HUMAN BONE MICROSTRUCTURE STUDIED BY COLLAGENASE ETCHING

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Bone samples from the iliac crest of patients with no signs of bone disorder were treated with collagenase to remove the collagen component and so allow detailed observation of the mineral hydroxyapatite. Both polished and unpolished surfaces were studied in the scanning electron microscope and they showed that the mineral component of bone is composed of small rounded units about 10 nm across which are fused together to form larger spheroidal units roughly 100 nm in diameter. In the unpolished surfaces these 100 nm units are seen to aggregate to form columns approximately parallel to their neighbours and with numerous interconnections forming a continuous mineral phase. The polished sections also show the hydroxyapatite as a continuous phase of contiguous spheroids and the holes from which the collagen fibres were removed are clearly revealed. Lamellations in the surface are interpreted as resulting from adjacent layers of collagen fibres having orientations approximately perpendicular to each other.

The structure of the mineral component of bone has long been the subject of investigation: the techniques employed, however, have been widely criticised for introducing artefacts by either physical or chemical attack. Early investigations, involving line broadening and low angle diffraction experiments (Engström 1972), and transmission electron microscopy of thinly sectioned bone (Bocciarelli 1970; Cameron 1972; Jackson, Cartwright and Lewis 1978) have suggested the presence of a variety of needle and plate-shaped hydroxyapatite crystallites with dimensions varying between 2 nm and 150 nm. More recently Weiner and Price (1986) have studied the disaggregation of bone from various sources and concluded that the hydroxyapatite crystals are in a tabular or book-like form typically 45 nm long by 30 nm wide. On the other hand, a number of contemporary investigations have proposed a model in which spheroidal shaped particles about 100 nm in diameter aggregate to form the mineral component of macroscopic bone. Boyd (1972) observed such 'spheroidal particles' in fetal bone and Boyd and Sela (1978) isolated similar particles from solutions of NaOC1 used to wash bone and dentine. Sela (1977) reported observing 'calcospherites' 100 to 300 nm in diameter in osteogenic tumours. Pautard (1978) isolated similar particles from six-day-old mouse calvarial bone after a somewhat severe treatment including freeze drying, enzymatic attack and density fractionisation. Contiguous spheroidal particles have also been observed by Turner and Jenkins (1981) and Turner (1981) in bovine and human compact lamellar bone following removal of the collagen by ion etching.

Criticism that these techniques have damaged the mineral and introduced artefacts led us to use a more subtle biochemical etching procedure involving incubation of polished and unprocessed bone surfaces in a solution of collagenase. This technique of collagenase etching has been successfully used by Green, Isaac and Jenkins (1985, 1987a, b, 1988) to demonstrate the structure of bovine, fish and chicken bone and by Nokes, Green and Knight (1987) on human bone of archaeological origin. This present report is concerned with the application of this technique to normal human bone.

MATERIALS AND METHODS

Material from the iliac crest was chosen for investigation since this is the established site for biopsy in histological investigations of metabolic bone disease (Beck and Nordin 1960; Pödenphant et al. 1986). Transiliac samples of bone were removed post mortem by trephine (8 mm diameter) in a region 20 mm below the iliac crest. Specimens were collected from males 30 to 70 years of age who exhibited no signs of bone disorder.

Samples were sectioned longitudinally and polished using carbide papers and polishing alumina. They were then thoroughly washed in distilled water to remove any adherent alumina prior to boiling in distilled water for 30 minutes to denature the collagen and thus enhance the action of the collagenase. Aliquots (5 ml) of collagenase...
solution each containing 2100 units were made up in 0.05 m Tris buffer pH 7.4 (Sigma Chemicals Ltd: Type 1A). These solutions also contained 0.1 M calcium chloride, the Ca** ions being necessary for activation of the collagenase. The solutions containing the specimens were incubated at 37°C for one week to ensure adequate etching. Each bone segment was bathed in 5 ml of enzyme solution which was agitated twice daily. The efficacy of each batch of solution was tested by its ability to disintegrate small pieces of rat tail tendon which were placed in the enzyme solution with at least one of the bone specimens in each batch. Following incubation the specimens were coated with gold and examined in the scanning electron microscope. For the low magnification photographs shown in Figures 1, 2, 5, 6, 7, 10 and 11 a Jeol 35C was used at an accelerating potential of 30 kV. For the higher resolution pictures of Figures 3, 4, 8, 9, 12 and 13 a Jeol 120C Temscan operating in the scanning mode at 100 kV was used.

RESULTS

Figure 1 shows an area of polished, collagenase-etched, cortical human bone. Lamellation, observed as a series of alternating light and dark layers may be readily observed, as may a number of osteocytic lacunae. Figure 2 illustrates a polished surface at higher magnification. The alternate dark and light banding can here be discerned to arise from areas which contain holes, up to approximately 200 nm in diameter (light areas) alternating with areas which exhibit few such holes (dark areas). These holes were produced by the removal of collagen fibres of up to 200 nm in diameter due to the action of the collagenase etching process. The holes from which the
collagen has been removed can be seen more clearly in Figure 3. These micrographs also reveal the mineral as a continuous phase which is largely independent of and surrounding the collagen fibres. The mineral phase can be seen to be composed of spheroids about 100 nm in diameter. Figure 4 is a high resolution micrograph demonstrating the structure of the mineral spheroids. It can be seen that the spheroids are themselves made up of smaller units, approximately 10 nm in diameter.

Figure 5 is a low magnification electron micrograph showing both polished and unpolished surfaces of part of a trabecula. Figure 6 reveals that the polished surface of this trabecula exhibits a similar banding structure to that observed in the polished section of cortical bone. It appears in this case however that the arrangement of the observed lamellation is not as regular as in the former example. On closer examination (Fig. 7), it can be seen that this lamellation is also due to areas containing holes resulting from the removal of collagen fibres, which in vivo ran approximately perpendicular to the plane of section, alternating with areas which have fewer holes. Figure 8 once more demonstrates a mineral phase which is made up of spheroids approximately 100 nm in diameter, which aggregate to form a continuous structure independent of and surrounding the collagen fibres. Figure 9 in common with Figure 4 reveals that the 100 nm spheroids are composed of smaller units approximately 10 nm in diameter which have coalesced.

Figure 10 is a low magnification micrograph of an unpolished region of the trabecular surface. The preferred orientation of the observed mineral rods probably indicates the in vivo direction of the collagen fibres in this area and thus is also related to the stress distribution in the bone. In the higher magnification photograph of

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**Fig. 5**
Scanning electron micrograph at low magnification of a sectioned trabecula which has been treated with collagenase solution. The polished surface is in the centre of the picture and to the right is an unpolished area.

**Fig. 6**
At this higher magnification the polished trabecular surface reveals a lamellar structure. To the right of this micrograph an unpolished surface is seen.

**Fig. 7**
A higher magnification of a polished trabecular surface showing more detail of the lamellations and revealing numerous holes in the light lamellae from which collagen fibres have been removed. Dark lamellae contain fewer holes.

**Fig. 8**
At yet higher magnification the polished trabecular surface is seen as a continuous matrix of mineral with holes (typically about 200 nm across) from which collagen fibres have been removed by the action of collagenase. The mineral matrix is composed of spheroidal particles 100 nm across which are fused together.
Figure 11 the material is seen to consist of a series of columns interdispersed with holes oblique to the surface and about 200 nm across; the columns are arranged approximately parallel to one another. Figure 12 shows these columns as rows of aggregated spheroids roughly 100 nm in diameter which are contiguous and form a continuous mineral phase. Figure 13 once more demonstrates the small units 10 nm in diameter which make up the larger spheroids.

**DISCUSSION**

The classically held view of the ultra-structure of bone is of discrete needle or plate-like crystals intimately linked with the tropocollagen molecules, and it has been suggested that they occupy the 'gap regions' in the collagen. This model is difficult to reconcile with the known mechanical properties of bone, since the model

This highest resolution micrograph of polished and collagenase etched trabecular bone shows the smallest units of mineral as rounded particles about 10 nm across, which coalesce to form the 100 nm spheroids.

A micrograph of a typical unpolished surface of collagenase etched trabecula.

A more detailed photograph of the unpolished trabecular surface showing the columnar or rod-like arrangement of the mineral.

This higher resolution micrograph of the unpolished collagenase treated trabecular surface shows the mineral to consist of spheroids which fuse into approximately parallel columns about 100 nm across with numerous interconnections.

At this highest resolution the 100 nm spheroids on the unpolished trabecular surface are seen to be composed of smaller rounded units approximately 10 nm across.
envisages the mineral crystals merely stiffening the collagen and not forming a continuous phase in their own right. The failure of this model becomes readily apparent if a bone which has been treated above the temperature at which collagen denatures is examined. The denaturation of the collagen would be expected to disrupt totally the structure of bone without a continuous mineral phase, thus destroying its mechanical integrity. In practice this clearly does not occur.

The model that this study supports is one in which the mineral component is made up of spheroids about 100 nm in diameter which coalesce to form a continuous mineral phase largely independent of the collagen. The spheroids themselves are composed of aggregated smaller contiguous rounded units some 10 nm in diameter. This arrangement of mineral is seen in both polished and unpolished surfaces (see Figs 4, 9 and 13), and is therefore not an artefact generated by the polishing process. On the unpolished surfaces, it is observed that the 100 nm units coalesce to form rows of fused spheroids producing a plexiform of columns 100 nm across with numerous interconnections.

The holes observed in the mineral phase are produced by the removal of the collagen due to the action of the collagenase, and therefore indicate the in vivo position and direction of the collagen fibres. The lamellation observed in, for instance, Figures 1, 2, 6 and 7 is reminiscent of the lamellar structure of bovine secondary osteons which have been studied in some detail using the same technique. It has been demonstrated by Green et al. (1987a) that the lamellation in bovine secondary osteons is a result of the direction of collagen fibre orientation changing through approximately 90° from one lamella to the next. While such detailed study has not yet been carried out on this human bone, the marked similarities in lamellar structure observed in both materials make it highly likely that they arise from the same phenomenon. The lower regularity and presence of more holes than previously observed in some of the dark lamellae is probably because these sections are not cut at specific orientations, so that collagen fibres are not precisely perpendicular and parallel to the cut surface in alternate lamellae. It appears from examination of the micrographs of the unpolished surfaces of the trabecular bone that the mineral phase is stacked into columns with the collagen interdispersed between them. This is compatible with the polished sections through the trabecular bone in which the mineral is seen to surround the holes from which collagen fibres have been removed by the collagenase.

**Conclusions.** The mineral component of normal human cortical and trabecular bone forms a continuous phase, largely independent of the collagen fibres. The smallest particles observed are rounded and about 10 nm in diameter. These aggregate to form larger spheroidal units approximately 100 nm in diameter which are seen in the unpolished surfaces to form highly interconnected columns 100 nm across. It is very likely that the directions of the columns are governed by the collagen fibre orientations which in turn are related to the in vivo stress distribution. In the polished surfaces, holes from which collagen fibres up to 200 nm in diameter have been removed are clearly visible and the lamellar structure is explained as alternating orientations of collagen fibres.

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**REFERENCES**


