ELECTRON MICROSCOPY OF CARTILAGE AND BONE MATRIX AT THE DISTAL EPIPHYSEAL LINE OF THE FEMUR IN THE NEWBORN INFANT*

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Plates 84 and 85

This study of rapidly growing bone and cartilage matrix is an extension of previous work on the relationship of apatite crystals to collagen in bone (Robinson, 1952; Robinson and Watson, 1952, 1954; Watson and Robinson, 1953). It is part of a wider survey of the ultramicroscopic organization of a growing epiphysis and metaphysis, which will establish a base line for future study of abnormalities of growth in this region. Knowledge of human epiphyseal growth has depended on light microscopy with its inherent limitations in resolution. This has prevented any understanding of the arrangement of crystals in the matrix and encouraged the idea that bone and epiphyseal cartilage matrix have the same structure. In the epiphysis under discussion, at least, this is not the case and it is to be expected that, with the examination of more tissue, further information will be elicited especially in regard to the epiphyseal growth region of bones in rickets and scurvy.

Materials and Methods

Tissue was taken from the distal end of the femurs of infants who died in the neonatal period. It was obtained at autopsy, usually some 6 to 12 hours after death, during most of which period the body was refrigerated. After the bones were freed of soft tissue, sagittal slices about 0.3 mm. thick were cut through the epiphysis and 5 mm. of metaphysis and placed in ice-cold veronal buffered osmic acid (Palade, 1952). This procedure usually took about 30 minutes and the tissues were then allowed to remain in the osmic acid for a further hour. After fixation the slices were dehydrated in ice-cold acetone (Karrer, 1956) and impregnated with a partially polymerized mixture (Borysko, 1955) of four parts butyl methacrylate and one part of methyl methacrylate to which had been added ½ per cent by weight of 2, 4-dichlorobenzoyl peroxide. The tissue was left in the methacrylate syrup in a refrigerator overnight. It was then embedded, a modification of the flat embedding technique (Borysko and Sapranauskas, 1954) being used to allow for easy orientation of the tissue and subsequent alignment for cutting particular areas. The methacrylate was polymerized at 60°C. (Borysko,

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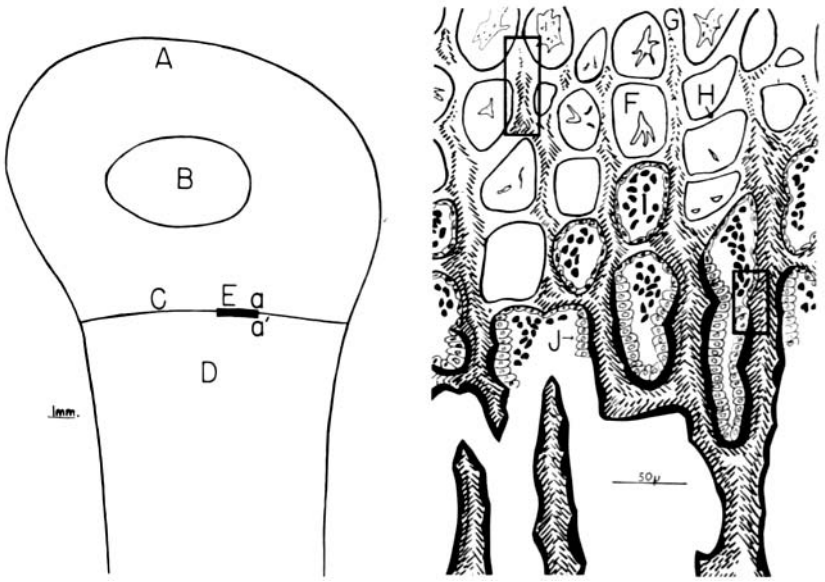
1955) for 3 days. To remove any residual acetone or moisture the embedded tissue was subsequently kept overnight under reduced pressure (less than 1 mm. of mercury). The area examined was limited by a line about 0.1 mm. distal and 0.3 mm. proximal to the furthest penetration of capillaries into the epiphysis from the metaphysis (Text-fig. 1) and any part could be localized fairly accurately due to the optical clarity of the flat plastic. As a final check of position thin slices were cut free hand from the part of the block selected and examined by phase microscopy.



TEXT-FIG. 1. The diagram on the left represents a sagittal section through the distal end of the femur. *A* is the articular cartilage, *B* the ossification center of the epiphysis, *C* the epiphyseal line, and *D* the metaphysis. The tissue examined for this paper is to be found in zone *E* and thin sections have been cut through *E* in the plane of *a*-*a'*.

The right-hand diagram is an enlargement of the epiphyseal area through *a*-*a'*. *F* is the capsule of one of the degenerating cells in a column of chondrocytes and *G* the intercolumnar bar of matrix. *H* is one of the crossbars of matrix separating the cells in the column. *I* is a capsule which has been penetrated by a capillary and *J* a site of osteoblastic activity. The shaded areas in the columns represent calcified cartilage and the filled areas bone. Osteoblasts have not been shown covering all the bone surface in order to reduce the complication of the diagram. The small rectangle at the top of the diagram represents the location of Fig. 1 and the lower one represents that of Fig. 8.

Thin sections less than 0.1 mm. square were cut with a Servall microtome (Porter and Blum, 1953) using specially selected glass knives (Cameron, 1956) and picked up on 100 mesh Athene grids covered with a carbon-coated formvar film (after Watson, 1955). They were examined in an RCA EMU 2 microscope fitted with externally centrable objective and condenser apertures.

OBSERVATIONS

In the process of bone growth at the epiphyseal line, tissue originally composed of columns of chondrocytes separated by horizontal and vertical bars

of extracellular cartilage matrix is partly converted into calcified cartilage matrix. Capillaries, supporting tissue, and osteogenic cells penetrate the lacunae of cartilage cells, remove some of the cartilage matrix, and lay down extracellular bone matrix on the remnants of the calcified cartilage matrix. The changes in the extracellular cartilage and bone matrix and their possible mechanism are to be discussed in this paper. For clarity the matrix can be divided into zones, namely: (1) uncalcified cartilage matrix, (2) cartilage matrix undergoing calcification, (3) completely calcified cartilage matrix (possibly undergoing absorption), (4) calcified cartilage matrix next to which bone matrix is formed by osteoblasts.

In a newborn infant these changes can be observed in the femur in a zone about the distal epiphyseal line measuring less than 0.5 mm. in extent in the long axis of the bone. Text-fig. 1 shows this area.

1. Uncalcified Cartilage Matrix (Figs. 1 to 3).—In a relatively thick section this matrix is seen (Figs. 1, 2) to be made up of a felt work of fibers running more or less longitudinally in the center of the bar of matrix but also sweeping around the edge of the chondrocyte capsules at the periphery of the bar (Fig. 1-*A*, 1-*B*). In thinner sections (Fig. 3) the fibers occur singly or in small groups all widely spaced in a matrix relatively less dense to electrons. Those few running in the plane of section have a thickness of the order of 100 to 200 A and discrete fibers may form groups up to 1250 A thick (Fig. 3-*A*). There is a suggestion of periodicity of about 200 A along some of the fibers (Fig. 3-*B*). Throughout the section round structures can be seen varying from 100 to 500 A in diameter (Fig. 3-*C*). The larger ones have an osmiophilic border; however, no fiber of this diameter has been cut in its long axis.

2. Cartilage Matrix Undergoing Calcification (Figs. 1 to 7).—Calcification commences in the intercolumnar bars of matrix about the level of the third chondrocyte capsule distal to the metaphysis (Text-fig. 1). The chondrocytes are in the last stages of degeneration or have almost completely disappeared at this level. Crystals measuring 250 to 750 by 50 to 75 A are deposited singly or in small groups (Fig. 4), between the center and edge of the bar of matrix (Fig. 1) without linear relationship to its fibers. More distally, the crystal clusters increase in size and the peripheral crystals tend to have a radial arrangement (Fig. 5), but the central crystals are quite haphazardly placed. In these clusters the crystals seem to be in a setting which is denser than the surrounding matrix. This is probably caused by a heaping up of the tissue during cutting or possibly due to inorganic material. The clusters continue to increase in size (Fig. 6) until within a space of 40 to 50 micra towards the metaphysis there is a solid mass of crystals about midway between the center and edge of the bar of the matrix (Figs. 1, 7). The central zone is incompletely calcified (Fig. 1) and remains so after ossification has commenced. Examination of the more densely calcified areas does not reveal any increase in crystal size or orientation in a particular direction. However, those crystals at the edge of cal-

cification appear to have their long axes arranged more or less radially to the main mass. This arrangement has no apparent relationship to the general direction of the matrix fibers.

3. Completely Calcified Matrix (Figs. 8-A, 9).—Within the column of cells the matrix crossbars disappear before the invading capillaries, seemingly without undergoing calcification. Except at its center each intercolumnar bar of matrix becomes fully mineralized at about the point of furthest penetration of these capillaries but the calcified mass has an irregular edge (Figs. 8, 9), different from the smooth sweep of the fibers forming the margin of the capsule. This edge appears unaltered until ossification commences and no evidence of absorption of calcified cartilage matrix could be found.

4. Zone of Earliest Bone Deposition (Figs. 8, 10 to 13).—There is little space between the place of penetration of the cartilage by capillaries and the beginning of bone formation where osteoblasts are first observed to cover the calcified cartilage matrix. The first indication of osteoblast activity (Fig. 10) is the formation of a loose network of fibers (Fig. 10-*C*) between cell (Fig. 10-*A*) and calcified cartilage (Fig. 10-*B*). The fibers of this osteoid, when running in the plane of section, can be recognized as collagen (Fig. 11) and are 400 to 600 A in thickness. No thin fibers such as in cartilage could be demonstrated. Calcification occurs soon after fiber formation, as the uncalcified zone is usually less than 1.5 micra in thickness. The crystals are laid down upon or close to the fibers (Figs. 10-*D*, 11-*F*, 11-*G*) but without any definite linear relationship to the fiber axis, and without the radial arrangement noted in cartilage. However, the density of some of the cross bands may be due to mineral deposition. This density is markedly increased in one area of the fiber in Fig. 11-F but this may be an artifact. The crystals are of the same order of size as those in calcified cartilage. The change from the initial light to a more complete calcification of the osteoid seems to be more sudden and diffuse than that found in the cartilage matrix. In the outermost region of the more fully calcified osteoid (Fig. 12) many of the crystals line up parallel to the fibers, but without obvious relationship to the periodicity. However, within 2 micra of the uncalcified matrix more definite linear orientation can be seen and the calcification seems to be most dense over the major periods of the underlying fibers (Fig. 13). Whereas the uncalcified fibers are only loosely arranged and, even when undergoing initial calcification, are not packed, in an area about 2 to 3 micra from the osteoblast they are close together and show some period alignment (Fig. 13). As far as can be determined there is no obvious change in crystal size at this stage.

The calcified cartilage forming the scaffolding for the new bone formation does not appear to be changed at this level of bone apposition. If there is any alteration in crystal size or position it is not very great.

The junction between the two tissues is not obvious in calcified sections but following decalcification a distinct line of demarkation (Fig. 14) can be seen.

This has the appearance of a "double membrane" (Fig. 14-*A*), each wall of which is separated by about 700 to 1200 A and encloses a reticular structure.

DISCUSSION

We are aware of one previous paper in which is discussed the electron microscopical appearance of newly formed human bone (Robinson and Watson, 1955). The bone described was from the lateral rib cortex of a 2-day-old infant. The long axis of the inorganic crystals at the calcification front tended to parallel the fiber long axis, but about 1 micron deeper in the calcified osteoid the inorganic material was most dense over the major bands of the collagen and the size of the crystals tended to be smaller. The collagen in newly formed osteoid when decalcified had a suggestion of a 100 A fine period and the diameter of these fibers was 380 to 530 A although a few about 150 A wide were seen.

Martin and Jackson have described some aspects of the fine structure of the tibia in the chick embryo. Martin (1954) found in the epiphyseal cartilage of the tibia that there was a network of fine fibers without a distinctly recognizable periodicity although occasionally bands 400 A apart were seen. She suggested that this fine type of fibril might be a reflection of the rapid rate of bone formation and that, where growth is slower, the fibrils might be different. Jackson (1954) described tibial periosteal bone formation in the chick embryo. She stated that no dividing line could be distinguished between osteoblasts and the fibers of the newly formed bone and that the fibers immediately next to the cartilage matrix apparently originated from this matrix. Neither of these conditions existed in the material examined by us.

The main fact brought out in this paper is that two closely related structures, both of which calcify, have a widely differing architecture. Much attention has been paid in recent years to the mechanism of calcification and many of the ideas put forward have been based on studies of cartilage, or of osteoid and cartilage together. There may be a tendency to generalize (Gutman and Yu, 1950) and to assume that what happens in one happens in the other. Morphologically, the deposition of inorganic salts in the two areas differs in several ways. Calcification in cartilage appears after the degeneration of the cells, whereas in bone it occurs close upon the laying down of the collagenous matrix by osteoblasts. Secondly, the crystals are laid down at a distance from the edge of the cell capsule in cartilage but within a fraction of a micron of the bone cells. This delayed potential for calcification is further emphasized by the space between the level of cartilage matrix formation and the level of its calcification. Thirdly, the nidus of calcification appears to be on or closely related to the fibers in osteoid. This may also be true in cartilage, but it is not demonstrable. The initial crystal arrangement in bone at the epiphyseal line is as irregular as that in cartilage, but the crystals in the osteoid soon take on a pattern of orientation in relation to the collagen fibers. This coalignment of inorganic

crystals and fibers is not observed at any stage of cartilage matrix calcification. Perhaps the difference is related to the greater mass of fibers per unit volume of osteoid and the larger size of the individual fibers. Due to this mass relationship they may have some orienting effect on the crystals in bone. These variations suggest that the mechanism of calcification in the two tissues could be somewhat different.

It has been the custom to describe the extracellular organic matrix as collagenous in both bone and cartilage (Maximov and Bloom, 1951). This is probably true but the difference in organization is quite striking. The fibers of osteoid are 2 to 5 times the diameter of those in cartilage and have an obvious periodic structure not seen in the cartilage fibers. Martin (1954) has suggested that in the embryonic chick tibia the rapidity of growth in the epiphysis may determine the smallness of the cartilage fibers. It is conceivable that in more slowly growing or resting epiphyses they may subsequently be shown to be larger. We have no direct observations bearing on this, but it should be pointed out that where epiphyseal growth is rapid the growth of the bone following the epiphysis is also rapid. One therefore might expect the osteoid fibers to be small also but that is not the case in the material studied. They are much larger than those in the cartilage.

Another difference in the organization of the osteoid and epiphyseal cartilage matrix is the packing of the fibers. Those in cartilage are widely separated before and after calcification but there is a marked change in the arrangement of the osteoid fibers (Fig. 13) a short time after crystal deposition starts. This difference is quite well demonstrated in decalcified sections. The means by which the bone collagen is reorganized following initial calcification is not known, although it might be assumed that within the first 2 to 3 micra of the osteoblast both organic and mineral elements of the newly formed bone tissue may be undergoing very rapid and simultaneous analysis and synthesis. Tracer studies (Neuman and Weikel, 1955) indicate that the crystals are very stable unless absorbed in the remodelling process but this concept does not really apply to the zone close to the osteoblast. This region may in a few minutes undergo considerable regrouping before it becomes comparatively stable as the calcification front moves forward with the cell. It is unlikely that cellular remodelling involving osteoclastic activity in its usual histological sense could account for the change. It is possible that the looseness of the fibers (Fig. 10) may be changed immediately before calcification. At the moment of initial calcification they might become more closely packed, but this has not been observed up to the present.

From a volume standpoint, in the cartilage matrix, the space between the fibers is greater than that occupied by the fibers; in osteoid within 2 to 3 micra of the osteoblast the reverse situation obtains. Since this is the space in which the cement substance is believed to be located, there is reason to presume that

there is a greater volume of cement substance in a unit volume of epiphyseal cartilage than in an equal volume of osteoid.

In previous studies on bone (Robinson and Watson, 1955) it was suggested that although the bulk of the inorganic crystals was between the fibers, there may have been some mineral in the fibers at the band regions. It becomes obvious from these investigations that in epiphyseal cartilage the crystal deposits occur primarily *between* the fibers in the cement substance space. Using microradiographs Owen (1955) found calcified epiphyseal cartilage remnants in the diaphyseal sections of long bones of the rabbit. The calcified cartilage had more calcium mass per unit volume than even the most completely calcified bone tissue and therefore was distinguishable because of its greater absorption of x-rays. The explanation would appear to be that there is more space for crystal deposition between the fibers in cartilage matrix than there is between the fibers in an equal volume of osteoid.

SUMMARY

An examination of the fine structure of cartilage and bone matrix at the distal epiphyseal line of the femur of a newborn infant has revealed the following information.

Cartilage matrix is composed of a network of widely spaced fibers without obvious periodic banding. Calcification is first seen about the level of the third chondrocyte capsule distal to the furthest penetration of the capillaries. It starts as a haphazard deposition of crystals which have no obvious relationship to the location of the fibers. The process of calcification is completed before ossification commences but the central zone of matrix remains only partly mineralized.

Bone matrix is formed over a bar of calcified cartilage. Fibers, recognizable as collagen, are deposited in a loose network in a narrow zone between the osteoblasts and cartilage. These fibers are 2 to 5 times as wide as the fibers in epiphyseal cartilage. Calcification then begins in the osteoid, crystals being first laid down irregularly on or close to the fibers. As they increase in number, the crystals tend to line up along the fibers and eventually are arranged so that the periodicity of the underlying collagen is emphasized. In such an area the fibers are more tightly packed than when uncalcified.

There is no change observed in the calcified cartilage at this level.

The extracellular matrices of this epiphyseal cartilage and bone can be distinguished from one another in the electron microscope.

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EXPLANATION OF PLATES

PLATE 84

FIG. 1. Calcification of an intercolumnar bar of cartilage matrix. The progress from uncalcified matrix to heavy calcification can be followed from the upper to the lower part of the picture and at higher magnification in Figs. 2 to 7 which are from sections of a similar area. *A* and *B* are chondrocyte capsules. × 4,800.

FIG. 2. The network of fine fibers can be seen in this relatively thick section. × 22,000.

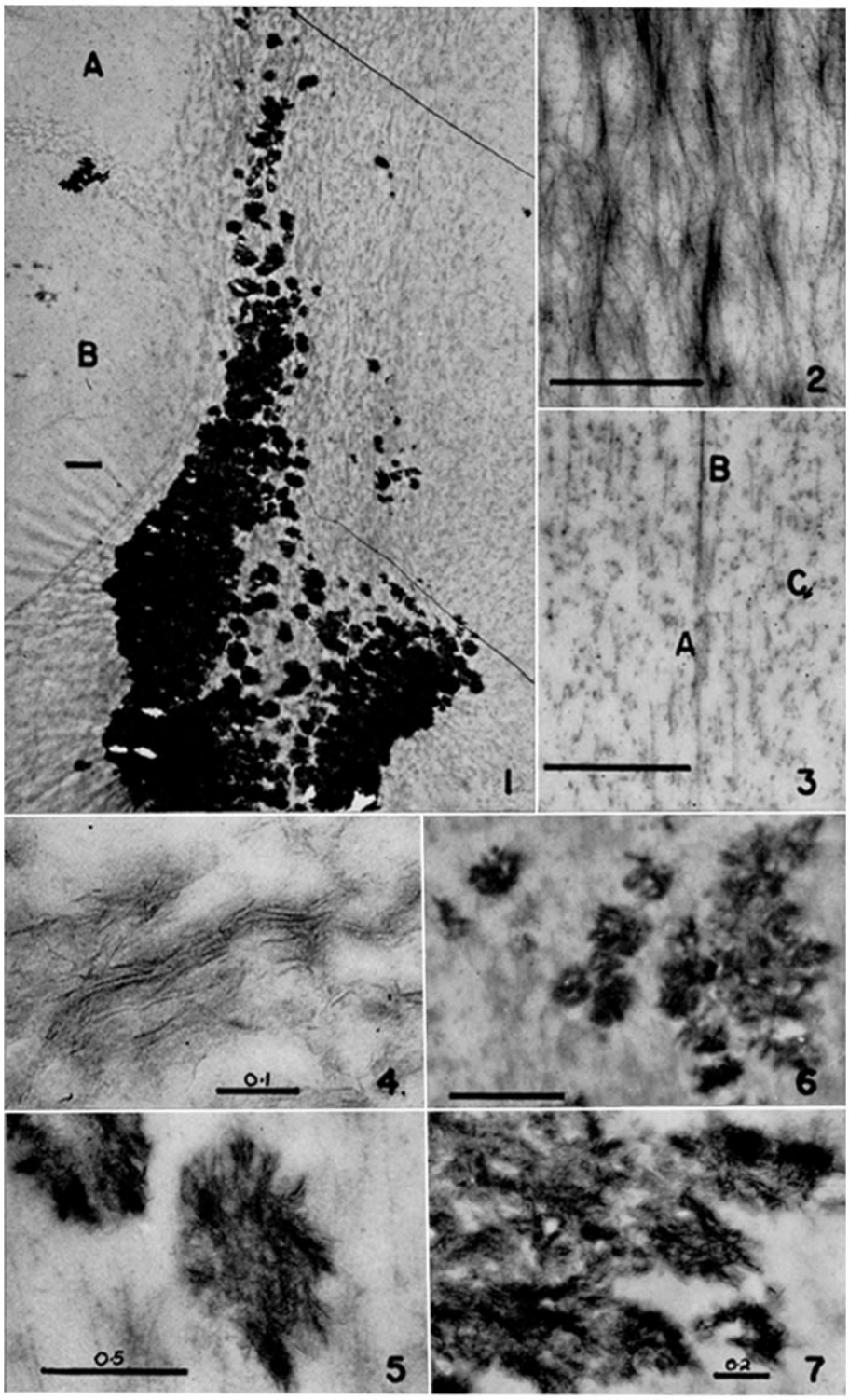
FIG. 3. Fine fibers singly and in groups (*A*) in a thinner section. There is a suggestion of periodicity at *B* and *C* is a fiber cut in cross section. × 21,000.

FIG. 4. Early crystal formation. × 120,000.

FIG. 5. A cluster of apatite crystals which are haphazardly arranged in the center and have a tendency to a radial arrangement at the edge. There is no apparent alignment with the fibers. × 42,000.

FIG. 6. Clusters of crystals coalescing. × 16,500.

FIG. 7. A densely calcified area. × 40,000.



(Robinson and Cameron: Distal epiphyseal line of femur in newborn infant)

PLATE 85

FIG. 8. The cartilage matrix is now completely calcified (*A*). The capsule has been invaded by a capillary *B* and ossification *C* has commenced between the osteoblasts *D* and the calcified cartilage matrix. × 4,800.

FIG. 9. The fully calcified cartilage matrix. The chondrocyte capsule is on the left and one or two fibers remain unobscured by the crystals. The crystals have a completely haphazard arrangement. The margin of the calcified area is irregular in outline. × 22,000.

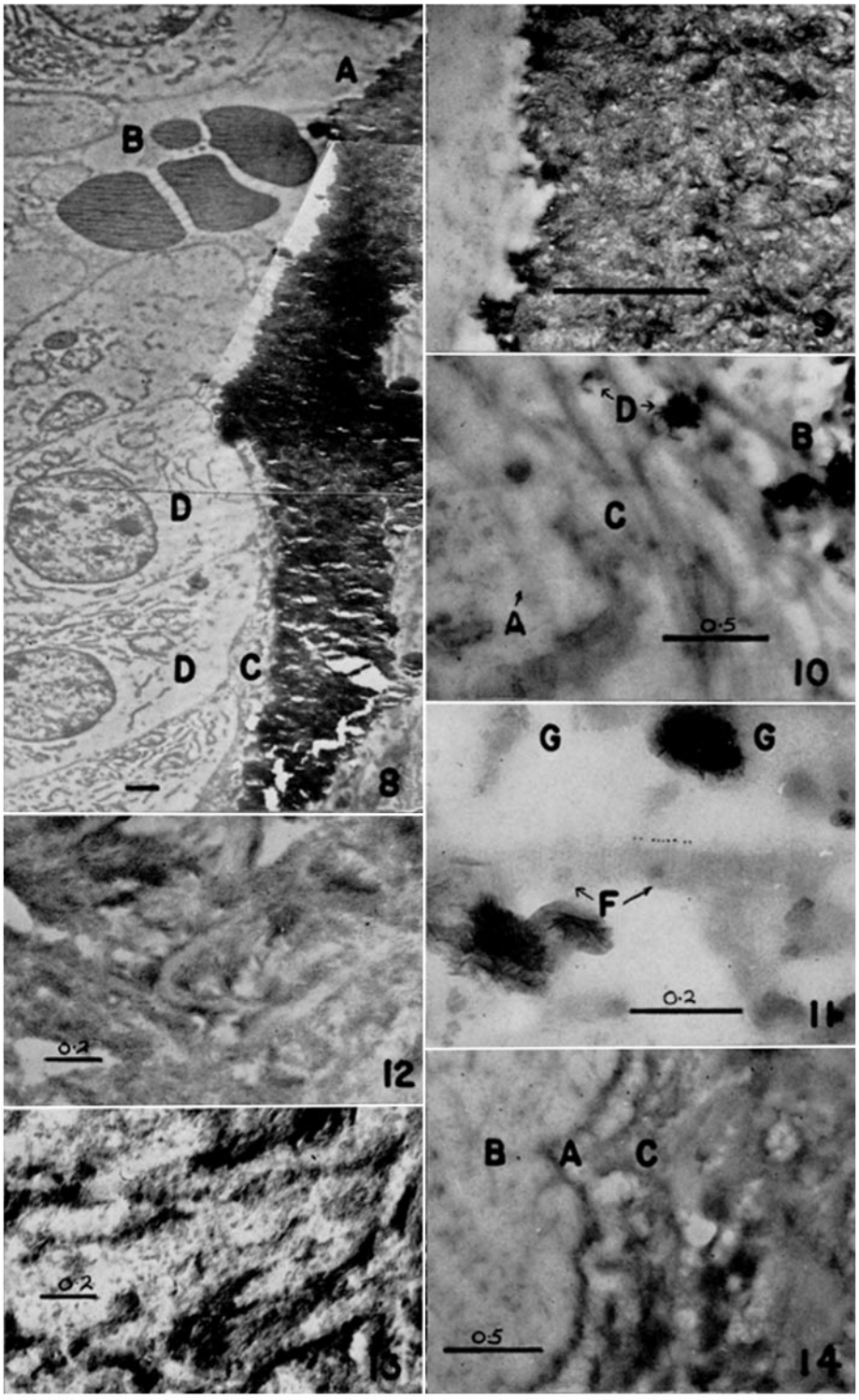
FIG. 10. The beginning of ossification. The border of an osteoblast is visible at *A* and the margin of the calcified cartilage matrix at *B*. Between is a loose network of collagen fibers *C* with some small foci of calcification *D*. × 30,000.

FIG. 11. This shows an enlargement of one collagen fiber. The *A B C D* bands and a suggestion of the *E* band are visible. This is rather different from the fibers in Fig. 3. There is some increase in the density of the bands at *F* and clusters of crystals at *G*. × 80,000.

FIG. 12. Newly formed bone. The crystals are lined up along the fibers but they have no periodic arrangement. × 40,000.

FIG. 13. The crystals are now arranged so that the periodicity of the underlying fibers is emphasized. The fibers are so aligned that the periods on each are in phase. × 42,000.

FIG. 14. The junction *A* between cartilage matrix *B* and bone matrix *C* in a decalcified section. The "double membrane" is apparent. × 28,000.



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