A Study of Fibrogenesis in the Leech, *Hirudo medicinalis*

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With four plates (figs. 1–4)

SUMMARY

The fibrocytes and the fibres of *Hirudo medicinalis* have been studied with the electron microscope. The fibrocyte can be shown by other methods to contain triglyceride droplets, phospholipid droplets, and mitochondria, all of which can be recognized in the electron micrographs. In addition, a 'vesicular' component has been observed just below the cell surface.

The collagenous 'cortex' of the fibre is tubular, and can be shown to contain a long process of the cell which secretes it. There are many small fibrils about 250 Å in diameter comprising the cortex of the fibre; they lie along the fibre axis, and often are associated in pairs or groups of three or four. These fibrils sometimes show a poorly defined periodicity of 300 Å.

It seems that the fibrils 'shred off' from the surface of the fibrocyte. The process shows some resemblance to that described in the chick by other authors.

ALTHOUGH the existence of fibres in the connective tissues of leeches has been known for a long time, little work has been published on them in the last twenty years. Recently these fibres have been studied structurally and histochemically in the leeches *Glossiphonia complanata* and *Hirudo medicinalis* (Bradbury, 1957, 1958).

The fibres appear to be composed of a 'cortex' and a 'medulla'; the latter seems to be a long process of the fibrocyte cytoplasm. It has been shown by means of histochemical tests and by X-ray diffraction studies that the cortex of the fibre is composed of collagen or a similar protein. No evidence was obtained from the previous work that would help to elucidate the question whether the fibre was secreted extra- or intracellularly, and whether the collagen of this invertebrate resembled that of vertebrates in possessing a submicroscopic periodicity. This last point is of some interest, as a report has been published describing the occurrence of unstriated collagen in the earthworm cuticle (Reed and Rudall, 1948), whilst, on the other hand, collagen obtained from certain cephalopod molluscs seems to be of the typical vertebrate structure (Schmitt, Hall, and Jakus, 1942).

The electron microscope has been used for many studies of fibrogenesis in vertebrates, particularly by Porter (1951), Wyckoff (1952), and Martin (1953). Very little attention has been paid to similar problems in invertebrates, however, and it is the purpose of this paper to give the results of a study of the fine structure of the fibres and fibrocytes of the leech *H. medicinalis*.

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Material and Methods

Pieces of connective tissue were dissected out of the animal and placed immediately in ice-cold 1% osmium tetroxide solution with 0·22 M sucrose and 0·035 M veronal buffer at pH 7·3 (Caulfield, 1957). After dehydration in graded alcohols, the tissue was embedded in *n*-butyl methacrylate. Sections were cut on a Porter–Blum microtome; only those sections showing silver or gold interference colours were examined. After being mounted on Smethurst High-light 3-mm grids covered with a carbon film, the sections were studied in a Siemens Elmiskop I, operated at 60 kV.

The methacrylate was removed from some sections by immersing them overnight in redistilled ethylene dichloride, a technique suggested by Dr. Porter. These sections were then lightly shadowed with platinum before examination. In an attempt to obtain dissociated fibres, connective tissue from the leech was blended in a high-speed blender in the minimal quantity of formaldehyde solution for periods of several minutes. Drops of the resulting homogenate were dried down on to grids, shadowed with gold-palladium or platinum, and examined.

Structure of the Fibrocyte

As shown by the light microscope, this cell is bipolar and roughly spindle-shaped, with two long processes extending from it for over 100 μ on each side of the cell-body. From studies of the living cell by phase-contrast and interference microscopy, and from the histochemical studies already quoted, it is possible to list the cytoplasmic inclusions with some certainty.

Besides the nucleus, it is easy to detect the mitochondria, which are small, rod-shaped, and rather few in this cell. They are generally dispersed towards the periphery of the cell. The remaining two types of inclusion are both lipid in nature; one is a group of small lipochondria which are clustered round the nucleus and can be shown to contain phospholipid. The other type of lipid droplet is much larger (up to 3 μ in diameter) and very numerous. These fat droplets occupy most of the cytoplasm of the fibrocyte, and form the most conspicuous object in the cell; they seem to be composed almost entirely of triglyceride.

In the electron micrographs each of these types of cytoplasmic inclusion may be recognized (figs. 1; 2, A). The triglyceride droplets are very obvious, and it is noticeable that they appear badly shrunken, so that they have crenated edges. Studies of this type of droplet in living cells show them to be apparently perfectly spherical, so that this crenation is probably an artifact.

Fig. 1. (plate). A fibrocyte cut in longitudinal section.

Fig. 2 (plate). A, a fibrocyte cut in transverse section, showing all the cytoplasmic inclusions. Note the vesicular component just below the cell membrane.

B, a tangential section through the cortical region of one of the connective-tissue fibres; the small fibrils are clearly visible.

C, isolated fibrils from the connective tissue, showing some ill-defined surface periodicity. Shadowed with gold-palladium.

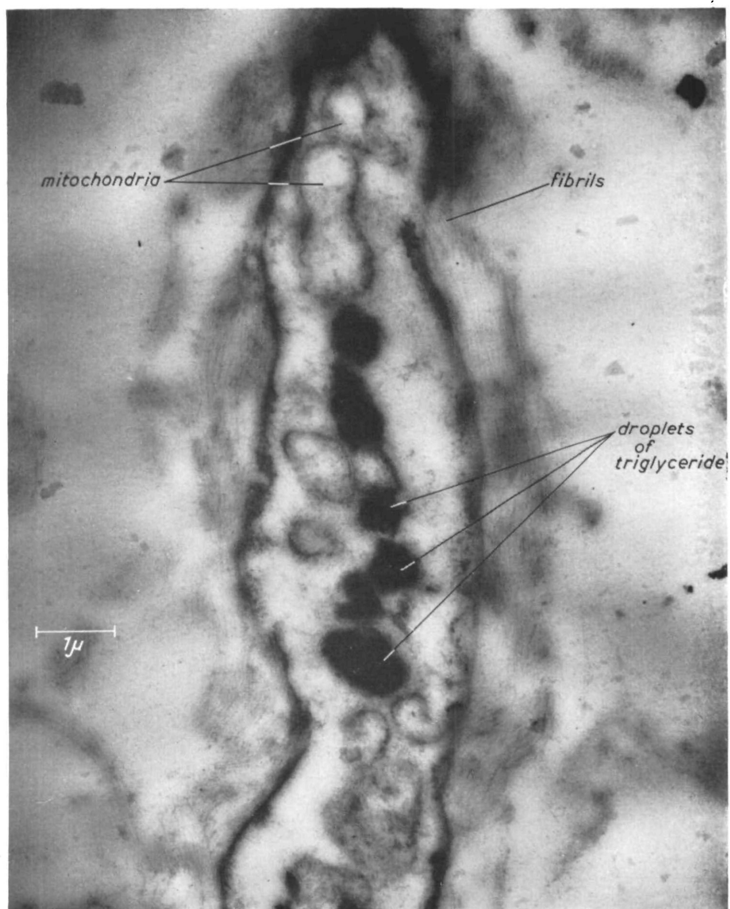

FIG. 1

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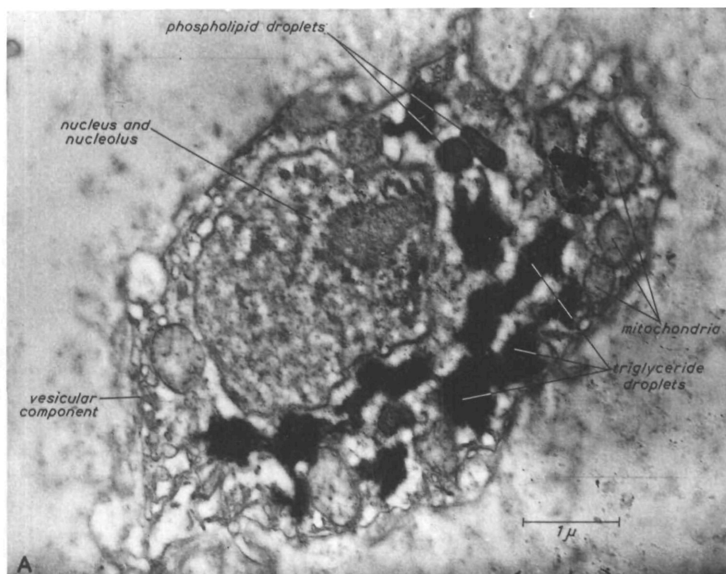

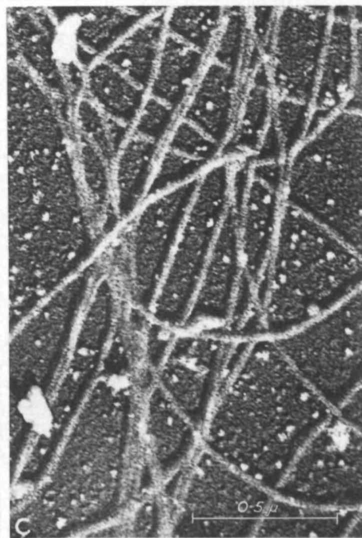

FIG. 2

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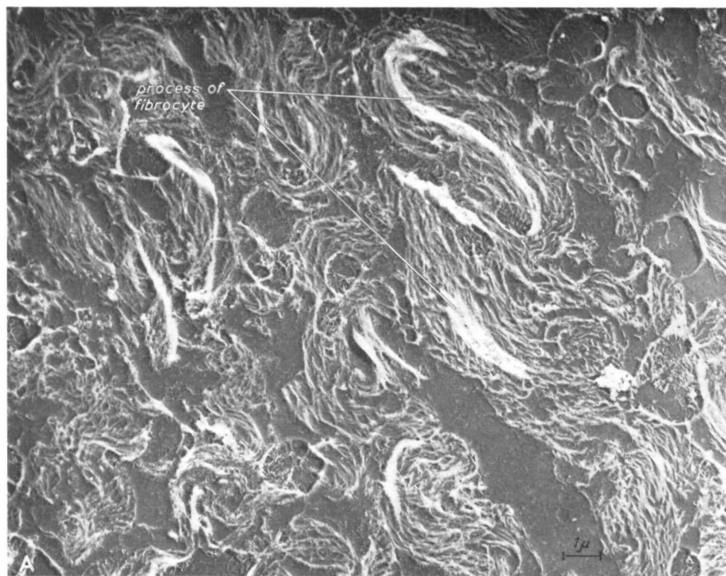

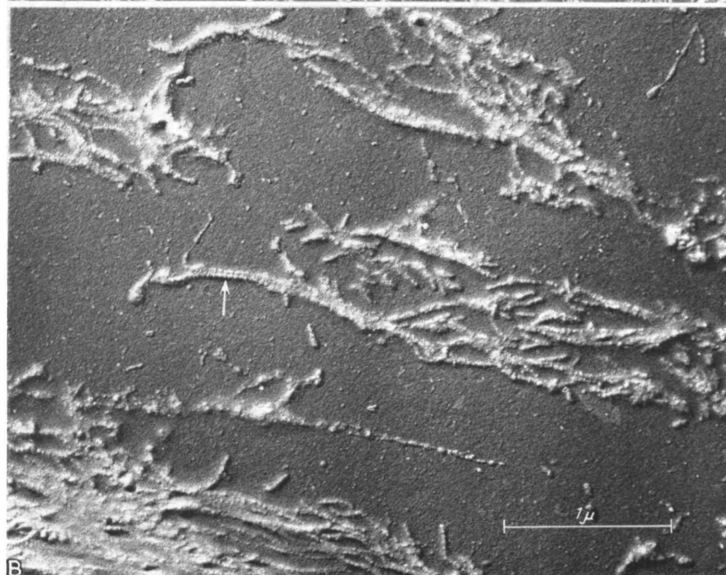

FIG. 3

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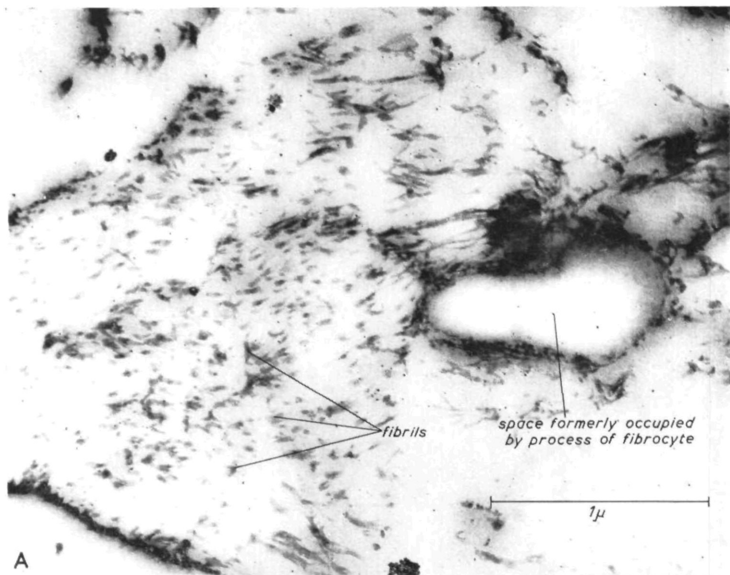

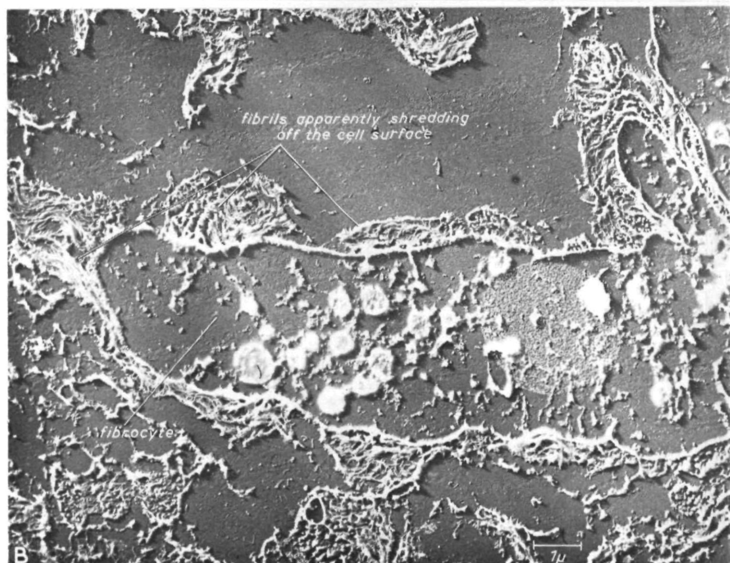

FIG. 4

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The triglyceride droplets are very electron-dense, probably because unsaturated lipid reduces the osmium tetroxide of the fixative.

Mitochondria are generally characterized in electron micrographs by the double membranes of the cristae; in the present material, however, it was found that the cristae were very poorly developed, or were poorly preserved by the technique of fixation used. It was possible to identify the mitochondria by comparing their shape, size, and position in the cell with those of mitochondria studied in living cells and in cells prepared with a standard mitochondrial technique.

The droplets which contain phospholipid are electron-dense, but less so than the triglyceride droplets. They are not greatly distorted by the fixation and embedding, but usually preserve the spherical shape seen in the living cell. No internal structure is visible, but there is some suggestion of an external membrane, which may be double.

One further cytoplasmic component is apparent in the electron micrographs which cannot be detected in either the living or the fixed cell, when studied with the light microscope. Immediately below the cell surface a complex of vesicles may be seen (fig. 2, A). It is not certain whether the vesicles seen in transverse section are isolated, or are continuous down the long axis of the cell. In one cell, where the plane of section was tangential to the cell surface, and just below it, the vesicles seem to be dilatations of a continuous system of canals or small tubes. This vesicular component of the fibrocyte cytoplasm will be considered further in the discussion.

The Structure of the Fibre

The ground substance of the leech connective tissue can be seen by light microscopy to contain numerous fibres, interlacing in all directions. When the connective tissue is examined under the lowest powers of the electron microscope a similar fibrous appearance may be seen, though as the sections are only about 200 Å thick, very little of each individual fibre may be visible (fig. 3, A). The fibres seemed to contain some sub-microscopic structure, so photographs were made at higher magnification. The actual collagenous material of the fibre forms the apparent cortex which is seen with the light microscope; it can now be seen to consist of numerous fibrils, approximately 250 Å in diameter, roughly orientated with their long axes parallel to the fibre axis. It appears from fig. 2, B that the fibrils associate in pairs or groups of three or four. It is probable that the space between the fibrils or groups of fibrils is occupied in life by the ground substance of the connective tissue.

Fig. 3 (plate). A, connective tissue of *Hirudo*, showing many fibres. In several places the process of the fibrocyte is apparent. Section shadowed with platinum.

B, connective-tissue fibrils; the arrow indicates two fibrils lying together, showing the periodicity. Shadowed with platinum.

Fig. 4 (plate). A, section through a single fibre. Note the space formerly occupied by the cell process, and the numerous fibrils, here mostly seen in tangential section.

B, fibrocyte, showing the fibrils apparently 'shredding' off the cell surface. The cytoplasmic inclusions are very poorly preserved. Section shadowed with platinum.

This would explain the positive periodic acid / Schiff reaction of the fibre which was noted in the previous paper (Bradbury, 1958).

It is not possible to measure the length of these fibrils in thin sections, so an attempt was made to isolate them by blending. Some fibrils found are shown in fig. 2, C, and from measurements of their diameter (about 300 to 500 Å) it seems that they represent not only individual fibrils, but also associated pairs. The fibrils are very long; the longest which it was possible to measure was over 3 μ in length and may well have been much longer in the living fibre. In fig. 2, B, C, which represent preparations lightly shadowed with platinum or gold-palladium, some suggestion is given of a surface periodicity or banding. Further sections, from which the methacrylate had been removed, were then soaked in distilled water for several hours in an attempt to remove any ground substance which might have been obscuring detail of the surface periodicity. The result is shown in fig. 3, B, where it is clear that the fibrils do show a poorly defined periodicity at an interval of 300 Å.

It was found that if tissue was fixed in 10% formaldehyde and subsequently embedded and sectioned in the normal manner, then only the fibrils of the fibre cortex were preserved, most of the fibrocyte cytoplasm and its inclusions being removed during dehydration and embedding. In one of these preparations a single fibre was seen in transverse section (fig. 4, A); again the association into small groups of fibrils is well marked, and particularly obvious is the space which in life would be occupied by the long process of the fibrocyte.

Discussion

From consideration of the results described above, it seems reasonable to postulate that the fibrils which form the cortex of the connective-tissue fibre are actually produced at the cell surface. In sections from which the embedding medium has been removed and which have then been shadowed, this effect is very striking (fig. 4, B); the fibrils seem to shred off from the cell surface. A similar impression is obtained from pictures of fibre formation in the chick, which was studied by Wyckoff (1952), and fig. 8 in his paper is very similar to the appearance which is found in the present study. The material comprising the fibrils is presumably secreted just below the cell surface, and it seems reasonable to suppose that the 'vesicular' component noticed in the leech fibrocytes is active in this process. At present it is not possible to apply histochemical methods to the study of the levels of organization which are revealed by the electron microscope, so we are not able to form any more definite conclusions. It is interesting to speculate on the basis of the histochemical work on this cell which has been referred to previously. A striking feature was the presence in the cytoplasm of both diffuse and granular PAS-positive material which was also metachromatic, and of diffuse phospholipid. In view of the observations of Jackson (1955) that PAS-positive granules are extruded in chick fibrogenesis, it may be that in the leech fibrocyte the acid mucopolysaccharide is contained within the structure of the vesicular component, which may itself be primarily composed of phospholipid.

A further point of similarity between the process of fibrogenesis in this leech and in the chick is that in the latter, both Porter (1951) and Wyckoff (1952) consider that the cell surface plays the most important role in organizing the fibrils of collagen outside the cell. On the basis of the present work, it is possible to suggest that this is also true for the fibrogenesis of Hirudinea.

Although some periodicity has been detected in the fibrils composing the cortex of the connective tissue fibres of the leech, the periodicity is not the 640 Å banding which is characteristic of mammalian collagen, but a figure which is about half of this. The periodicity of leech fibrils is never very well marked, in contrast to vertebrate material, and often it is not visible on all of the fibrils, but only on the thickest. The periodicity may, however, be masked by the presence of ground substance. The presence of periodicity in the fibrils of a leech is rather interesting in view of the report by Reed and Rudall (1948) that the collagenous fibres of the earthworm cuticle are not striated. It is worth noting that the periodicity of 300 Å which is measured for these fibrils agrees rather closely with the periodicity of 270 Å which is found for some embryonic chick fibrils of approximately the same diameter (Martin, 1953; Porter, 1951). The significance of this observation is not apparent, but presumably it is concerned with the degree of organization of the fibril at the molecular level. In the vertebrates, stages of periodicity of 110 Å, 270 Å, and 640 Å are successively reached with increase in the diameter of the fibrils, whilst in the leech it seems that as the maximum diameter of the fibril is about 300 Å, the periodicity never attains the value found in the largest (and presumably the most highly organized) mammalian fibrils. No fibrils were noticed with a periodicity of less than 300 Å.

It seems then that in the leech *Hirudo*, the cortex of the fibre is collagenous and surrounds the process of the fibrocyte which secretes it. This cell process remains inside the cortex throughout the life of the animal. In this work, and in the previous study with the light microscope, fibres lacking the cellular process down their centre were never seen. Recently work has been published (Wyckoff, 1952) which seems to show that some mammalian fibres are, in fact, hollow tubes. It may be that these mammalian fibres are secreted in a similar manner to those of the leech, but the cell and its process do not persist into adult life.

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