A Cytological and Histochemical Study of the Connective-Tissue Fibres of the Leech, *Hirudo medicinalis*

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With two plates (figs. 1 and 4)

SUMMARY

The fibres and the connective-tissue ground-substance of *Hirudo medicinalis* resemble those of the rhynchobdellid leech *Glossiphonia complanata* in general form and chemical composition. The fibres show a differentiation into 'cortex' and 'medulla'. The 'cortex' is found to contain arginine, and acid mucopolysaccharide but no tyrosine or lipids; these results suggest that it is collagenous. Confirmation of this supposition is provided by the X-ray diffraction pattern. The 'medulla' is an extension of the cell-body of the fibrocyte.

The cytoplasm of the fibrocyte is found to contain three types of inclusion: mitochondria; spherical lipochondria about 1μ in diameter, which seem to consist of phospholipid; and larger triglyceride droplets. The cytoplasm also contains diffuse phospholipid and granular accumulations of acid mucopolysaccharide.

The connective tissue ground-substance resembles that of *Glossiphonia* in its properties and chemical composition, though there seems to be a higher proportion of acid mucopolysaccharide in the ground-substance of *Hirudo*.

THE presence of fibres in the connective tissue of leeches has been noted by many authors since Ray Lankester (1880) first figured them in his paper on the histology of *Hirudo medicinalis*. Bourne (1884) and Scriban and Autrum (1934) in particular deal with their general structure, but as far as is known, no detailed histochemical study has been carried out on the fibres of this species of leech. Recently an account was published (Bradbury, 1957) describing the histochemistry of similar fibres which occur in the rhynchobdellid leech, *Glossiphonia complanata*, together with details of the ground-substance in which they are embedded. It is the purpose of this paper to give a comparable account of the fibres, fibrocytes, and intercellular matrix in the gnathobdellid leech *H. medicinalis*.

MATERIAL AND METHODS

Though the medicinal leech no longer occurs abundantly in this country, specimens may readily be obtained from dealers and kept alive in aquarium jars in the laboratory for many months. This species is much larger than the pond leech *G. complanata*, and it has the advantage that the connective tissue may be dissected out so that the living cells may be studied. In the present work, small pieces of connective tissue were removed and placed in 0·75% saline solution so that any pieces of adherent muscle or gut-wall could be removed before the connective tissue was mounted in the saline. Light pressure

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on the coverslip was sufficient to spread the tissue thinly enough for satisfactory phase-contrast microscopy. Interference and dark-ground microscopy were also used in the study of the fibrocytes. For supravital staining, neutral red chloride, methylene blue, dahlia violet, and Janus green B were used. The dyes were dissolved in distilled water, which was then diluted with saline to give the required concentration, according to the technique adopted in our laboratory (Baker, 1949; Chou, 1957). It was found that a period of 20 min. in the dye gave satisfactory results, though in the case of Janus green the tissue often had to be left as long as one hour in order to achieve any staining.

Details of the histochemical tests applied to fixed material are listed in the appendix.

The Connective-Tissue Fibres

The connective-tissue fibres may be seen in any transverse section coloured by suitable techniques, or in spreads of connective tissue studied by phase-contrast or interference microscopy. The fibres are single and interlace extensively in the connective-tissue ground-substance which fills the bulk of the body. In all essential details, the fibres of *Hirudo* appear identical with those found in the other species of leech. They are very long, about 1–2 μ in diameter, and show the 'cortex' and 'medulla' appearance noted before (Bradbury, 1957). There is no branching, and no tendency for the fibres to run together (fig. 1, A), as in the tendons of vertebrates. From a careful study of sections it seems that the tendency noticed in *Glossiphonia* for the fibres to be orientated obliquely to the long axis of the body, and to show 'crossing over' with fibres on a different plane, is not very marked in the medicinal leech. It seems that the fibre distribution is much more random, which may, perhaps, be partly responsible for the very marked difference in 'body tone' which is found in these two species; for *Hirudo* always appears much more limp than *Glossiphonia*. It was not possible to observe the mode of termination of the fibres.

The staining reactions of the fibres were those typical of collagen, though it was noticed that there was no birefringence of the fibres when they were studied with polarized light; they did swell, however, on treatment with acetic acid. Such reactions only provide presumptive evidence of the nature of the fibre, so further study was by means of more specific tests.

Histochemistry of the fibre cortex

As shown in the appendix, there was no positive reaction to any of the tests for lipids. It was suspected that some lipid material could be present in the fibre in such a condition that any reaction with the standard colouring agents for lipids would be prevented; the sections were therefore treated by the

Fig. 1 (plate). A, fibres in the connective tissue of *Hirudo*, photographed with the interference microscope. Note the cortex and the random arrangement of the fibres.

B, a fibrocyte, showing the numerous triglyceride droplets in the cytoplasm. This was a living preparation photographed with the interference microscope.

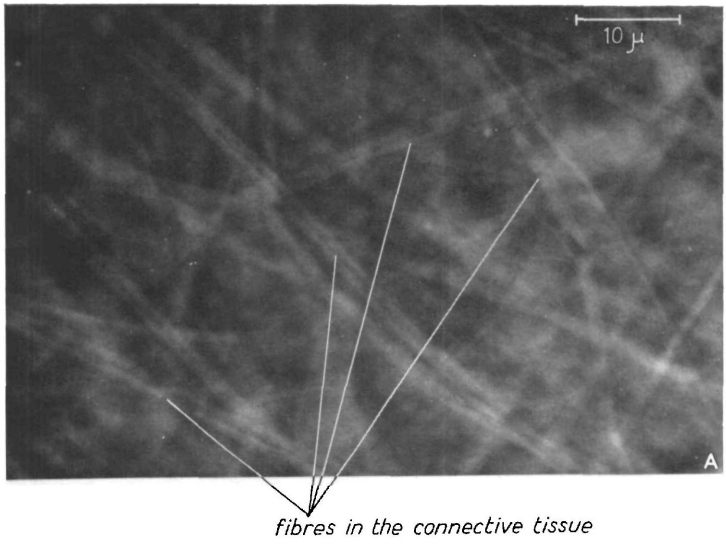

FIG. 1

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techniques of Ciaccio (1926) in order to unmask any lipids. After fixation in Da Fano's fluid or in Cajal's fluid it was not possible to demonstrate any lipid reaction in the fibre 'cortex'. As suggested by Ciaccio, tissues were fixed in formaldehyde and sections were then incubated in a solution of phenol (1% in distilled water) for 24 h, a procedure which he considered to break most associations of lipid and protein. As there was still no positive reaction from the cortex of the fibres, it was concluded that they do not contain any appreciable amount of lipid.

Proteins and amino-acids

As in the previous study of the fibres of *Glossiphonia* only three methods were tried, namely the Sakaguchi test for arginine (Baker, 1947), the Hg / nitrite test for phenols, especially tyrosine (Baker, 1956), and the coupled tetrazonium reaction (Danielli, 1947; Pearse, 1954). Positive results were obtained from the Sakaguchi test, but no reaction could be obtained with the Hg / nitrite test. There was a positive reaction with the coupled tetrazonium technique, which was slightly increased by the application of mild heat and benzoylation. This is considered to be suggestive of the presence of collagen or some related protein (Pearse, 1954). It is also of interest to note that these histochemical tests suggest that the quantity of arginine is much greater than that of tyrosine, a further point which lends support to the hypothesis that collagen is present in the cortex of these fibres.

In an attempt to measure the basiphilia of the cortex, the methylene blue extinction technique of Pischinger (1926) and of Dempsey, Singer, and Wislocki (1950) was used. This method can be adapted to furnish an approximate indication of the iso-electric point of a tissue component. In the present material, it was found that the cortical material of the fibres ceased to take up the dye at pH 5, which is remarkably close to the value for the iso-electric point of collagen (pH 4·85) as given by Pearse (1954).

Carbohydrates

The fibre cortex gave a positive reaction to the periodic acid / Schiff (PAS) test, appearing slightly more coloured than the ground-substance, which also gave some reaction. The PAS reaction was also positive after the slides had been incubated in saliva at 37° C, so that the result was not due to the presence of glycogen. As no lipid material could be detected, it seems unlikely that the reaction was given by glycolipids, but was probably caused by the presence of mucopolysaccharide in or on the fibres.

When the sections were stained with toluidine blue, it was found that there was a slight metachromasy of the cortex, which could be abolished by a treatment with hyaluronidase (a solution of 'Rondase' 1 mg / ml) for 1 h at 37° C. This suggests that some part at least of the PAS reaction is due to the presence of an acid mucopolysaccharide. As the specificity of this enzyme has not been established with certainty, it is not possible to be more precise, though

Pearse (1954) considers it probable that such a substance would be either hyaluronic acid itself, or chondroitin sulphate of type A or C.

Miscellaneous tests

Although it was not thought likely that there would be any striking accumulation of nucleic acids in the cortex of the fibre, the Feulgen and pyronin / methyl green tests were carried out. With the Feulgen test for deoxyribonucleic acid (DNA) there was a positive result in the nuclei of the fibrocytes, but it was noted that this reaction was only feebly positive, as in most of the other somatic nuclei. When the pyronin / methyl green technique of Jordan and Baker (1955) was employed, it was found that both the ground substance, the cortex of the fibres, and the cytoplasm of the fibrocytes coloured with the pyronin. In order to discover whether this reaction was, in fact, due to the presence of ribonucleic acid (RNA), the 'treated-saliva' technique was used (Bradbury, 1956). It has been shown that the basiphilia due to RNA can be abolished by incubating slides in a bath of treated saliva; in the cortical material of the fibres and in the cells it was found that the basiphilia resisted the incubation, and was not diminished, so there does not seem to be any RNA present in this material. It seems likely that the strong affinity for pyronin may be attributed to the presence of large amounts of acid mucopolysaccharides, as these substances would be expected to show a strong basiphilia. It is also perhaps significant that the epidermal mucous-cells containing large quantities of strongly metachromatic material, which can be shown to be acid mucopolysaccharide, also colour strongly with pyronin.

As it proved possible to extract pieces of connective tissue, it was decided to try to obtain the X-ray diffraction pattern of the fibre. It has been shown by numerous workers (e.g. Astbury, 1938; Champetier & Fauré-Fremiet, 1938) that proteins of the collagen group have a very characteristic X-ray diffraction pattern, so that here is a further criterion for the identification of collagen, which has the advantage of being independent of a chemical reaction.

Small pieces of tissue were excised and placed in distilled water; all adherent muscle was removed. In order to remove all salts and as much of the pigmented tissue as possible, both of which would interfere with the diffraction pattern of the protein, the tissue was washed for 72 h in running water and then in distilled water (several long washes). The tissue was dried down on a glass slide and finally lifted off this surface with a sharp scalpel. The exposure to the X-ray beam lasted about 24 h.

The resulting diffraction pattern was that of a protein of the collagen type; it is shown in fig. 2 together with the diffraction pattern of human articular cartilage (chiefly collagen and ground-substance) for comparison. It is seen that the same rings are present in both, though in the leech preparation there is an extra ring due to the pigment; the degree of orientation of the collagen is only small. Both of these diffraction patterns correspond very closely to the 'supercontracted' type of collagen diffraction pattern, noted by Astbury in the reference already quoted.

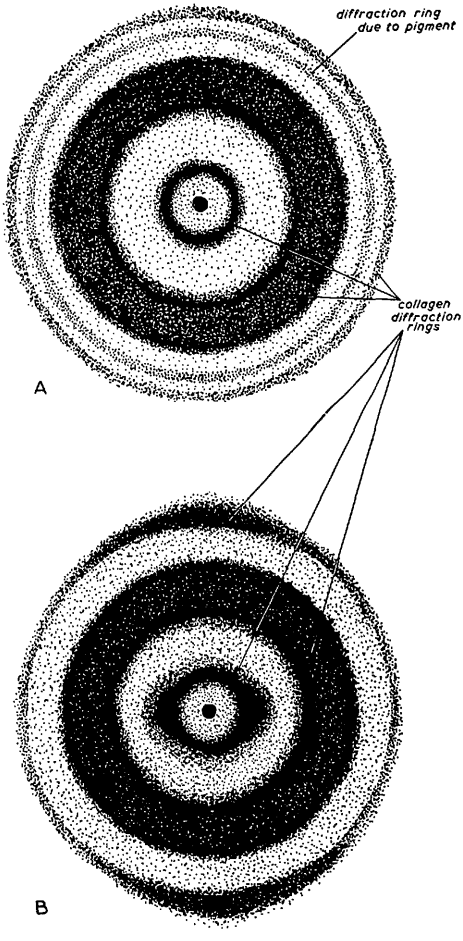

FIG. 2. A, the X-ray diffraction pattern of the connective tissue of *Hirudo*. Note the three collagen rings and the additional ring which is due to the presence of pigment. B, the X-ray diffraction pattern of human articular cartilage for comparison. Only the three collagen rings are present. Both figures are semi-diagrammatic.

On the basis of this evidence, together with consideration of the histochemical and other studies, it seems possible to say with certainty that the cortex of the connective-tissue fibres of *Hirudo* is a collagen-type protein, and that it is associated with some quantity of an acid mucopolysaccharide, either incorporated into its substance, or adsorbed on to its surface.

The Body of the Fibre-cell

General

The shape and general structure of the cell-body is shown in fig. 1, B and in a diagrammatic form in fig. 3; it is usually between 20 and 24 μ in length

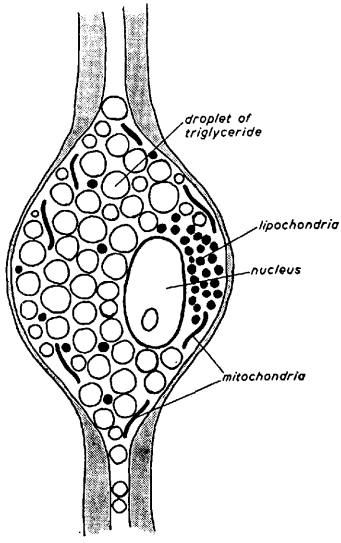

FIG. 3. A diagram of the structure and cytoplasmic inclusions of the fibrocyte of *Hirudo*.

with a diameter of about 10 to 15 μ. The ends of the cell are drawn out into the very long processes which form the medulla of the fibres. As was noticed in the other species of leech, the cortex of the fibre is continued over the cell-body, though it is thinner in this region. The cell has one nucleus, usually oval, measuring about 5 to 8 μ along its greatest length; there is often a well-defined nucleolus.

When these cells were studied alive, by both phase-contrast and interference microscopy, it was found that three types of cytoplasmic inclusions could be distinguished. The most obvious of these are the spherical, highly refringent droplets which are very densely packed and occupy almost the

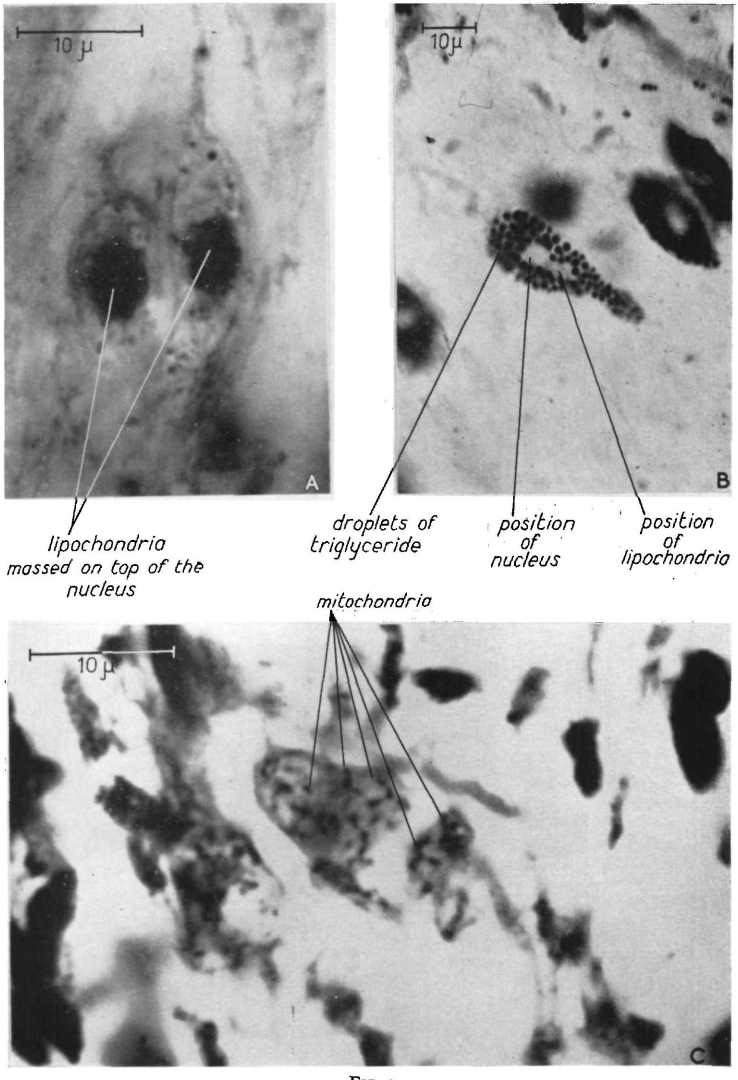

FIG. 4

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whole of the cytoplasm (figs. 1, B; 4, B). They are variable in size but their diameters seem to fall in the range 2 to 5 μ. With the interference microscope it was found possible to measure the refractive index of these droplets by the method outlined in a previous paper (Bradbury, 1957), when the mean value of several measurements gave a result of 1·486, which strongly suggests that they are lipid in nature and do not have much water associated with them (Ross and Chou, 1957).

A further type of cytoplasmic inclusion is very difficult to see on account of the great number of larger droplets, but if a preparation is well flattened either by gentle pressure on the coverslip or by allowing it to dry up slightly, then many small droplets become visible in the cytoplasm near the nucleus (figs. 3; 4, A, B). They seem to be spherical and do not exceed 1 μ in diameter; although they have a higher refractive index than the cytoplasm they are not so strongly refractive as the larger droplets in the same cell. It is noticeable that they alone colour with vital dyes such as neutral red and dahlia violet, thus contrasting sharply with the larger droplets which remain uncoloured by these techniques. It seems that these small droplets differ significantly from the other cytoplasmic inclusions, a supposition which is supported by the observation that only these small objects will impregnate with silver or osmium in the Aoyama or Mann-Kopsch techniques.

The third type of cytoplasmic inclusion is the mitochondria. They are not very numerous, but with phase-contrast they may be seen to be scattered around the periphery of the cell. They are short rods, either straight or slightly curved, measuring about 0·5 μ in diameter and about 2 μ in length. It has been possible to study these in fixed preparations by the use of the Hermann / post-osmication technique (Baker, 1957). A typical appearance is shown in fig. 4, C. The mitochondria will colour with Janus green or Janus black, though only feebly and after a long exposure to the dye.

Histochemistry

One of the chief differences from the similar type of cell in *Glossiphonia* is that in the present work it was found that the cytoplasm of the fibrocyte of *Hirudo* loses its affinity for methylene blue at a higher pH. The M.B.E. value is approximately pH 4. The cytoplasm also contains much more carbohydrate than the corresponding cytoplasm in the other species of leech. The PAS-positive substance is found to occur in the cytoplasm both in a diffuse and in a particulate form, the latter being very noticeable just below the cell surface, where small PAS-positive granules tend to aggregate. This observation is especially interesting in view of reports (e.g. by Jackson, 1955) that fibrogenesis in some avian tissues is accompanied by the secretion of droplets of

FIG. 4 (plate). A, two fibrocytes, showing the lipid droplets massed on top of the nucleus. Acid haematein technique.

B, fibrocyte coloured with Sudan black after fixation in Da Fano's fluid. The triglyceride drops are clearly seen together with the space formerly occupied by the lipochondria.

C, mitochondria in a fibrocyte. Baker's Hermann / post-osmication technique.

PAS-positive material. In the present study the PAS-positive substance was found to be strongly metachromatic. It thus seems very probable that again the reaction may be attributed to the presence of highly sulphated acid mucopolysaccharide.

The cytoplasm also gives a strong, diffuse reaction with Sudan black. Taken in conjunction with the presence of PAS-positive substances, this is perhaps suggestive of the presence of a cerebroside. After fixation in cold acetone, however, the cytoplasm failed to take the Sudan colouring agents. It thus seems unlikely that the PAS reaction and the sudanophilia is due to the presence of a cerebroside. It was found that both types of droplet coloured with Sudan black, so that their lipid nature may be regarded as established. It was, however, difficult to study them because the diffuse lipid in the cytoplasm obscured their outline. Fixation in Da Fano's fluid followed by coloration with the Sudan dyes proved helpful in this respect. It was found that neither the cytoplasmic lipid nor the smaller lipid droplets were preserved, only the large spherical lipid drops colouring strongly (fig. 4, B). Nile blue was found to colour these drops pink. This suggests that they are neutral lipid (Cain, 1947). No reaction with this dye could be detected in any other component of the cell. There was a definite though slight coloration of the large lipid drops with the performic acid / Schiff technique and also with the PAS reaction, but it was found that in the latter test they also coloured with the Schiff's reagent when the preliminary oxidation with periodate was omitted, so that it is not possible to draw any conclusions from these observations. As the coloration of the lipid drops was most marked after fixation in formaldehyde, it may be that the formaldehyde reacted with lipid to form a PAS-positive complex, as Wolman and Greco (1952) suggested. Alternatively, the reaction may be the result of the oxidation of unsaturated lipids by atmospheric oxygen, with production of aldehydic groupings. Support for this hypothesis is provided by the observation that the reaction was much more pronounced when the sections were allowed to stand for some time before being placed in Schiff's reagent.

The large fat drops did not give any positive reaction with the acid haematein test, nor with any other test which was used in the present study, so that it seems probable that these drops are mostly triglyceride; the measured refractive index is in agreement with this hypothesis. The small lipid droplets, however, colour very strongly with the acid haematein reaction (fig. 4, A), as do the mitochondria. If this technique is used in conjunction with a lipid-soluble colouring agent such as Sudan IV, the small lipid droplets and the mitochondria appear blue, whilst the larger triglyceride drops are coloured red. The diffuse lipid noted in the cytoplasm of this cell also coloured with the acid haematein reaction, so it is presumably a phospholipid.

It is apparent that the fibrocytes of the two species of leech bear a great deal of structural resemblance to each other; there are, however, differences in their chemical constitution. The fibre-producing cell in *Hirudo* has large drops of triglyceride, a cytoplasm that has not a very low iso-electric point

and contains much acid mucopolysaccharide and diffuse phospholipid; that of *Glossiphonia*, on the other hand, contains no triglyceride, but only phospholipid droplets and mitochondria, together with much RNA. The latter substance does not seem to occur in the same type of cell in *Hirudo*.

The Ground-Substance

In unstained sections and spreads of living tissue examined by phase-contrast, the ground-substance appears perfectly homogeneous, though it may be recognized in standard histological preparations by its coloration with dyes such as light green and aniline blue. The ground-substance also shows a strong affinity for metallic ions, as was shown by Bensley (1934). In *Hirudo* it is found that fixation of the ground-substance is difficult; when coagulant fixatives such as Zenker's fluid are used the ground-substance no longer appears homogeneous but the basiphil material contained in it is found to have aggregated into small spherical droplets which alone colour and react to histochemical tests. It was found that this aggregation did not occur with formaldehyde fixation, so that for all studies on fixed material this was used as the standard fixative.

The refractive index of the ground-substance was measured in a preparation of living connective tissue by use of the interference microscope. The value was found to be 1·353, which agrees with the value of 1·35 found for the ground-substance of the pond leech.

Histochemistry

The ground-substance was found to have an M.B.E. value of pH 4, considerably less than that of the fibres. No lipids could be detected in it, even after all the unmasking procedures suggested by Ciaccio had been tried. There was a feeble reaction to the coupled tetrazonium test, no result with the Sakaguchi test, but a strong positive with the Hg nitrite test for tyrosine. The PAS reaction was positive, and there was strong metachromasy, which could be suppressed by hyaluronidase treatment. The conclusion must be that here again is a site of concentration of acid mucopolysaccharide. It is possible that there is a neutral mucopolysaccharide present as well; but as the original metachromasy of this tissue is so intense, it is not possible to decide whether there is any increase on sulphation, and at present no conclusion can be reached on this point.

Discussion

The most striking feature of this study is the general similarity between the two species of leech in their connective-tissue fibres, fibrocytes, and ground substance. The similarities are perhaps most marked in the fibres: in both species they show the cortex and medulla, they run singly in the ground substance, and as far as can be ascertained by histochemical methods they are composed of a collagen or a similar protein. The cells which secrete the fibres show minor differences, such as the presence of triglyceride fat drops in the

fibrocytes of *Hirudo*. It seems probable that this is part of the adipose reserve of the body, for similar fat is found to accumulate in the cells of the crop wall. This difference is correlated with the absence, in *Hirudo*, of the adipose cells which form such a conspicuous feature of the connective tissue of *Glossiphonia*.

The other major difference is that *Hirudo* has relatively much more ground substance than the rhynchobdellid leech, and that the ground-substance has a much larger content of acid mucopolysaccharide. Increase in the amount of ground-substance may be supposed to be a consequence of the greater degree of coelom reduction found in the jawed leeches. It is also noticeable that the general body 'stiffness' of *Hirudo* is much less than that of *Glossiphonia*, a fact which was pointed out by Bourne (1884). This he attributed to differences in the amount of connective-tissue substance, but it may well be related in some way to the observed differences in the proportions of acid and neutral mucopolysaccharides. In this we may find some clue to the special functions of the connective-tissue ground-substance, such as direct control of the cellular environment, and it may well be that such activities are influenced by the presence of these two types of substance.

No observations made during the course of the present work throw any light on the process of fibrogenesis, especially on the question whether the cortex is intra- or extracellular. A further study of this tissue by means of the electron microscope has been undertaken in order to attempt to answer this question.

I wish to acknowledge my great debt to Dr. J. R. Baker, for his continued encouragement and helpful advice. My thanks are also due to Professor Sir Alister Hardy for continued facilities for research in his department, and to Dr. K. Little and Miss Maureen Westerdon for undertaking the X-ray diffraction study of the fibres. The work was carried out during the tenure of a Senior Hulme Scholarship of Brasenose College, Oxford, and of a grant from the Department of Scientific and Industrial Research.

REFERENCES

AOYAMA, F., 1929. Z. wiss. Mikr., **46**, 489.
ASTBURY, W. T., 1938. Trans. Faraday Soc., **34**, 378.
BAKER, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Ibid., **87**, 441.
—— 1947. Ibid., **88**, 115.
—— 1949. Ibid., **90**, 293.
—— 1956. Ibid., **97**, 161.
—— 1957. Ibid., **98**, 29.
BENSLEY, S. H., 1934. Anat. Rec., **60**, 93.
BOURNE, A. G. 1884. Quart. J. micr. Sci., **24**, 419.
BRADBURY, S., 1956. Ibid., **97**, 323.
—— 1957. Ibid., **98**, 29.
CAIN, A. J., 1947. Ibid., **88**, 363.
CALLEJA, C., 1898. Z. wiss. Mikr., **15**, 322.
CHAMPETIER, G., and FAURÉ-FREMIET, E., 1938. J. chim. Phys., **35**, 223.
CHOU, J. T. Y., 1957. Quart. J. micr. Sci., **98**, 47.

CIACCIO, C., 1926. Boll. soc. Biologia sper., **1**, 47.
DANIELLI, J. F., 1947. Symp. Soc. exp. Biol., **1**, 101.
DEMPSEY, E. W., SINGER, M., and WISLOCKI, G. B., 1950. St. techn., **25**, 73.
FEULGEN, R., and ROSSENBECK, H., 1924. Z. phys. Chem., **135**, 203.
GOMORI, G., 1952. *Microscopic histochemistry.* Chicago (University Press).
HERXHEIMER, G. W., 1901. Deut. med. Woch., **36**, 607.
JACKSON, S. F., 1955. Nature, **175**, 39.
JORDAN, B. M., and BAKER, J. R., 1955. Quart. J. micr. Sci., **96**, 177.
LANKESTER, E. R., 1880. Ibid., **20**, 307.
LISON, L., 1953. *Histochimie et cytochimie animales.* Paris (Gauthier-Villars).
PANTIN, C. F. A., 1948. *Microscopical technique.* Cambridge (University Press).
PEARSE, A. G. E., 1954. *Histochemistry, theoretical and applied.* London (Churchill).
PISCHINGER, A., 1926. Z. Zellforsch., **3**, 169.
ROSS, K. F. A., and CHOU, J. T. Y., 1957. Quart. J. micr. Sci., **98**, 341.
SCRIBAN, J. A., and AUTRUM, H., 1934. 'Hirudinea' in Kukenthal and Krumbach's *Handbuch der Zoologie.* Berlin (De Gruyter).
WEIGL, R., 1910. Bull. Intern. Acad. Sci. Cracovie, Ser. B (no vol. no.), 691.
WOLMAN, M., and GRECO, J., 1952. St. techn., **27**, 317.

APPENDIX

Results

Test	*Reference*	*Ground substance*	*Fibre*	*Fibrocyte cytoplasm*	*Large lipid drops*	*Small lipid drops*
Picro-indigo carmine	Calleja, 1898	blue	blue	orange	—	—
Masson . .	Pantin, 1948	green	green	reddish	—	—
Basiphilia . .	—	+	—	—	—	—
Acetic acid . .	—	—	swells and dissolves	—	—	—
Methylene blue extinction . .	Pearse, 1954	pH 4	pH 5	pH 4	—	—
Refractive index .	—	1·353	—	—	1·486	—
Aoyama . .	Aoyama, 1929	O	O	O	O	+++
Mann Kopsch .	Weigl, 1910	+	+	O	O	+++
Neutral red . .	—	O	O	O	O	+++
Methylene blue .	—	O	O	O	O	+++
Sakaguchi . .	Baker, 1947	O	++	+	O	—
Hg nitrite . .	Baker, 1956	+	O	+	O	—
Coupled tetrazonium	Pearse, 1954	O	++	++	O	—
C.T.+benzoylation	,,	O	+	O	O	—
C.T.+dinitrofluorobenzene . .	,,	O	+	O	O	—
C.T.+performic acid	,,	O	O	++	O	—
Sudan black . .	Baker, 1944, 1949	O	O	++	+++	+++
Sudan IV . .	Herxheimer, 1901	O	O	O diffuse	+++	+++
Sudan black after Da Fano's fluid .	—	O	O	O	+++	O
Sudan black after phenol . .	Ciaccio, 1926	O	O	+ diffuse	+++	++
Sudan black after Cajal's fixative .	—	O	O	+ diffuse	+++	++

Results (cont.)

Test	*Reference*	*Ground substance*	*Fibre*	*Fibrocyte cytoplasm*	*Large lipid drops*	*Small lipid drops*
Sudan black after cold acetone	—	O	O	O	O	O
Nile blue	Cain, 1947	O	O	O	pink	—
Acid haematein	Baker, 1946	O	O	+	O	+++
,, control	,,	O	O	O	O	O
Liebermann	Lison, 1953	O	O	O	O	—
Windaus	,,	O	O	O	O	—
Pseudo-plasmal	,,	O	O	O	++	—
Performic acid Schiff	Pearse, 1954	O	O	O	+++	—
PAS	,,	++	+++	+++	+	—
PAS control	,,	O	O	O	+	—
PAS after saliva	,,	+	++	+++	+	—
Toluidine blue	,,	+++	+	++	O	—
Toluidine blue+hyaluronidase	,,	O	O	O	O	—
Feulgen	Feulgen and Rossenbeck, 1924	O	O	O	O	—
Pyronin / methyl green	Jordan and Baker, 1955	++	++	++	O	—
P/Mg saliva control	Bradbury, 1956	++	++	++	O	—
Gomori alkaline phosphatase	Gomori, 1952	O	O	O	O	—
Gomori acid phosphatase	,,	O	O	O	O	—

Key: +++ Strong reaction.
++ Moderate reaction.
+ Weak reaction.
— No observation.
O Negative reaction.