The Haemocytes and Connective Tissue Formation in an Insect, *Rhodnius prolixus* (Hemiptera)

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**Summary**

The amoebocytes, which form the most abundant type of blood-cell in *Rhodnius*, contain rounded, oval, or rod-shaped inclusions which stain by the periodic acid / Schiff method. These are believed to be neutral mucopolysaccharides. The amoebocytes apply themselves to the basal membranes, which likewise are PAS-positive, and appear to contribute to these membranes by discharging their contents. They insinuate themselves into the developing muscles and give rise to the sheaths of connective tissue by which the muscle fibres are surrounded. And they collect around deposits of injected Indian ink, producing similar sheets of PAS-positive material, presumably mucopolysaccharide.

The chitinous endocuticle is PAS-negative (except in a few special regions such as the neck and the conjunctival membranes of the limbs). It becomes positive during digestion by the moulting fluid. The tracheae react similarly.

Other PAS-positive structures which are not produced by the amoebocytes are the striated border of the mid-gut, the basement membrane of the gut, the perilemma around the ganglia and nerves, and the ground substance within the ganglia.

**Introduction**

In a recent paper on the role of the haemocytes during growth in *Rhodnius* (Wigglesworth, 1955), it was suggested that the most abundant type of blood-cell, the phagocytic 'amoebocyte', plays an essential part during the initial stages of moulting when the moulting hormone is being secreted. These cells apparently intervene between the neurosecretory cells of the brain and the thoracic gland.

An obvious possibility was that the product of the neurosecretory cells, after being transferred to the corpus cardiacum and discharged into the blood, might be transported to the thoracic gland by the amoebocytes. Sections of the brain and corpus cardiacum, stained with the chrome-haematoxylin method of Gomori (1941), were therefore prepared.

As in other insects the neurosecretory cells were found to be filled with blue-black deposits; material with the same staining properties was present between the cells in the medial part of the corpus cardiacum (fig. 1); and around the corpus cardiacum and adjacent tissues there were amoebocytes with round, oval, or rod-shaped inclusions which stained in the same way (fig. 1, c).

The chrome-haematoxylin stain is in no way specific for the neurosecretory product; other structures staining blue-black in these same sections are the
perilemma around the ganglia and nerves, the material separating the neurones within the ganglia, the finer tracheal branches, the basement membranes, and the connective tissue sheaths around and between the muscle fibres.

Sections stained with fuchsin-paraldehyde (Gabe, 1953) show the neurosecretory cells, the inclusions in the amoebocytes, and the connective tissue structures all staining deep red (table 1). On the other hand, in sections stained with Masson's trichrome (method of Foot), whereas the neurosecretory substance stains red with the fuchsin, the inclusions in the amoebocytes, and the connective tissues, stain green.

| Table 1 |
|-----------------|-----------------|-----------------|-----------------|
| **Stain**       | **Neurosecretory product** | **Amoebocyte inclusions** | **Connective tissues** |
| Chrome-haematoxylin | blue-black | blue-black | blue-black |
| Fuchsin-paraldehyde | deep red | deep red | deep red |
| Masson's trichrome | red | intense green | intense green |
| PAS              | —          | +            | +             |

So far as they go these staining results suggest that the inclusions in the amoebocytes may be the precursor substance of the connective tissues and basement membranes, and that the amoebocytes may be concerned in the formation of these structures. This was the view put forward by Lazarenko (1925). The object of the present work was to reinvestigate this possibility.
The role of the haemocytes in connective tissue formation has been studied (i) by observing the behaviour of the blood-cells at all stages of the moulting process, and (ii) by using the periodic acid / Schiff method (after removal of glycogen with ptyalin) as a more specific stain for the substance of the connective tissues. Although the PAS test is given by a wide variety of substances (Pearse, 1953), the PAS-positive connective tissues of insects are presumably composed of some mucopolysaccharide, and on this assumption they will be referred to as such throughout this paper. Attempts to characterize the substance further by the use of toluidine blue after various treatments (Pearse, 1953) gave inconclusive results.

For the general study of the haemocytes during moulting, the abdomen has been cut along the margins and the isolated tergites and sternites with the adherent fat body and haemolymph, fixed in Bouin’s or Carnoy’s fluids, stained with the ferric trioxyhaematein of Prenant, and mounted whole. For the staining of connective tissues the insects were fixed in neutral formaldehyde or in Carnoy and sections of the abdomen and thorax stained by the periodic acid / Schiff procedure after treatment with filtered saliva, and counterstained with light green.

The haemocytes in Rhodnius

A short description of the haemocytes in Rhodnius has been given elsewhere (Wigglesworth, 1955). For the most part these cells are not floating freely in the circulating haemolymph but are loosely adherent to the basement membranes, often in large temporary aggregations. As a result, the numbers of cells in blood withdrawn from the body cavity are exceedingly variable: usually the first drop contains a fair number of cells, but subsequent drops very few. The number of free cells can be increased if the insect is immersed in water at 55°C for 5 minutes (Yeager, 1938), but the blood-cell count is still equally variable and probably much below the true figure.

The following cell types are recognized: (i) proleucocytes, the multiplying precursor forms (fig. 2, j); (ii) amoebocytes (fig. 2, A-I); (iii) oenocytoids (fig. 2, k); (iv) lipocytes (fig. 1, D); (v) large granular cells; (vi) large non-granular cells (fig. 2, l). Types (v) and (vi) are relatively few in number; they are only feebly phagocytic and contain no inclusions. The oenocytoids (iii) are rounded or oval disk-shaped cells, non-phagocytic, with homogeneous cytoplasm and without pseudopodia. The lipocytes (iv) do not seem to differ from small detached fat-body cells.

The amoebocytes (ii) are much the most abundant type: in drawn blood they are about ten times as numerous as the oenocytoids, which come second in abundance. They are highly pleomorphic: rounded, spindle-shaped, irregular, or spread out in an attenuated form so as to merge imperceptibly with the basement membrane. When examined in the fresh state they show numerous filamentous pseudopodia (Wigglesworth, 1955, fig. 5). They are active phagocytes. This is the only type with which this paper is concerned.
Wigglesworth—The Haemocytes and

The inclusions in the amoebocytes, staining with chrome-haematoxylin, fuchsin-paraldehyde, and the light green in Masson's trichrome, have already been noted. In the fresh state these inclusions can be seen as clear glassy droplets which stain supravitaly with gentian violet, taking on a slate-blue tint. They stain pale pink or mauve with toluidine blue; they are only faintly stained with Prenant's ferric trioxyhaematein and with the bromophenol blue stain for protein (Mazia and others, 1953). They do not stain with Sudan black B nor with osmium tetroxide, nor do they give an argentaffin reaction. They give a strongly positive PAS reaction unchanged by treatment with ptyalin, but this reaction is not so intense as in the glycogen deposits of the epidermis or fat-body cells. The inclusions do not stain with alcian blue (Pearse, 1953). They are not strongly basiphil: after treatment with methylene blue at pH 4.0 the cytoplasm of the amoebocytes stains strongly, but the inclusions are unstained. They are presumably composed of neutral mucopoly saccharide material of some kind.

Within a day after feeding, in the 4th-stage larva, many proleucocytes can be seen undergoing mitosis. Multiplication continues, and by 3 days after feeding the amoebocytes are becoming very numerous. Besides the mucopolysaccharide inclusions a few contain colourless vacuoles. By 4 days after feeding the amoebocytes are abundant; many are enlarged and are now filled with the clear vacuoles which seem to be connected with the beginning of moulting (Wigglesworth, 1955). Mitosis in the haemocytes continues until at least the
ninth day after feeding: they become increasingly numerous as moulting proceeds.

**The formation of connective tissue**

At about 6 days after feeding, when mitosis in the epidermis is at its height, the haemocytes are often applied in great numbers to the basement membrane below the epidermis. Fig. 2, A–I shows some of the forms which the amoebocytes assume at this time. A and B are very common types with a relatively small amount of mucopolysaccharide in the cytoplasm. C is a type frequently seen in side view on a nerve or trachea. D is a less common type with extensive massed deposits of mucopolysaccharide. It can be noted in C and D (and the same can be seen in fresh unstained cells) how the deposits seem to be in process of extrusion on to the surface of the cell. E shows a not uncommon condition where nearly all the mucopolysaccharide seems to be extruded on to the surface of the cell and to be merging with the basement membrane. F is an extreme form in which there is only some diffuse PAS staining at one end of the cell, apparently fusing with the basement membrane. G, H, and I represent a very common type in which the cell is spread out in stellate form on the surface of the basement membrane; here again some of the deposits seem to be in process of extrusion and in places seem to merge with the basement membrane, which has the same staining properties with PAS. (This figure contains for comparison a proleucocyte (j), an oenocytoid (k), and a large spindle-shaped non-granular cell (l); all these are devoid of mucopolysaccharide inclusions.)

The general impression to be obtained from these observations is that the amoebocytes are contributing material to thicken the basement membrane. The same impression is gained from observing the muscle insertions. Fig. 3, A shows the attachment of a small muscle to an apodeme in the thorax. Amoebocytes are aggregated around the insertion and among them are free droplets of PAS-positive secretion which are clearly derived from the haemocytes and which appear to be in process of fusing to form the muscle sheath. This sheath is continuous with the basement membrane below the epidermal cells.

More convincing evidence of the part played by the amoebocytes in forming the connective tissues is afforded by the fact that these cells (and no other blood-cells) insinuate themselves between the muscle fibres; here they become exceedingly flattened and clearly give rise to the connective tissue sheaths by which the fibres are surrounded. Fig. 3, B shows a longitudinal section of a small thoracic muscle in the 4th-stage larva during this process. Fig. 4 shows a transverse section of a dorso-ventral abdominal muscle in the 4th-stage larva, in which all stages in the transformation from amoebocytes to sheaths can be followed.

The deposition of these sheaths is most active in the final stages of moulting when the development of the muscles is complete. It is particularly evident in the thorax of the 5th-stage larva when it moults to the adult, for at this time there is a great development of thoracic muscles. In sections of such muscles,
containing 200 fibres or more, almost every cleft where three or four fibres come together is occupied by an amoebocyte laden with mucopolysaccharide inclusions, as in fig. 4. Taken altogether this must represent a very large population of amoebocytes dispersed through the substance of the muscle. Later, when the sheaths are fully formed, cells with discrete inclusions can no longer be seen; perhaps they break down and disappear.

Another circumstance under which the formation of connective tissue sheaths by the amoebocytes becomes evident is after the injection of foreign matter into the blood. The injection of Indian ink is followed by an enormous increase in the numbers of amoebocytes. Eventually, groups of cells laden with the ink become aggregated and surrounded by other haemocytes containing no ink, and these form rather fragile sheaths around the aggregations. Fig. 5 shows a section through the margin of one of these deposits. The surrounding haemocytes contain great quantities of 'mucopolysaccharide'; sometimes this appears to be discharged into the intercellular spaces in the form of

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**Fig. 3.** A, insertion of a small muscle into an apodeme in newly-moulted 5th-stage larva of *Rhodnius*: PAS and light green. Amoebocytes containing PAS-positive inclusions apparently forming muscle sheath and basement membrane. The blood-cells without inclusions are oenocytoids. B, longitudinal section of small muscle at same stage as A: PAS and light green. Amoebocytes with inclusions flattened between the muscle fibres.
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droplets or strings; sometimes the cells are drawn out into filaments in which the 'mucopolysaccharides' form elongated deposits. Both processes lead to the formation of concentric sheaths of non-cellular material.

FIG. 4. Transverse section of tergo-sternal muscle in abdomen of 4th-stage larva one day before moulting: PAS and light green. Amoebocytes around and between the muscles giving rise to PAS-positive connective tissue sheaths.

FIG. 5. Connective tissue sheath and amoebocytes around a foreign body. The foreign body (a clump of cells filled with Indian ink) lies at the upper part of the figure but is not included in the drawing. PAS and light green.

Distribution of periodic acid Schiff staining in Rhodnius

It is certain that the amoebocytes do not have a monopoly in the formation of PAS-staining material. The striated border of the mid-gut, which is strongly positive, must be formed by the epithelial cells. The basement membrane of the intestine is likewise positive; this is separated from the body cavity and
the blood-cells by the longitudinal and circular muscle fibres; it seems likely that this layer also must be a product of the epithelium. The same applies to the Malpighian tubules.

The contents of the neurosecretory cells are PAS-negative. (Rehm (1955) describes the ground substance of the neurosecretory cells in Lepidoptera as being PAS-positive; she gives evidence that this reaction is due to bound lipoids.) The sheaths separating the neurones within the ganglia, which are weakly PAS-positive, are presumably the product of neuroglia cells. And the tough homogeneous 'perilemma' which forms the sheath around the ganglia and nerves, and is strongly PAS-positive, is almost certainly a product of the 'perineurium cells' below (Scharrer, 1939). Indeed, it is sometimes possible to see PAS-staining inclusions in these cells. But amoebocytes laden with 'mucopolysaccharides' can often be seen applying themselves to the surface of nerves and ganglia; it is possible that they contribute to the formation of these sheaths.

The same argument applies to the basement membrane below the epidermis of the cuticle and of the tracheae. This may well have a dual origin, with both epidermal cells and amoebocytes contributing to it. There must presumably be a rather close chemical relation between the substance of the basement membrane and the chitin-protein which is the main product of these cells. It is interesting to note that (contrary to the usual statements in textbooks) the chitinous endocuticle over the greater part of the body in Rhodnius does not stain by the PAS method. But there may well be differences in different insects and in different regions of the same insect (cf. Richards, 1952); for in Rhodnius the flexible membrane of the neck and some of the conjunctival membranes around the thoracic appendages are sharply marked off from the remainder of the cuticle in being PAS-positive.

During the deposition of the chitinous cuticle there is plenty of glycogen within the epidermal cells, but after this has been removed with ptyalin no PAS-staining material has been observed; whereas at this same time amoebocytes filled with 'mucopolysaccharide' are applying themselves to the surface (fig. 2, A–I). During the process of digestion which takes place before the old skin is shed, the inner layers of the cuticle do stain with PAS, and so likewise does the corresponding layer of the old tracheae. It would seem that during the digestion of chitin reactive groups are exposed.

Discussion

The connective tissues of insects are generally inconspicuous and little attention has been given to them. They have been briefly described in Sialis by Ochsé (1946), who showed that in this insect all the basement membranes and tissue sheaths could be stained by a silver impregnation method; they are described in the gut of grasshoppers by Riedel (1946) and in blowfly larvae by Waterhouse (1950). But none of these authors considers the formation of this tissue. The only detailed work on that subject is by Lazarenko (1925) who
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used supravital methylene blue to show how widespread these thin sheets of connective tissue are in the larva of Oryctes. Lazarenko also implanted tubes of celloidin into the larvae and observed that these objects became enclosed in capsules formed by spindle-shaped cells which later fused to a syncytium in which connective tissue fibres appeared. From these observations he inferred that the normal connective tissues of insects are probably formed in the same way. The cells in question he regarded not as blood-cells but as special mesenchyme cells from the tissue spaces. An alternative view is that the tracheal cells unite with one another to form a fenestrated membrane which constitutes the 'peritoneal membranes' and indefinite connective tissues of insects (de Sinéty, 1901; Riede, 1917; Remy, 1925; Dreher, 1936). Membranes of this type may well exist, but they certainly do not include the basement membrane in Rhodnius (Wigglesworth, 1954) nor the other membranes considered in this paper.

The encapsulation of foreign bodies by blood-cells in insects has been described by many authors. Clumps of tubercle bacilli are encapsulated by conglomerations of leucocytes in Galleria (Metalnikov, 1908); but according to Hollande (1930), in this cyst formation there is nothing comparable with the connective tissue formed by the fibroblasts of vertebrates. Similar observations were made by Iwasaki (1927), Boese (1936), Rooseboom (1937), and others after the introduction of Indian ink, parasites, &c. Lartschenko (1933), who describes the encapsulation of the eggs of parasites in the larva of Pieris, accepts the view of Lazarenko. But Ermin (1939), who gives a useful survey of the literature on the blood-cells of insects, again emphasizes that the conglomerated sheets of blood-cells which form in Periplaneta around injected Indian ink or around dead yeast-cells, are not really comparable with the connective tissue of vertebrates.

The results reported in the present paper agree with these conclusions. Fig. 5 shows a small part of the capsule formed around blood-cells laden with Indian ink. It is clear that these cells are not only forming a cellular sheath; they are giving rise to free membranes of 'mucopolysaccharide'. But these membranes are very fragile structures, easily dispersed, and not to be compared with fibrous tissue. Precisely the same mechanism of formation is seen in the normal connective tissue around and between the muscle fibres.

The cells which take part in this process are the commonest type of haemocytes; they are here called amoebocytes. Whether they are free in the blood or adherent to the tissues, they are always recognizable by their 'mucopolysaccharide' contents. It seems unnecessary to separate them as connective tissue-cells. Indeed, these cells in Rhodnius are now recognized as having at least three functions: they are the most actively phagocytic of the haemocytes; in the early stages of moulting they seem to play some essential part in the production of the moulting hormone by the thoracic glands; and in the later stages of moulting they liberate the 'mucopolysaccharide' material which forms or contributes to the connective tissues and basement membranes.
REFERENCES

REMY, P., 1925. Contribution à l'étude de l'appareil respiratoire et de la respiration chez quelques invertébrés. Nancy (Vagner, 220 pp.).
SINETY, R. DE, 1901. La Cellule, 49, 119.