ELECTRON MICROSCOPE OBSERVATIONS OF THE GROUND SUBSTANCE OF INTERSTITIAL CONNECTIVE TISSUE

by

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Previous observations led to the conclusion that the so-called ground substance of interstitial connective tissue is not amorphous but consists of a system of delicate membranes in which fine fibrils lie embedded[1]. It was further suggested that hyaluronic acid was not essential to this lamellar structure but probably bore the same kind of relationship to it as water-proofing material does to a fabric. The electron micrographs shown here would seem to add support to these views.

METHODS

A mouse of 25 g was killed by sharp extension of the neck. A median incision was made through the skin of the trunk and extended along the ventral aspect of the limbs on one side. The flap thus outlined was gently pulled away from the trunk and pinned on to a cork mat. About 1 ml of physiological saline was injected beneath the fascia so exposed. In this way the fascia was swollen from paper thickness into a bleb about 1 cm high (Ranvier's "Boule d'oedème"). Pieces of the bleb about 5 mm wide were snipped off with scissors and immersed in about 500 ml of distilled water adjusted to pH 10.3 with NaOH. A similar bleb was raised in the fascia of the opposite side by the injection of hyaluronidase in physiological saline (Benger's "Hyalase" 10 units/ml). Similar pieces were removed from this and immersed for ten minutes in hyaluronidase solution. These also were transferred to distilled water at pH 10.3.

It has been shown previously that at pH 10.3 the tissue will undergo a considerable hydration in which the ground substance alone takes part[2]. The mechanical separation of the components of the tissue caused by the injection together with the hydration at this pH produced a degree of swelling which permitted pieces of tissue to be handled which when dry were sufficiently thin for use in electron microscopy.

The principal problem in mounting the specimens was that of spreading them thinly enough for the electron beam to penetrate easily, without stretching the tissue to such an extent that gross distortion of structure was brought about. This was finally achieved by the following very simple technique. After the pre-treatment described a piece of tissue was examined under a dissecting microscope, any masses of fat present were removed, and the tissue was given a final quick rinse in distilled water. Still under the dissecting microscope, the specimen was placed on a grid in a drop of water and gently opened out till it was more or less fully extended without any stretching whatsoever. As much of the water as possible was then carefully drained off with strips of filter paper. It was observed that if the tissue is greatly stretched at once, it shows a tendency to retract when the tension is removed, but after a few minutes it is dry enough to remain stretched (owing to adhesion to the surface of the slide), but yet moist enough to be extensible. At this stage the tissue can be extended quite easily to form a thin lamella which, under the electron microscope, shows little or no gross damage to the tissue substance or structure. The spread was then left to dry thoroughly. We found that drying for twelve hours in a desiccator removes all water inclusions between the spread, the grid and the slide.

The question of removing the mounted specimen is not straightforward, as any attempt merely to cut round the grid with a scalpel and lift it away with tweezers usually separates the specimen from the grid. We have found the following technique simple and satisfactory. Four radial scratches

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are made in the tissue at the edge of the grid and a small dab of glue put on each. These hold the tissue firmly to the grid. When the glue is dry, a ring of tissue is removed from around the grid by radial strokes of a fine needle or tweezer point (*e.g.* watchmaker's Dupont No. 7 tweezers). By this means the grid can be completely isolated from the surrounding spread without any disturbance of the mount. It can then be lifted with tweezers and inserted in the specimen holder of the microscope.

Alternatively and preferably, if with short outward radial strokes the tissue round the outer edge of the grid is scraped away when it is dry and whilst the tissue on the grid still is not, it will be found that the grid and accompanying tissue, after drying, can be lifted from the slide for mounting without disturbing the latter (Fig. 1).

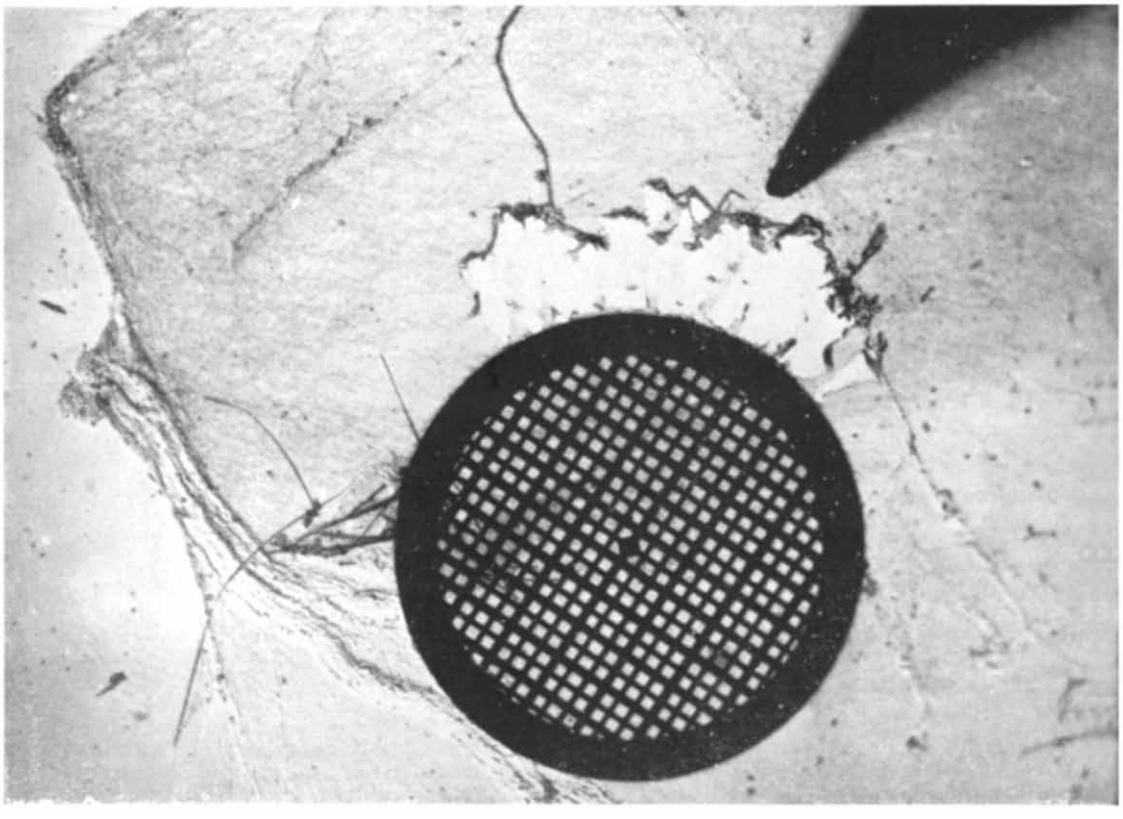

Fig. 1. The method of removing the mounted specimen. The point of the tweezers is clearly visible in the top right-hand corner. × 20.

RESULTS

The structure of normal tissue

Spreads of untreated tissue show that the interfibrillary substance (as seen by light microscopy, Fig. 2) consists, in fact, of a membrane of otherwise amorphous material in which fibres are embedded (Fig. 3). Superimposed on the picture of the membrane are the dense stripes representing the thick collagen fibres observed in the light microscope. Fig. 4 shows an enlargement of a small area of Fig. 3.

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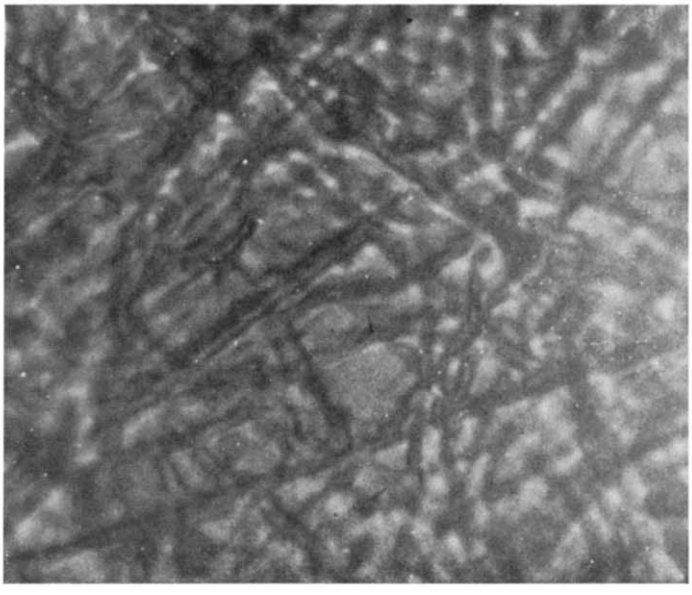

Fig. 2. Photomicrograph of tissue spread as used for electron microscopy. The spaces containing the ground substance are clearly visible. × 1400.

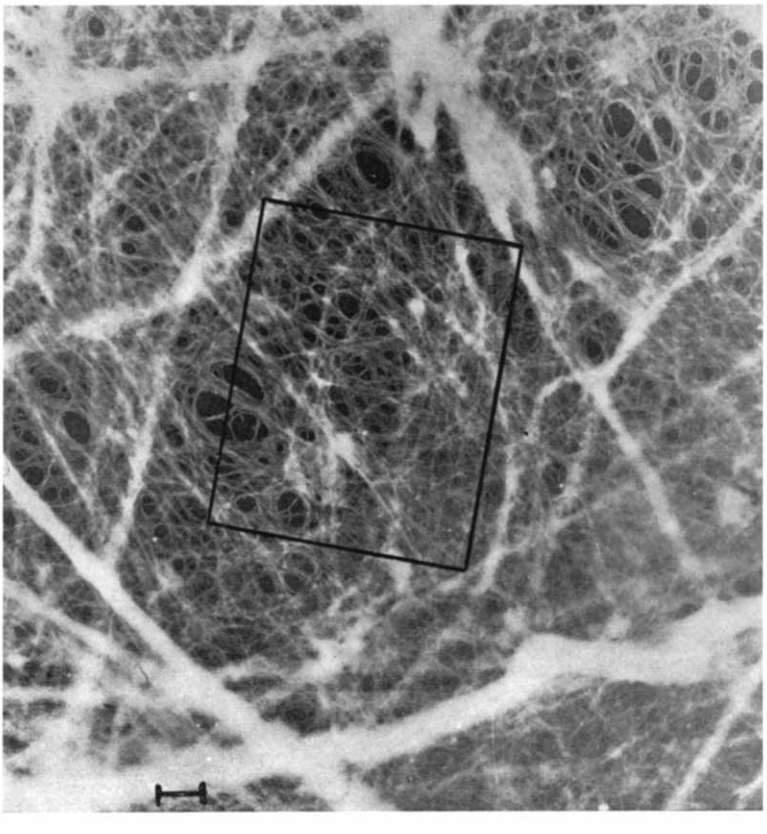

Fig. 3. Electron micrograph of a spread of untreated fascia. The membranous structure with embedded fibrils is clearly visible. The large holes are artefacts.

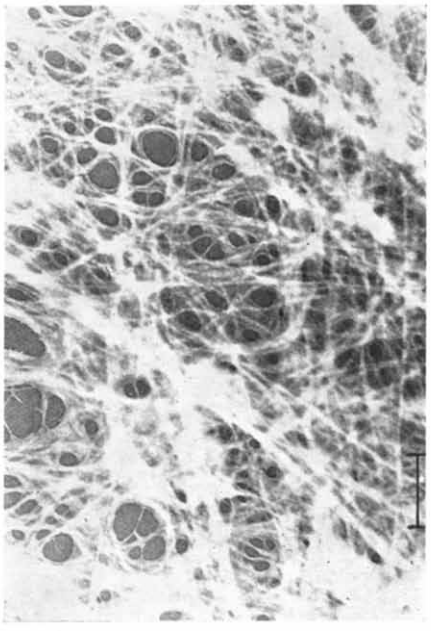

Fig. 4. An enlargement of the area in the rectangle in Fig. 3. The typical 640 A cross banding of collagen is suggested in several parts of the print but is nowhere clearly visible.

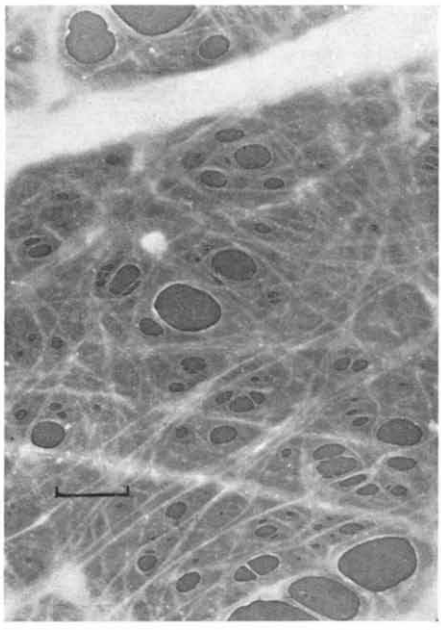

Fig. 5. Electron micrograph of a spread of hyaluronidase-treated fascia. The membranous structure, with embedded fibrils, is clearly visible, though the 640 A periodicity of collagen is not observable. The large holes are artefacts.

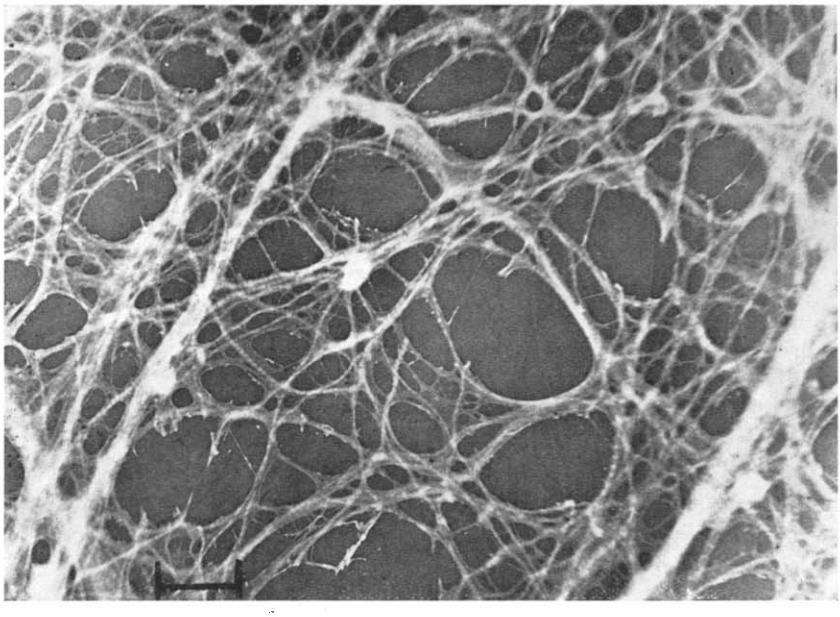

Fig. 6. Electron micrograph of a spread which has been slightly overstretched. The membranous structure has almost completely disappeared, but it is now possible to observe the 640 A cross banding of the collagen.

The structure of the hyaluronidase-treated tissue

Rather surprisingly, the hyaluronidase-treated tissue shows no difference from the untreated tissue. A spread appears as the membrane and thick fibre complex described above, the membranous component consisting of amorphous material and thin fibrils (Figs. 5 and 6).

The nature of the fine fibrils

It was not possible to establish with complete certainty the nature of the fibrous matter. From its general appearance it would seem to be collagen, and the characteristic banding of the collagen fibre is observable in some parts of some photographs (Figs. 4 and 6), but the majority of fibres did not show this banding and cannot, therefore, be positively described as collagen without further evidence.

An attempt was made to obtain further evidence by examination of untreated, trypsinated and hyaluronidase-treated teased specimens, but in all three cases the fibres appeared to be amorphous, or to have a periodicity of much less than the expected 640 A. In some instances the fibres appeared as collagen fibres embedded in an amorphous sheath but this was not always the case (Fig. 7).

The bearing of the results on this particular question may be summarised as follows: if the not unreasonable assumption is made that they are coated with a sheath of amorphous material, in no case is the appearance of the fibres incompatible with their being collagen. But the fact that the typical banding is not always observable makes it impossible to state categorically that all the fibres are collagen fibres on the evidence submitted here.

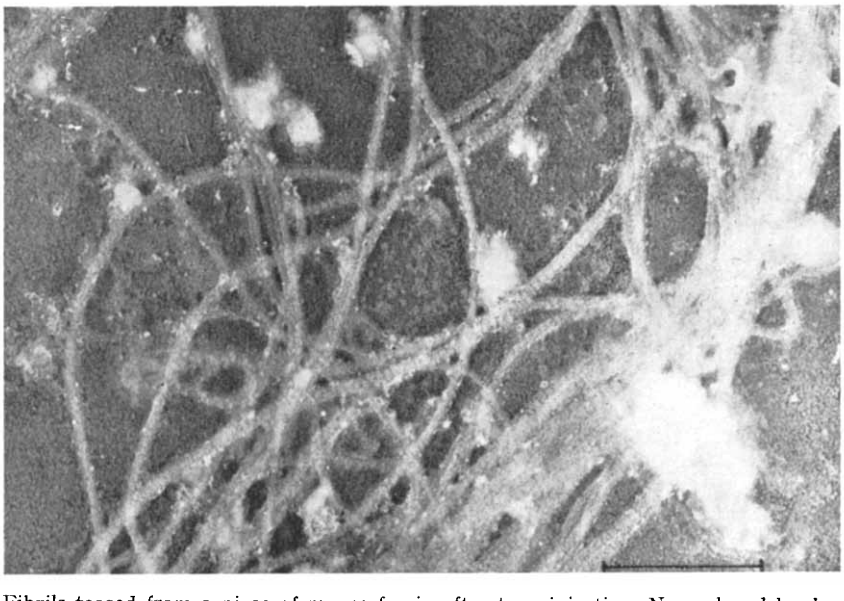

Fig. 7. Fibrils teased from a piece of mouse fascia after trypsinisation. Normal and hyaluronidase-treated tissues show the same appearance and so are not reproduced here.

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The nature of the amorphous material

The smallest fibres visible would seem to be embedded in and even to merge into a thin layer of structureless substance. Whether this substance has the same chemical composition as the fibres or whether it is composed entirely differently could not be determined. The possibility that its structureless appearance may be to some extent an artefact due to drying is considered later.

The activity of the hyaluronidase solution

A membrane of fascia was removed from a freshly killed mouse and mounted as an occluding membrane over the end of a glass tube in the way previously described[3]. The rate of flow of saline through the membrane at a pressure of 4 cm water was 0.42 μl/sec. After about 0.5 ml of the hyaluronidase solution had been allowed to flow through the membrane, the rate of flow quickened at once to 10 μl/sec.

DISCUSSION

The term connective tissue ground-substance is generally considered to refer to material lying between those fibrils which may be rendered visible by ordinary microscopic methods. In the present instance, fibrils of this kind could be identified in the specimens examined, both by an ordinary microscope and also in the fluorescent screen of the electron microscope itself. There can be no doubt therefore that the membranes and fibrils to be seen in the electron micrographs do actually occupy the precise situation in which ground substance may be supposed to exist.

An essentially lamellar structure of the ground-substance of interstitial connective tissue has been postulated previously[4,5]. One reason why this view has so far failed to obtain general recognition would seem to be the prevalence of histological techniques involving the making of thin sections. It is obvious that such methods cannot reveal fine membranous structures as such. It has been shown too that the membranes react sensitively to slight changes in chemical environment[6] and must certainly be disrupted by many of the usual reagents. So far as the examination of fresh, unfixed tissue is concerned it has been shown that the ground substance, at physiological conditions of pH and salt concentration, combines avidly with water[2]. This hydration renders invisible its structured components even when examined under dark ground illumination. They can, however, be brought into prominence by gentle dehydration[1]. It will be appreciated therefore that the examination of fresh tissue does not in itself offer an easy method for the study of ground-substance. The demonstration of the essentially membranous nature of ground-substance has so far depended upon special techniques of unestablished validity. It is probably for this reason more than any other that this conception of its structure has failed to obtain general acceptance.

In view of the recent tendency to identify the ground-substance with hyaluronic acid[7] it is of interest that the fine membranes and fibrils which appear to constitute the ground-substance are as prominent in the electron micrograph of a specimen which had been adequately treated with hyaluronidase as they are in the micrograph of untreated tissue. It must be concluded that hyaluronic acid is not an essential component of these structures.

The question as to how a sample of hyaluronidase which failed to cause any change in the appearance of tissue under the electron microscope was, all the same, capable of

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increasing its permeability so greatly, remains unanswered. It is clear that hyaluronic acid must be associated with the visible components of the tissue in a way which determines its permeability to water but the nature of this association remains undetermined. It has been suggested by one of us[3] that at a molecular level the ground-substance consists of a protein network with hyaluronic acid in its interstices. No indication of such an arrangement is to be seen in the present photographs, but this does not invalidate the conception. A network of such a kind could very well exist in the tissue in its normal hydrated state and become obscured by the general coagulation which accompanies drying. It is probable, too, that the network postulated is too small to be resolved by the electron beam. The large holes in the membranes are artefacts since some were seen to form during the process of photography. They are in any case too large for macromolecules to impact in them in the way suggested.

SUMMARY

Electron micrographs of interstitial connective tissue show that the bulk of the ground-substance consists of minute membranes composed of fine fibrils embedded in an apparently structureless substance.

Hyaluronic acid does not appear to take any part in the composition of these structures.

RÉSUMÉ

Une étude du tissu conjonctif interstitiel à l'aide du microscope électronique a montré que la majeure partie de la substance de base de ce tissu consiste en de toutes petites membranes composées de fibrilles fines qui sont entourées d'une substance apparemment dépourvue de structure.

Il ne semble pas que l'acide hyaluronique prenne part à la constitution de ces structures.

ZUSAMMENFASSUNG

Aufnahmen des Zwischenbindegewebes mit dem Elektronenmikroskop zeigten, dass der grösste Teil der Grundsubstanz aus winzigen Membranen besteht, die aus feinen, in eine scheinbar strukturlose Masse eingebetteten Fibrillen zusammengesetzt ist.

Hyaluronsäure scheint an der Zusammensetzung dieser Strukturen nicht beteiligt zu sein.

REFERENCES

[1] T. D. DAY, *Lancet*, ii (1947) 945.
[2] T. D. DAY, *J. Physiol.*, 109 (1949) 380.
[3] T. D. DAY, *J. Physiol.*, 117 (1952) 1.
[4] W. FLEMMING, *Arch. mikr. Anat.*, 12 (1876) 391.
[5] E. LAGUESSE, *Arch. Biol.*, 31 (1921) 173.
[7] T. D. DAY, *J. Path. Bact.*, 59 (1947) 567.
[6] The ground substance of the mesenchyme and hyaluronidase, *Ann. N.Y. Acad. Sci.*, 52 (1950) 943.

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