum albumen can also enter the oocytes. The use of fluorescein-labelled rabbit serum would appear to demonstrate this in *Rhodnius*, but is open to the criticism that, as *Rhodnius* are fed on rabbits, some of the proteins of the rabbit serum may enter the egg or crop, and these may enter the eggs. This seems unlikely, however, as van Sande & Karcher (1960) report that in Triatomidae the haemolymph protein pattern is independent of the host. It was not realized at the time of fluorescein labelling that dialysis does not remove all unattached fluorescent material present (Fothergill & Nairn, 1961), and so there is the possibility that part of this material may have become attached to some of the proteins of the female *Rhodnius* haemolymph. The selective nature of the absorption by the oocytes of *Rhodnius* is exhibited by the detection of only a trace of one of the two major 5th-stage haemolymph proteins in the eggs. The possibility that these 5th-stage proteins are being absorbed in quantity and converted into insoluble protein is excluded by the failure to obtain strongly fluorescing eggs with 5th-stage haemolymph labelled with fluorescein, and the only important difference between nymphs and females was in the two adult-specific proteins.

Some egg protein is formed outside the ovary of *Hyalophora cecropia* (Telfer, 1954), and Laufer (1960) showed that this protein is synthesized in the fat body and stored there in large amounts. In *Rhodnius*, egg proteins are not stored in the fat body, but seem to originate there. Vanderberg (1963) had concluded from his labelling experiments that protein synthesis in the fat body of *Rhodnius* was important. Similar strong labelling was found in *Schistocerca gregaria* (Hill from Wigglesworth, 1964).

Although the usual sequence in insects, as found in *Rhodnius*, appears to be that amino acids from the gut go to the fat body and then as egg protein to the ovary, the recently published results of Roth & Porter (1964) on *Aedes aegypti* give preliminary evidence that the gut may be fulfilling the role of synthesizing some of the yolk proteins in this insect.

**Quantitative changes in the haemolymph**

In *Rhodnius* protein concentration in the haemolymph increases after feeding, but there are no large changes in the protein fraction that includes the adult-specific proteins. The increase in protein concentration after feeding occurs after the increase in blood volume, and represents a return of the concentration to the level before feeding. Thus apart from the presence of the adult-specific proteins in the haemolymph, the changes found do not reflect egg production. This differs from the situation in the only two insects available for comparison.

The haemolymph in *Hyalophora cecropia* acts as a store of protein as this insect does not feed as an adult (Telfer, 1954). Therefore, as egg production proceeds, egg protein in the haemolymph is depleted. In *Schistocerca gregaria* the protein concentration of the haemolymph rises during feeding and oocyte development starts when a
concentration of 4 g./100 ml. is reached (Hill, 1962). During yolk deposition one of the proteins of the haemolymph greatly increases relative to the others and, since this protein also increases upon ovariectomy, it may represent protein destined for oocytes. The difference between Schistocerca and Rhodnius may be explicable by the hormonal control of metabolism. In Schistocerca protein synthesis is under the control of the neurosecretory cells (Hill, 1963), and the corpora allata are thought to control the absorption of protein by the oocytes (Highnam, Lusis & Hill, 1963). However, in Rhodnius the corpus allatum controls yolk protein synthesis in the fat body (Coles, 1964). If it is assumed that the corpus allatum also affects oocyte absorption, then with one hormone affecting the two processes, no marked change in the concentration of yolk protein in the haemolymph would be expected.

SUMMARY

1. There are two adult-specific proteins in the haemolymph of Rhodnius. They appear to be formed in the fat body.
2. The two proteins are absorbed by the oocytes and form the bulk of the soluble egg proteins.
3. The changes in the concentration of total protein in the haemolymph and of four protein fractions, as separated on cellulose acetate, do not reflect egg production. This may be a consequence of the hormonal control of reproduction.

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REFERENCES

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EXPLANATION OF PLATE

Photograph of developing eggs, after injection of female haemolymph proteins labelled with fluorescein into female *Rhodnius*.