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Crystal organization in rat bone lamellae

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The plate-shaped crystals of rat bone are arranged in parallel layers that form coherent structures up to the level of individual lamellae. The crystal layers of the thin lamellae are parallel to the lamellar boundary, whereas those of the thicker lamellae are oblique to the boundary. The basic structure of rat bone can be described as 'rotated plywood'; a structure hitherto unrecognized in either biologic or synthetic materials.

Bone; Collagen; Apatite crystal; Biomineralization

1. INTRODUCTION

Bone is composed of three major components: small plate-shaped crystals of carbonate apatite [1], water and macromolecules, of which type I collagen is the major constituent [2]. The manner in which the crystals and the collagen fibrils are organized in bone has not been resolved, even though it is known that they are intimately related, at least in newly-formed bone [3–5]. In the more easily studied mineralized collagen fibrils of turkey tendon, the crystals are arranged in parallel layers across the fibrils [6,7], with the crystal *c* axes aligned with the fibril lengths [8]. Here we report that the plate-shaped crystals of rat bone are also arranged in parallel layers within individual lamellae. The orientation of the crystal layers in the alternate thin and thick lamellae differs in that in the thin lamellae they are parallel to the lamellar boundaries and in the thick lamellae the layers are oblique to boundaries.

2. MATERIALS AND METHODS

We examined the diaphyses of rat tibia, as this bone undergoes little or no internal remodelling into Haversian structures [9]. Cortical bone of 4-, 10- or 15-month-old Wistar rat tibia was used. Specimens for examination in the scanning electron microscope (SEM) were mechanically fractured approximately transverse to the bone long axis. Slices of the bone were cut with a saw about 1–2 mm below the fracture surface, briefly washed with deionized water, sonicated for a few seconds, dried under vacuum, and examined using a Philips 515 SEM after coating with gold. For examination in the transmission electron microscope (TEM) a few milligrams of bone were lightly crushed in an agate mortar under liquid nitrogen and the powder was then suspended in about 0.5 ml deionized water and sonicated using a model 431A Heat Systems-Ultrasonics sonicator until a milky white suspension was obtained. A drop of the suspension was placed on a 400 mesh carbon-reinforced grid located on a glass slide. The grids and the glass slide were then cooled in liquid nitrogen vapour until the droplets froze. The slide was then placed in a vacuum desiccator, lyophilized, and examined in a Philips 400T TEM.

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3. RESULTS

The experimental strategy involved the use of the SEM to study surfaces fractured more or less transverse to the long axis of the bone and the TEM to study crushed bone particles, that were sufficiently small to enable the visualization of individual crystals at different tilt angles. Electron diffraction was used to determine their crystallographic orientations, particularly for larger particles that showed two distinct *c* axis orientations. Crystal *c* axes orientation indirectly provides information on the orientation of the collagen fibrils, as it is well known that the *c* axes of the crystals are aligned with collagen fibril axes [10]. The collagen fibrils in an individual lamella are all roughly oriented in one direction, but in adjacent lamellae are arranged more or less at right angles, but still parallel to the lamellar boundaries [11,12].

Fig. 1A shows an SEM low-magnification view of a transversely fractured surface of the tibia in the area adjacent to the forming periosteal surface. The first hundred or so microns from the periosteal surface fractured such that the lamellar structure is clearly observed. At higher magnification (Fig. 1B), the thick lamellae are seen to fracture along parallel cleavage planes that are oriented obliquely to the lamellar boundaries. In Fig. 2A and B the fracture surface is viewed from directly above the specimen in an area close to the periosteal surface revealing that a thin lamella, located between the thick lamellae, shows a lineation parallel to the lamellar boundaries. We interpret these cleavage patterns as due to the crystals being primarily organized in arrays of parallel layers, viewed approximately face-on in the case of the thick lamellae, and edge-on in the case of the thin lamellae. Parallel layers of crystals have

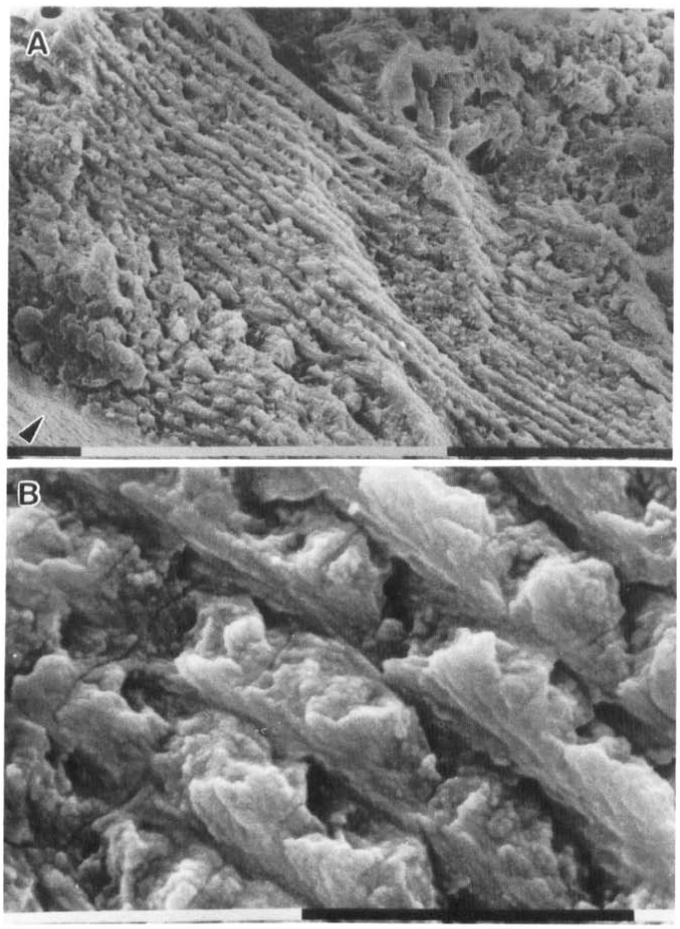


Fig. 1. SEM micrographs of the surfaces of a 10-month-old rat tibia fractured approximately transverse to the bone long axis. (A) An oblique view of this area adjacent to the periosteal surface (arrow) showing the tendency in this region for the fracture to reveal the lamellar structure. Scale bar = 0.1 mm. (B) A higher magnification view of the lamellar structure with the bone oriented in the same direction as in (A). The flat cleavage planes of the thick lamellae are arranged in parallel arrays, and are oriented obliquely to the lamellar boundaries. Scalr bar = 10 μm.

previously been observed in mineralized collagen fibrils from turkey tendons [7]. Similar cleavage planes, apparently along layers of crystals, have also been reported in more heavily mineralized bones [14]. Note that the thicknesses of the lamellae, and particularly the thin lamellae, can vary significantly (Fig. 2).

As the individual crystals cannot be visualized in the SEM, we studied small crushed bone particles in the TEM. By studying the thin edges of particles, rather than significantly thicker embedded and sectioned bone, we reduced the problems of interpreting the crystal organization due to the projection of the 3-dimensional structure onto the 2-dimensional film. Preparation artefacts are associated with both methods, but by making repeated observations on many particles, we hopefully reduced the potential for misinterpretation. Fig. 3 shows a representative example of a bone particle in which the crystal plates are all oriented more or less perpendicular to the electron beam. Note that the crystals appear larger close to the thin edges, as fewer layers are projected onto the photographic plate. We observed many such particles, irrespective of crushing conditions, sonication and drying from water or ethanol, indicating that the organization of plate-shaped crystals into parallel layers is common in rat bone.

Fig. 4A is an example of a larger particle in which the central thick portion shows a well-developed lineation, implying that the crystal layers are viewed more or less edge-on. The thin edges, however, show crystals oriented with their flat surfaces roughly perpendicular to the beam. An electron diffraction pattern from the thick area and one from the thin area show that the crystal *c* axis orientations are different. The diffraction pattern from an intermediate area shows a double orientation pattern (Fig. 4A insert). As the crystal *c* axes are aligned with collagen fibril axes and the collagen fibrils have different orientations in adjacent lamellae, we infer that the crystals in the particle are derived from at least two adjacent lamellae. Fig. 4B shows a schematic interpretation of Fig. 4A with the bone particle viewed in plane and in section. In the thicker lamella, the planes of the crystal layers are oblique to the lamellar boundary, but in the thinner basal portion the crystal layers are parallel to the boundary. This configuration is consistent with our interpretation of the SEM micrographs in terms of fracture occurring predominantly along parallel layers of crystals. We observed many particles with this type of two-layered structure. Crushed rat bone rarely breaks into fibrils, though we did occasionally see these in younger specimens with weak indications of the 640 Å collagen periodicity.

4. DISCUSSION

The organizational motif in which crystals are arranged in layers was first observed in mineralized collagen fibrils from turkey leg tendon [6,7,14]. A significant difference between tendon and bone is that in tendon all the collagen fibrils are aligned in one direction, whereas in bone this only occurs within individual lamellae. The preferred orientations of the collagen fibrils in alternate lamellae are different, and hence also the *c* axis orientations of the crystals associated with the fibrils. In the mineralized tendon the parallel crystal layers can be aligned within arrays of fibrils over distances of at least a micron [7]. In rat bone our observations suggest that the crystal layers are all more or less aligned in individual lamellae, some of which are a few microns thick (Fig. 2). The crystal layers are, however, aligned differently in alternate layers. For example, we envisage adjacent lamellae related by rotation of the fibrils and their associated crystal layers first through

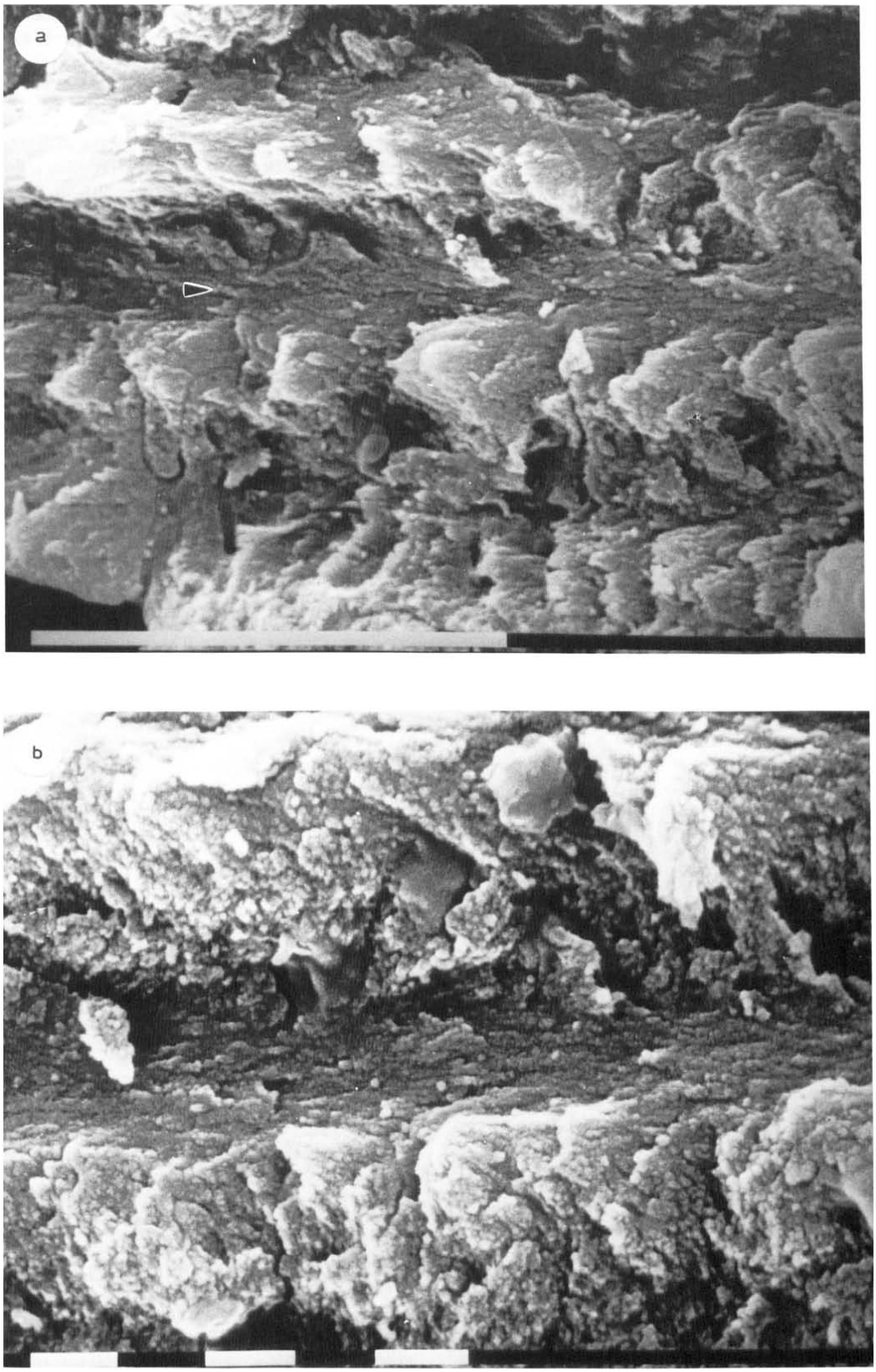


Fig. 2. SEM micrographs of the surface of a 15-month-old rat tibia fractured approximately transverse to the bone long axis, viewed from directly above the fracture plane. (A) The fractured surface shows the structure of two thick lamellae and one thin lamella (arrow). The thin lamella shows lineation parallel to the lamellar boundaries. Scale bar = 10 μm. (B) A higher magnification view of a thin lamella between two thick lamellae. The thin lamella shows lineation parallel to the lamellar boundaries. Scale bar = 1 μm.

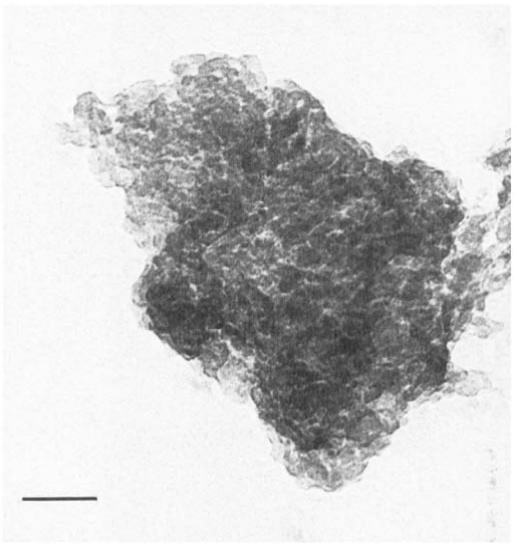
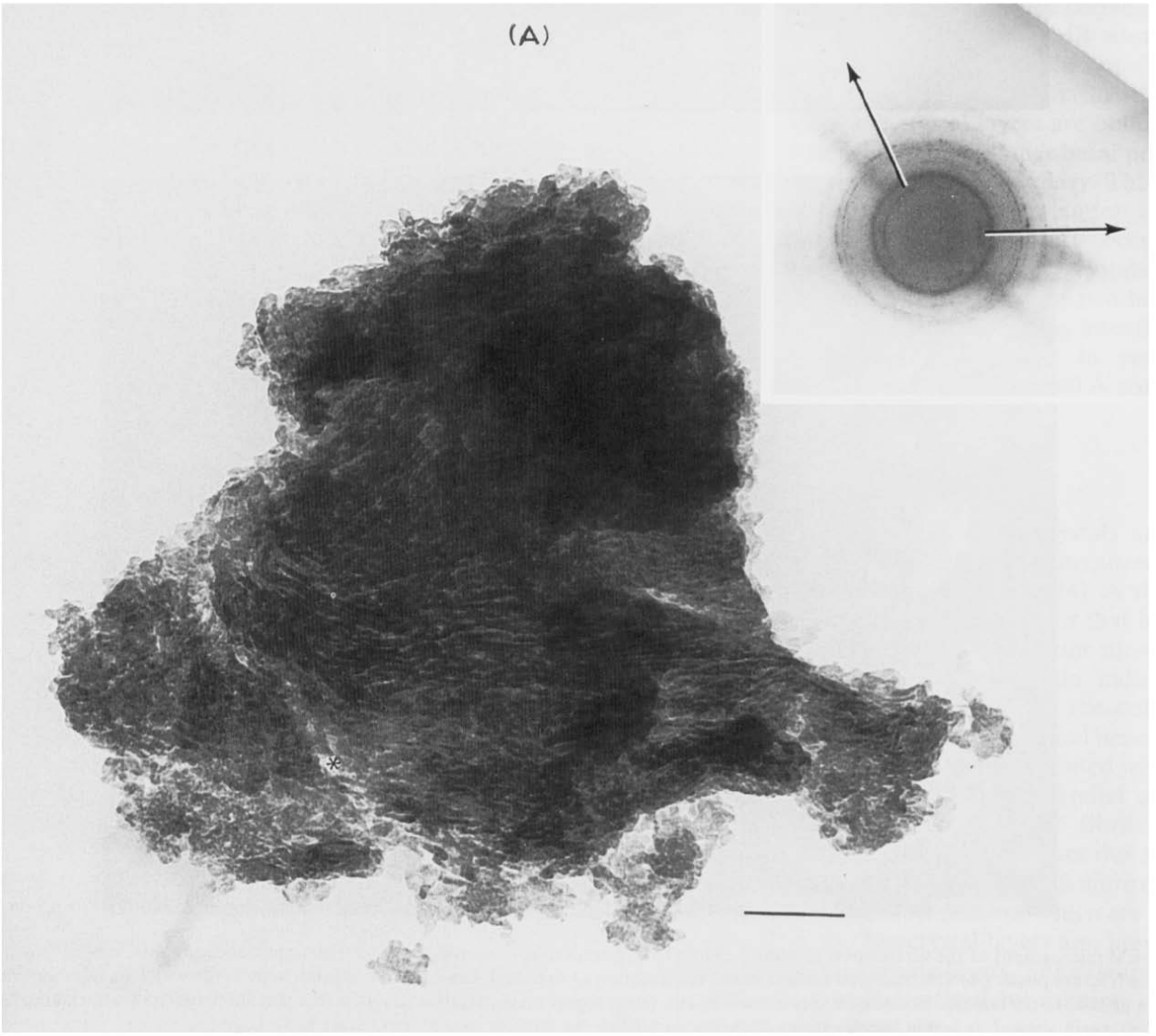


Fig. 3. TEM micrograph of a small crushed bone particle from a 10-month-old rat tibia. The particle is oriented such that the flat faces of the platey crystals are all more or less perpendicular to the electron beam. The beam has penetrated through several parallel layers of crystals in the central portion of the particle. Scale bar = 250 nm.

an angle ψ_1 parallel to the lamellar boundary, and then by rotation around their own fibril axes through an angle ψ_2, as schematically illustrated in Fig. 5. The final structure is schematically illustrated in Fig. 6. The term 'rotated plywood' serves to distinguish this structure from normal plywood. We note that secondary osteons are also composed of thick and thin lamellae [15], but it remains to be determined whether the rotated plywood structure is also characteristic of osteonal or Haversian bone.



The organization of the plate-shaped crystals into parallel layers is consistent with the 3-dimensional structure of type I collagen proposed by Katz and Li [16], in which the triple helical molecules are assembled such that the gap regions are contiguous in one direction [7]. This possible arrangement of the triple helical collagen molecules was also noted by Hodge [17], and direct experimental evidence supporting it was obtained from the stoichiometric analyses of the cross-links in bone collagen [18]. This type of structure is consistent with our measurements from electron micrographs such as Fig. 4 (but tilted with the layers parallel to the beam) showing that the distance between parallel layers viewed edge-on is about 50 Å. The arrangement of crystal layers interspersed with layers of collagen molecules is also consistent with X-ray [19] and neutron diffraction [20] observations of the triple helical molecules coming closer together with increasing mineralization [21].

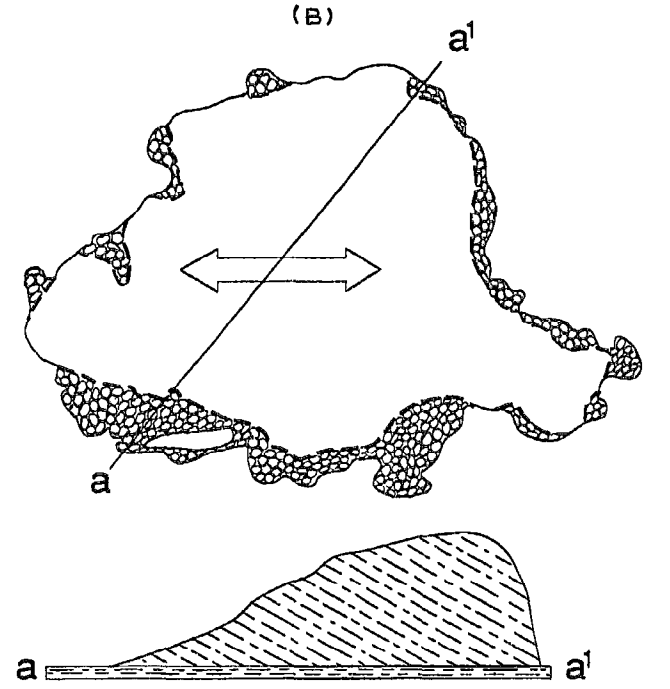


Fig. 4. (A, *left*) TEM micrograph of a relatively large crushed bone particle from a 10-month-old rat tibia. The central thicker part of the particle shows a well developed lineation, whereas the thin edges show areas in which the platey crystals are viewed approximately face-on. Scale bar = 200 nm. The inset shows an electron diffraction pattern from the thin and thick parts in the area centered around the asterisk. The arrows show two different predominant apatite *c*-axis orientations (arrows). (B, *above*) Schematic illustration of the structure of the bone particle imaged in Fig. 4A. The planar view (top) shows the central thick area with the lineation direction (arrow) and the thin areas around the periphery. The section a-a', following our interpretation, is shown at the bottom. The double orientation of the diffraction pattern implies that the particle is composed of crystals from two adjacent lamellae. In the thin layer at the particle base the crystals are oriented with their flat faces more or less perpendicular to the beam. In the thicker areas the lineation implies an approximate edge-on view of the crystal layers. The particular angle chosen is purely schematic.

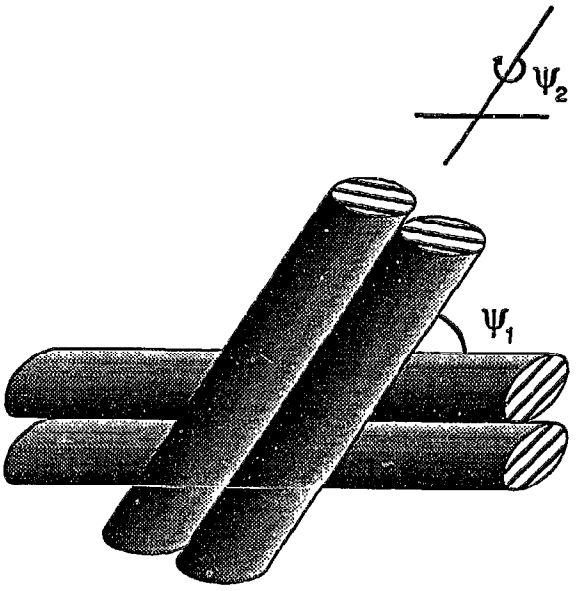


Fig. 5. Schematic illustration showing the basic elements of the structure. Each cylinder represents a collagen fibril with the orientation of the crystal layers shown on the ends. The structure can be defined in terms of the two angles ψ_1 and ψ_2.

We also note that the structural model of bone reported here implies that most of the crystals are within, or in close association with the collagen fibrils, as was originally observed for fish bone [5]. We have not observed any other major organizational motif for rat bone, that would imply a second locus of mineralization. The rotated plywood structure also pro-

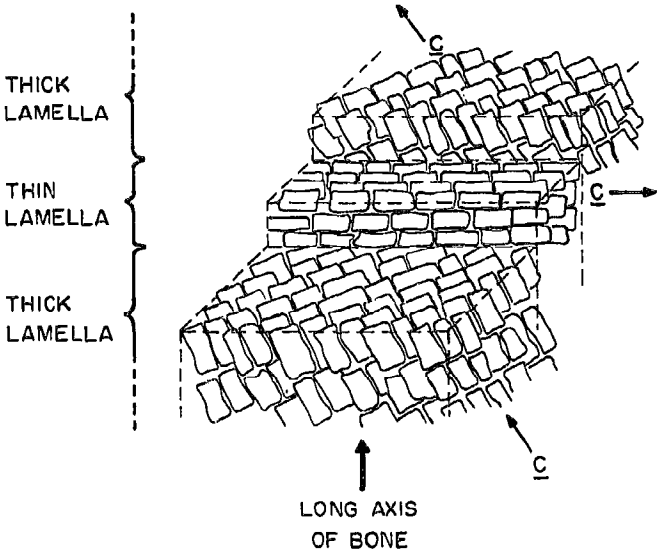


Fig. 6. Schematic illustration, not drawn to scale, of the rotated plywood structure of rat bone showing a thin lamella between parts of two thick lamellae. Each irregularly shaped rectangle represents a plate-shaped crystals. Aligned coplanar assemblies of these crystals form layers. The *c* crystallographic axial orientations of the crystals in the different lamellae, and the direction of the long axis of the bone are indicated by arrows.

vides an explanation for the often observed situation in embedded and sectioned bone photographed in the TEM, whereby the crystal plates are seen edge-on (as needles) and face-on (as plates) in the same local region. The projection of sections of the rotated plywood structure hundreds of Angstroms thick and more than likely oblique to the lamellae, would result in crystals of both orientations appearing in the same area.

The structural model presented here is based on a study of the cortical bone of rat tibia. In our effort to identify the basic underlying structure we have not emphasized the numerous local irregularities and the interface zones between lamellae [22]. Although there are a number of indications that the rotated plywood structure may be found more generally, this remains to be shown. We anticipate that careful comparative studies of bone structure at the level of crystal organization may well reveal fascinating adaptive strategies for bone function. An analysis of this structure in terms of its biomechanical properties is being prepared [23].

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