ELECTRON MICROSCOPE STUDIES OF COLLAGENOUS FIBRES IN BONE

by

A. V. W. MARTIN

Medical Research Council Biophysics Research Unit, King's College, London (England)

Collagenous fibres in bone were first demonstrated morphologically in the electron microscope (E.M.) by WOLPERS[1], who was able to show in fibres isolated from partly decalcified human bone a periodic structure similar to that of tendon collagen. More recently, a group of Swiss workers (KELLENBERGER AND ROUILLER[2]; RUTISHAUSER, HUBER, KELLENBERGER, MAJNO AND ROUILLER[3]; HUBER AND ROUILLER[4]) have used a replica technique to study the structure and arrangement of collagen fibres in polished and etched surfaces of human and canine bone.

Work on avian and mammalian bone now in progress in this Laboratory has two main objects: a. to find out whether the collagen of bone differs from that of other tissues; b. to ascertain what part the collagenous fibres play in embryonic and post-embryonic bone development.

The present communication is concerned only with the first question.

MATERIAL AND METHODS

Collagen was obtained from the following sources:

1. *Avian tissues.* Long bones, and the ossified and unossified parts of leg tendons of adult fowl and pheasant; neck tendon of fowl; long bones of 10-, 12-, 13- and 16-day fowl embryos.

2. *Mammalian tissues.* Long bones, leg tendon and tail of adult rat; long bones of adult mouse.

The tendon collagen was used for comparison with the fibres isolated from bone, and to study the effect on collagen of the reagents subsequently used for the decalcification of bone.

Preparation of tissues for the E.M. For examination in the E.M. the collagenous fibres of bone must first be freed from adhering inorganic salts. The following procedure was used;

1. Removal of periosteum, marrow and any cartilage present.

2. Fixation for about 12 hours in 70% alcohol or 5% formalin.

3. Decalcification in a 5% solution of trichloracetic acid (T.C.A.) in 10% formalin, or in a 6% solution of nitric acid. The time required varies with the specimen; 12–48 hours was usually satisfactory.

4. Treatment with a 5% solution of sodium sulphate to prevent swelling of the fibres; about 5–12 hours is sufficient.

5. Thorough washing in running water, followed by several changes of glass-distilled water.

6. Separation of collagenous fibres by scraping the bone with a sharp blade held at right angles to the bone surface, followed by teasing with a pair of needles under the dissecting microscope.

7. Centrifugation of the suspension for approximately 10 minutes at about 2500 r.p.m. with subsequent re-suspension of the precipitate in fresh glass-distilled water.

8. Transfer of one drop of the suspension to each of a series of formvar- or collodion-covered specimen grids.

The time needed for decalcification can be reduced by scraping and teasing the bone immediately after fixation, but this method calls for a rather elaborate and time-consuming washing procedure after decalcification. Embryonic bone fibres do not withstand the decalcification treatment described as well as does adult material. It is sometimes possible, however, to examine such fibres without chemical removal of the bone salts.

References p. 48.

All specimens studied in the present investigation were teased preparations, with the exception of a positive replica of the polished surface of a fowl bone made by KELLENBERGER's replica method[5]. The teased specimens were "shadowed" with palladium-gold or chromium at angles varying from 5 to 15°. The specimens were examined with a Siemens electron microscope (1938) at 50 Kv.

RESULTS

1. *Collagenous fibres of adult bone*

Undecalcified adult bone can be teased to show in the E.M. the presence of a fibrous component. To examine the fine structure of these fibres, however, it is necessary to decalcify the bone according to the method described above. Long bones of the adult fowl, treated in this way, reveal fibres with the well-known morphological characteristics of collagen.

The most conspicuous of these features are the slightly projecting twin bands of the dense portion ("A" band (SCHMITT); "D" band (WOLPERS)) of the repeating pattern. The whole of the period measures on the average about 660 A. As in other specimens of collagen studied by various authors, the dense A-band generally occupies $^2/_3$ of the total period. "Subbanding", as described by GROSS AND SCHMITT[6], and as observed by WOLPERS after treatment with certain chemicals and under some pathological conditions (WOLPERS[1,7]), was not observed after decalcification. In most fibres a filamentous fine structure can be seen running parallel to their long axis; this has been described for human skin collagen by GROSS AND SCHMITT[6]. Similar fibres can be demonstrated in avian bone by means of the replica method of KELLENBERGER *et al.*[3] (Plate I, Fig. 4).

It does not seem advisable at this stage to give measurements of the observed diameters of "fibres", as it is often impossible to decide whether isolated fibres or bundles are concerned. One type of arrangement which is met frequently in preparations of tendon collagen has not been seen in bone. It is shown in Plate I, Fig. 2, and consists of a number of fibres (or narrow bundles) tightly coiled around one another to form a thick cylindrical structure resembling a piece of rope.

If decalcified fowl bone is treated with acetone to extract the fat, and then digested with trypsin, a preparation is obtained consisting of collagen fibres with unaltered periodic structure, and differing from decalcified fowl bone not subjected to these treatments only in that larger fibre bundles are absent.

Of mammalian material, mouse bone was examined without decalcification. The inorganic layer covering the fibres appears to be of very even thickness, but the magnification used was insufficient to show whether or not it is built up of very small needle-shaped crystals as described in human bone by WOLPERS[1]. In adult rat bone, decalcification by the method described above caused fragmentation and swelling of the collagenous fibres, and partial destruction of their periodic structure. Where the cross-banding was still present the main spacing had not changed noticeably.

2. *Embryonic fowl bone*

Observations on embryonic bone are still of a very preliminary nature. So far, only untreated material has been examined, as the decalcification methods used with good results on the bone of the adult fowl destroy the collagenous fibres in embryonic specimens. The granular layer of bone salts covering these fibres is sometimes partly removed during the mechanical fragmentation of the specimen. Where this has occurred, collagen cross-bands become visible, but so far they have never been sufficiently well defined

References p. 48.

to allow accurate measurements. In some fibres "sub-bands" seem to be present, and the macroperiod of about 640 A is less marked. A detailed study of collagen fibres in embryonic bone will be possible only when a technique for isolating such fibres without damaging their structure has been developed.

3. *Collagen fibres from tendon (control observations)*

In order to compare the collagen fibres isolated from bone with those from other tissues, fibres from various tendons of the fowl and rat were examined with the E.M., both before and after the decalcification treatment described.

Avian tendon collagen, whether from the neck or from the calcified or uncalcified portion of the leg tendons, seems unchanged after treatment with T.C.A. (Plate I, Fig. 1, and Plate II, Fig. 7). Before "decalcification", uncalcified leg tendon of the fowl appears as in Plate II, Fig. 6; a comparison with Plate I, Fig. 3, shows the morphological similarity between these untreated fibres and the collagenous fibres isolated from avian bone by decalcification. In the ossified portion of avian leg tendons, the collagen fibres are covered by a layer of inorganic material. Often this is partly lost during the mechanical separation of the fibres, the periodic structure of which can then be studied in the E.M. (Plate II, Fig. 8). After decalcification, these fibres are indistinguishable from untreated collagen of the unossified portion of avian leg tendons, and from fibres of avian neck tendons.

On the other hand, collagen from rat tendon undergoes marked changes when "decalcified" according to the method used throughout this investigation. The flexibility of the fibres is destroyed so that teasing becomes very difficult and the fibres break when mechanical separation is attempted. The E.M. reveals a significant morphological difference between untreated collagen from the rat tail and "decalcified" fibres from the same source. The latter appear as flattened ribbons, ending abruptly in a break, and their cross-banding is blurred though still recognizable. The main periodic spacing of about 640 A has not changed noticeably, but apart from the alternating dense ("A"

PLATE I →

Fig. 1. Collagenous fibres from the ossified portion of leg tendon of the fowl. Decalcified with 6% nitric acid, followed by 5% sodium sulphate, after fixation with 70% alcohol The fibre bundles are flexible and easy to isolate; a few are flattened owing to the swelling caused by nitric acid; the periodic cross-banding is well-defined. Shadowed with palladium-gold. × 26,000.

Fig. 2. Collagen fibres from the unossified portion of leg tendon of the fowl, teased after fixation with 70% alcohol. Note the identical appearance of the fibres in this specimen and the TCA-treated pheasant tendon (Plate I, Fig. 3). The thick rope-like type of collagen bundle on the left, consisting of fibres or small bundles tightly twisted around one another, is found frequently in this material. Shadowed with palladium-gold. × 26,000.

Fig. 3. Collagenous fibres from adult fowl long bone, decalcified with TCA, followed by sodium sulphate. After decalcification the fibres were "cleaned" with Trypsin after pre-treatment with acetone. This procedure helps to break up larger bundles into smaller and relatively uniform elements but does not seem to affect the appearance of the periodic cross-banding. Compare this specimen with that shown in Plate II. Fig. 2. Shadowed with palladium-gold. × 26,000.

Fig. 4. Positive replica of collagenous fibres from a fowl long bone. The method employed was the celluloid-silicon monoxyde technique first described by KELLENBERGER[5]; the negative celluloid replica was shadowed with palladium-gold before SiO was evaporated onto it. A small area of bone surface near the centre of the diaphysis was polished on an Arkansas stone until the top layer had been removed and a highly polished surface had been achieved; this was etched with hydrochloric acid for about 3 minutes before the celluloid replica was taken. The arrangement of the fibres shows a high degree of orientation, and the fibres themselves do not differ appreciably from the teased material in Fig. 3. × 26,000.

References p. 48.

Fig. 1

Fig. 3

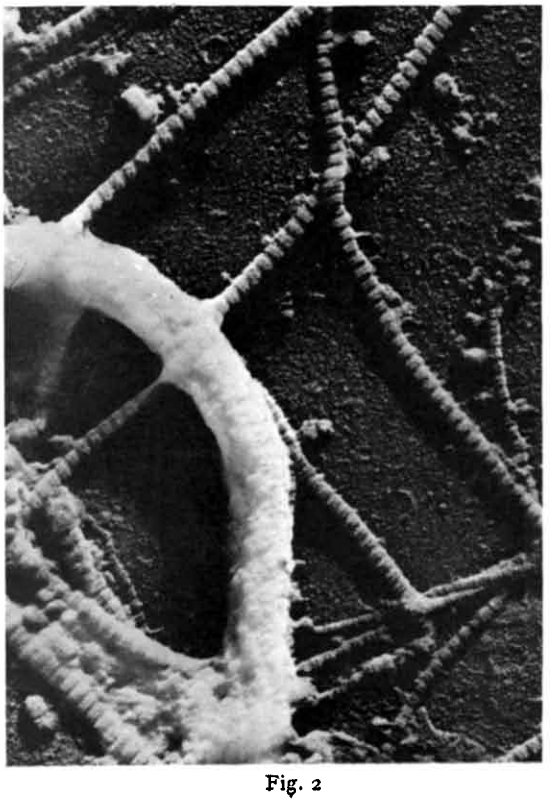
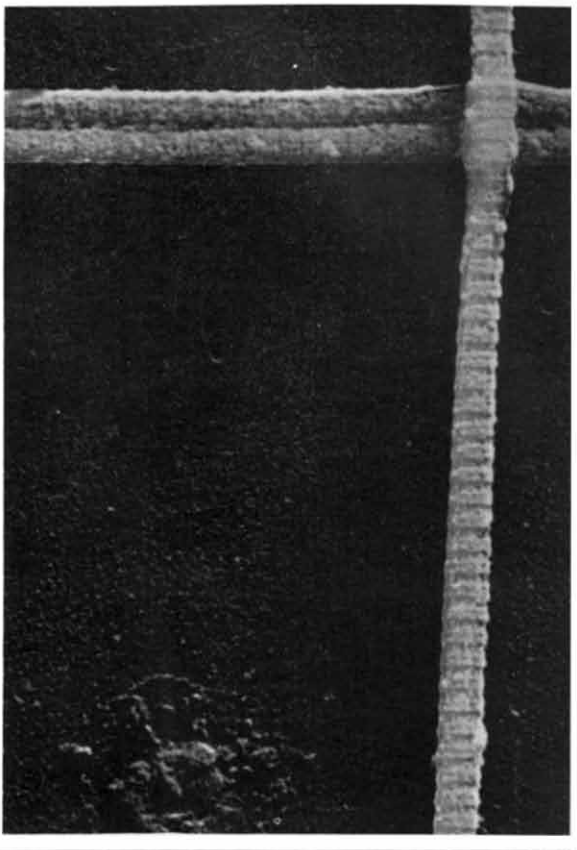


Fig. 2

Fig. 4

Fig. 6

Fig. 7



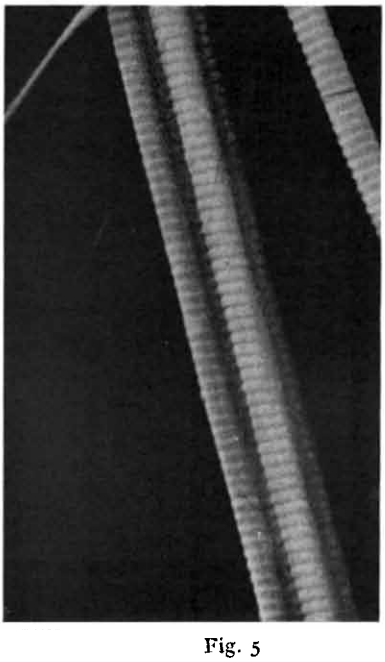


Fig. 5

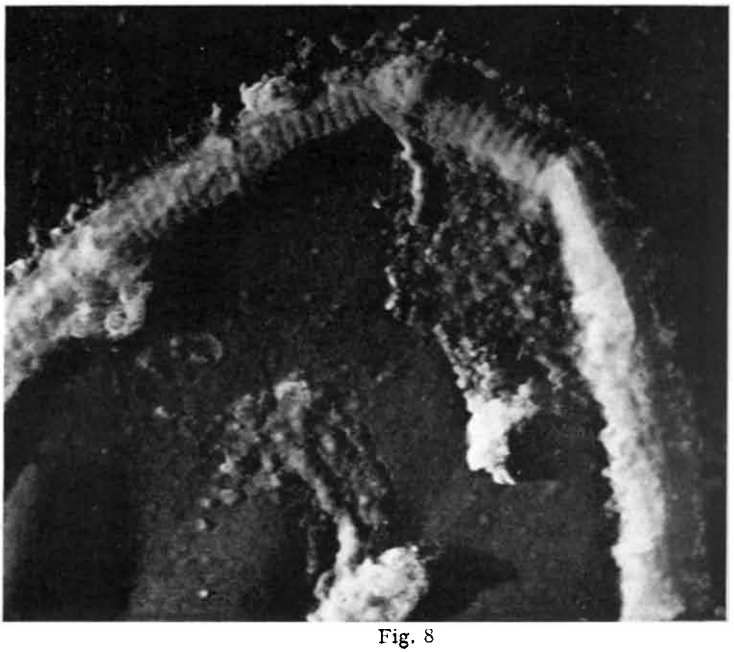


Fig. 8

bands of SCHMITT) and less dense regions ("B" bands) no structural features are visible.

Similarly, collagen from the leg tendons of the rat is damaged and partly destroyed by the decalcification treatment.

DISCUSSION

The observations recorded above show that the collagenous fibres from bone and tendon in the same species appear closely similar in the E.M. It was also found that collagen fibres from bone and tendon of the fowl and pheasant are not morphologically altered by the decalcification methods used in this study, while the tendons of the rat and mouse undergo severe changes when subjected to the same treatment. KELLENBERGER AND ROUILLER (personal communication) found that collagen in human and canine bone is severely damaged by decalcification, and it was mainly for this reason that they developed their present replica technique. This suggests that the reaction of rat collagen to decalcification may be characteristic of mammalian collagen in general.

The resistance of fowl collagen to such decalcifying agents as T.C.A. and nitric acid, which damage collagenous fibres from various mammalian species, may be due to some fundamental difference between fowl and mammalian collagen. In untreated material, however, no corresponding morphological difference can be detected in the E.M., a fact which must be borne in mind when discussing the significance of E.M. observations on collagen from fowl bone and tendon. The identical appearance of collagenous fibres from these two sources indicates no more than a similarity of structure at the level of magnification of the E.M.; it does not justify the conclusion that the fibres are identical in other respects, and chemical differences between them may well exist. Similarly, the fact that the E.M. does not reveal any change in fowl collagen after treatment with certain decalcifying agents does not prove that no chemical change has taken place.

In the literature only two kinds of morphological change in the collagen cross-banding (apart from some variation in the actual period spacing) have been described, and both types of change occur under widely varying conditions. Gross damage to the fibres, *e.g.* by heat or, in the case of mammalian collagen, by the action of decalcifying agents, results in partial or total disappearance of the cross-bands; sub-division of the 640 A period into 6 or 7 narrower bands has been observed in mammalian collagen after treatment with various tanning agents (SCHMITT[8]), after hydrogen peroxide (WOLPERS[9]), or after certain degenerative changes in the tissue (WOLPERS[1,7]). Although fowl collagen shows no change in its cross-banding after treatment with decalcifying agents, if it is

← PLATE II

Fig. 5. Rat tail collagen, teased after formalin fixation. The fibres and fibre bundles are very flexible and can be isolated easily and without damage. Shadowed with palladium-gold. × 27,500.

Fig. 6. Rat tail collagen, treated with 5% trichloracetic acid (TCA) followed by 5% sodium sulphate after formalin fixation. The fibres have lost their flexibility and have become so brittle that they break when teased. The fibre bundles appear flattened and their periodic cross-banding indistinct. Shadowed with palladium-gold. × 27,500.

Fig. 7. Collagen fibres from the unossified portion of leg tendon of the pheasant, treated with TCA and sodium sulphate after formalin fixation. The treatment was the same in every detail as that of the rat tail collagen in Fig. 2. In this material, however, none of the changes observed in TCA-treated rat collagen have occurred. The fibres have remained flexible, cylindrical in shape, and markedly cross-banded. Shadowed with palladium-gold. × 50,000.

Fig. 8. Collagenous fibres from the ossified portion of leg tendon of the fowl. Teased after fixation with 70% alcohol. The periodic cross-banding is partly obscured by a layer of bone salts. Shadowed with palladium-gold. × 27,500.

References p. 48.

subjected to the action of dilute acetic acid (about 0.002%) for several days "sub-bands" appear which are not visible in untreated material. This supports WOLPERS's view that sub-division of the cross-bands is the direct result of chemical treatment or of morbid changes in the organism, but the evidence also shows how unspecific this reaction is.

The periodic cross-banding of mature collagen, though a significant factor in distinguishing collagenous from non-collagenous fibres, does not therefore serve as an indicator of very small, though possibly important, differences between apparently identical fibres from different sources, or of structural changes on a near-molecular level.

The question of the difference between collagen in bone and in tendon, raised at the outset of this investigation, has thus been answered only in part. If such a difference exists at all it is not detectable by the present E.M. techniques, and information on the more strictly biochemical aspects of the problem must be obtained before the picture can be completed.

ACKNOWLEDGEMENTS

I wish to thank Professor J. T. RANDALL, F.R.S., Director of this Research Unit, and Dr H. B. FELL, F.R.S., Director of the Strangeways Research Laboratory Cambridge, for many suggestions and useful discussions.

SUMMARY

Collagenous fibres from adult fowl bone, unlike those from mammalian material, remain undamaged when isolated for electron microscopy by ordinary histological decalcification methods followed by mechanical fragmentation; the technique is described in detail. Bone collagen fibres thus obtained are examined in the electron microscope and compared with untreated and "decalcified" collagenous fibres from other sources.

RÉSUMÉ

Contrairement à ce que l'on peut observer chez les mammifères, les fibres de collagène d'os de poule adulte ne sont pas endommagées lorsqu'on les isole pas des méthodes histologiques ordinaires de décalcification suivies de fragmentation mécanique; la technique est décrite en détails. Des fibres de collagène d'os ainsi obtenues ont été examinées au microscope électronique et comparées à des fibres de collagène non traitées et à des fibres décalcifiées d'origine différente.

ZUSAMMENFASSUNG

Kollagenfasern aus Knochen vom erwachsenen Huhn werden, zum Unterschied von solchen aus Säugetierknochen, nicht zerstört, wenn man sie nach gewöhnlichen histologischen Methoden entkalkt und dann mechanisch isoliert; die Arbeitstechnik wird genau beschrieben. Auf diese Weise präparierte Knochen-Kollagenfasern wurden elektronenmikroskopisch untersucht und mit nicht vorbehandelten und entkalkten Fasern anderen Ursprungs verglichen.

REFERENCES

1 C. WOLPERS, *Grenzgebiete d. Medizin*, 2 (1949) 527.
2 E. KELLENBERGER AND CH. ROUILLER, *Schweiz. Z. Allgem. Path. und Bact.*, XIII (1950) 783.
3 E. RUTISHAUSER, L. HUBER, E. KELLENBERGER, G. MAJUS AND CH. ROUILLER, *Arch. Sci.*, 3 (1950) 175.
4 L. HUBER AND CH. ROUILLER, *Experientia*, VII (1951) 338.
5 E. KELLENBERGER, *New Methods for Biological Replicas*, (1948).
6 J. GROSS AND F. O. SCHMITT, *J. Exptl Med.*, 88 (1948) 555.
7 C. WOLPERS, *Franfurt. Z. Path.*, 61 (1950) 417.
8 F. O. SCHMITT AND J. GROSS, *J. Am. Leather Chem. Assoc.*, XLIII (1948) 659.
9 C. WOLPERS, *Arztl. Wochschr.*, 3 (1948) 644.
F. O. SCHMITT, C. E. HALL AND M. A. JAKUS, *J. Cell. and Comp. Physiol.*, 20 (1942) 11.
C. WOLPERS, *Das Leder*, 1 (1951) 3.
The last two references are not mentioned in the text.

Received July 8th, 1952