ELECTRON MICROSCOPE STUDIES OF CRYSTAL-COLLAGEN RELATIONSHIPS IN BONE*

IV. The Occurrence of Crystals Within Collagen Fibrils

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Plates 311 and 312

(Received for publication, May 29, 1957)

Earlier electron microscope studies have provided circumstantial evidence for the existence of crystals within the collagen fibrils[1] of bone matrix. In fully calcified bone matrix there are no areas which are not replete with crystals; a finding which indicates that there should be a deposition of crystals not only at the periphery of fibrils but within them as well (12). The position of crystals along collagen fibrils has been shown by Jackson in sections from incompletely calcified embryonic avian bone (4, 5). She suggests, when referring to longitudinally oriented collagen fibrils with attached crystals, that the section plane may pass through the fibrils. This implies that crystals could lie within the fibrils. The present communication reports the occasional finding of small crystals located within collagen fibrils during an early phase of calcification.

Materials and Methods

Immediately after the decapitation of twelve newborn white Swiss mice, random blocks from calcifying parietal bones and blocks from the central metaphyseal region of the tibia, where cancellous bone spicules are found, were fixed for 1 hour in 1 per cent isotonic buffered osmic acid at 0°C. These specimens were then gradually dehydrated in graded alcohol, and embedded in butyl methacrylate to which 1 per cent benzoyl peroxide had been added. Polymerization was achieved at 65°C. Sections were cut with glass knives on a Sjöstrand microtome (14), mounted on formvar-coated copper grids, and examined in an RCA EMU 2 microscope.

OBSERVATIONS

New calcification of bone matrix occurs at the periphery of bone spicules in normal animals. A layer of osteoblasts overlies the calcified spicule and the layer of calcifying bone matrix. In hematoxylin-stained sections for light micros-

* This work was supported by Grant A706 C-2 from the National Institutes of Health.

[1] Collagen fibrils are defined as fibrils of indeterminate length which show a characteristic periodicity of 640 A within which subbandings may be seen (1).

copy osteoblasts are seen to have an intensely basophilic cytoplasm. In the electron microscope this cytoplasm appears to contain densely packed lamellated intracellular membranes, the major portion of which appear to have small (150 A) granules attached to their outer surface (Fig. 2). Such membrane systems have been variously referred to as ergastoplasmic sacs (18), the rough-surfaced elements of the endoplasmic reticulum (8), and alpha cytomembranes (16). These membranes have been reported as a cytoplasmic component of most cell types and seem to be most prominent in cells that elaborate protein. The arrangement and abundance of these granular membranes in the osteoblast most nearly corresponds to the appearance of this component in the acinar cells of the pancreas (15, 7). The space between the smooth appositive surfaces of the granular membranes appears to contain an amorphous substance which is more dense than the embedding media (Fig. 3). It is interesting to note that various degrees of separation of the granular surfaced membranes are seen in different osteoblasts. Within the cytoplasm of osteoblasts there are in addition free 150 A granules, abundant mitochondria, and occasional granules with a single limiting membrane as described by Zetterqvist (19). Near the nucleus, smooth surfaced membranes and vacuoles corresponding to the Golgi apparatus are seen.

The relation of the cell surface membranes both to the amorphous substance between the appositive surfaces of the granular membranes and to the extracellular collagen fibrils is particularly difficult to delineate.

The osteoblast is separated invariably by a small distance from the calcified matrix. This area contains the "physiological osteoid" (9) or matrix which is becoming calcified. Within this area there are varying amounts of collagen fibrils, varying amounts of opaque interfibrous substance, and crystals in varying numbers indicating calcification. In normally calcifying bone matrix, collagen fibrils appear to be "vested" with an opaque coating a short distance from the osteoblast (less than 0.5 micron). Then within an exceedingly short distance these "coated" fibrils appear associated with inorganic crystals and the matrix is calcified. This has been referred to as the "calcification front" (12).

Occasionally two osteoblasts may overlie one another. In between such cells collagen fibrils are seen. In such an area, separated by one cell from the "calcification front," small crystals may be seen occasionally. Near the calcification front crystals are seen most commonly at the periphery of fibrils, between tangential fibrils, or in crystal aggregates sufficiently large to obscure underlying fibrils. In an area of Fig. 3 crystals also may be seen within transversely sectioned collagen fibrils (see inset). In two instances three crystals are seen within a single fibril (see arrows).

The smallest dimension of these crystals is approximately 50 A. Due to the presence of the opaque substance about calcifying fibrils it is difficult to determine their diameter.

DISCUSSION

The arrangement of osteoblasts about spicules of bone is well known. The present observations would seem to substantiate the hypothesis of Howard (3) that there is a syncytium of osteoblasts which could mediate metabolite transfer between the extracellular space and the calcified matrix. Electron microscope observations on the structure of the osteoblasts have been previously reported (5, 13), but the variable appearance of the spaces between the smooth surfaces of the "rough-surfaced components of the endoplasmic reticulum" or "alpha cytomembranes" in different osteoblasts has not been remarked. Increased distance between the smooth surfaces of these membranes may be associated with a phase of secretion. The physical relation of the newly formed extracellular collagen fibril to the intracellular amorphous substance which appears between the membrane systems remains elusive.

The dense or opaque haze which invests the collagen fibrils lying between an osteoblast and the calcification front has been noted in an earlier communication (12). It is suggested that this opaque substance, which is associated with the appearances of inorganic crystals, represents the earliest visible concentration of inorganic ions in the "physiological osteoid".

It may be of interest that in both Jackson's observations (5) and in those presented here, the site of the smallest crystal units attached to fibrils is between two cells rather than between a cell and the region of calcified matrix. That the structures described here at the surface of, and within, collagen fibrils are apatite is deduced from the following findings:

1. The structures are seen in sections from developing bone near the calcification front while they have never been observed in sections from other connective tissues.

2. The size, shape, and appearance of the structures seen here correspond to that of crystals seen in other electron microscope studies on bone in which diffraction techniques were also used (17, 5, 2).

3. The present techniques of preparation do not differ from those found to introduce the least artifacts for electron microscopy (6, 10, 19). No counterstains such as phosphotungstic acid were used nor were there any alterations in the fixative, dehydration, or temperature of embedding. The observed tissues were fixed at pH 7.3 and were not decalcified.

The possible geometrical relationships of the apatite crystals to collagen have been commented on in an earlier publication (12). The plane of section must be perpendicular to the long axis of the fibrils which contain crystals before it can be accepted that the crystals truly are within the fibril matrix. In Fig. 3 the majority of the fibrils appear to be transversely sectioned. The likelihood is small that the position of the crystals is a mechanical artifact caused by sectioning. It appears then that crystals may lie both at the surface and within collagen fibrils. The resolution achieved here does not permit further

characterization of the crystal size or axis dimension other than to state that the crystals within the collagen fibrils are approximately 50 A in profile.

The implications of these findings with regard to the calcification of bone and its water content will be discussed in a subsequent communication.

SUMMARY

Crystals are seen occasionally within the diameter of transversely sectioned collagen fibrils near the calcification front of newly formed bone.

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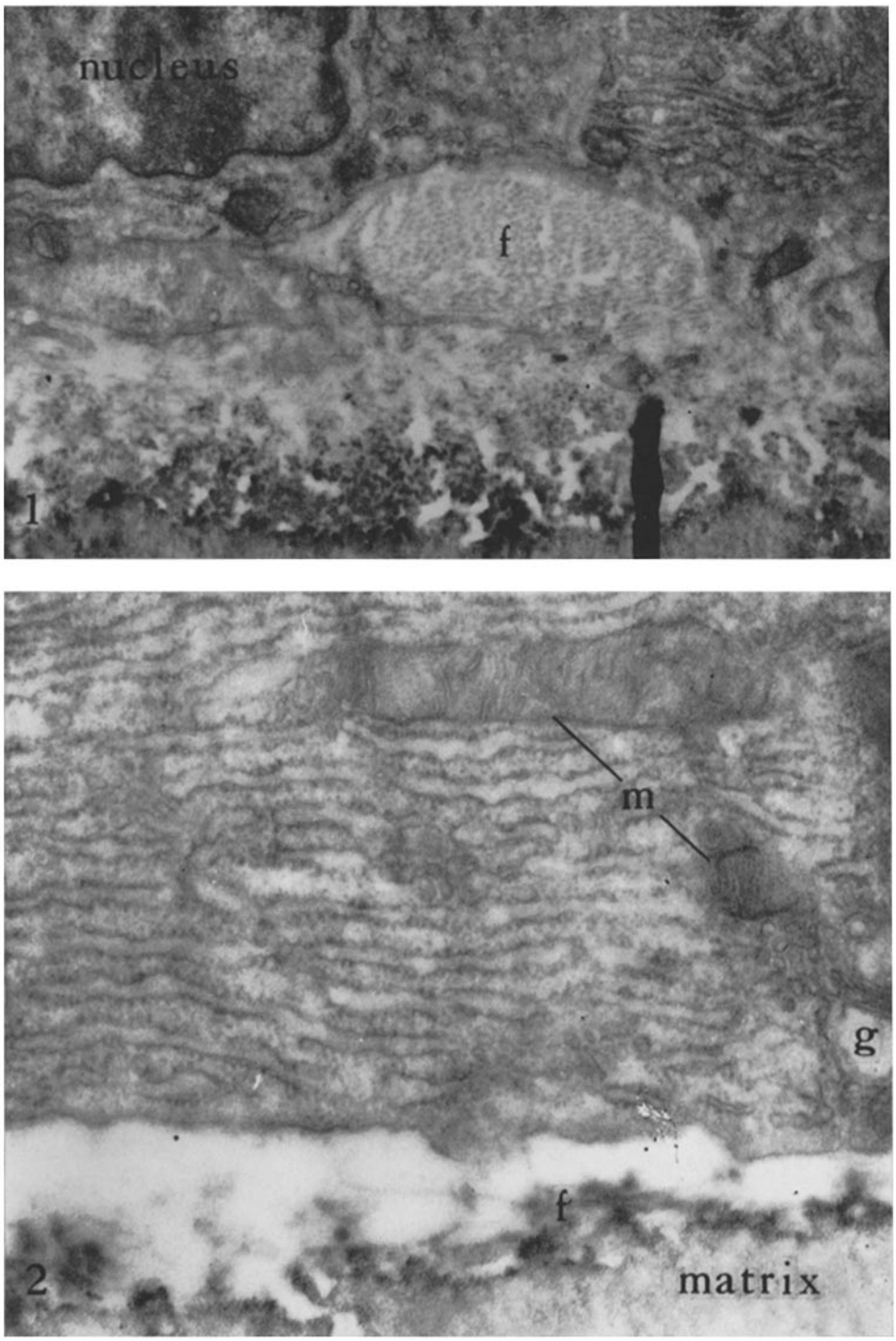
EXPLANATION OF PLATES

PLATE 311

FIG. 1. Survey electron micrograph of a portion of an osteoblast whose nucleus lies in the upper left corner. There is a large bundle of collagen fibrils (*f*) which lies partly surrounded by osteoblast cytoplasm. It is difficult to determine where the cell boundary ends. Physiological osteoid lies between the osteoblast and the calcified matrix which is at the bottom. In between the cell and the calcified matrix the collagen fibrils appear "vested" with an opaque coating. Magnification, 18,500.

FIG. 2. An electron micrograph of an area from an osteoblast which lies very close to the calcified matrix. A high degree of preferred orientation of the cytoplasmic membranes is apparent. In the right lower corner is a portion of the Golgi apparatus (*g*). Mitochondria (*m*) are also seen within the cytoplasm. A few collagen fibrils (*f*) lie between the cell and the calcified matrix. Magnification, 50,000.

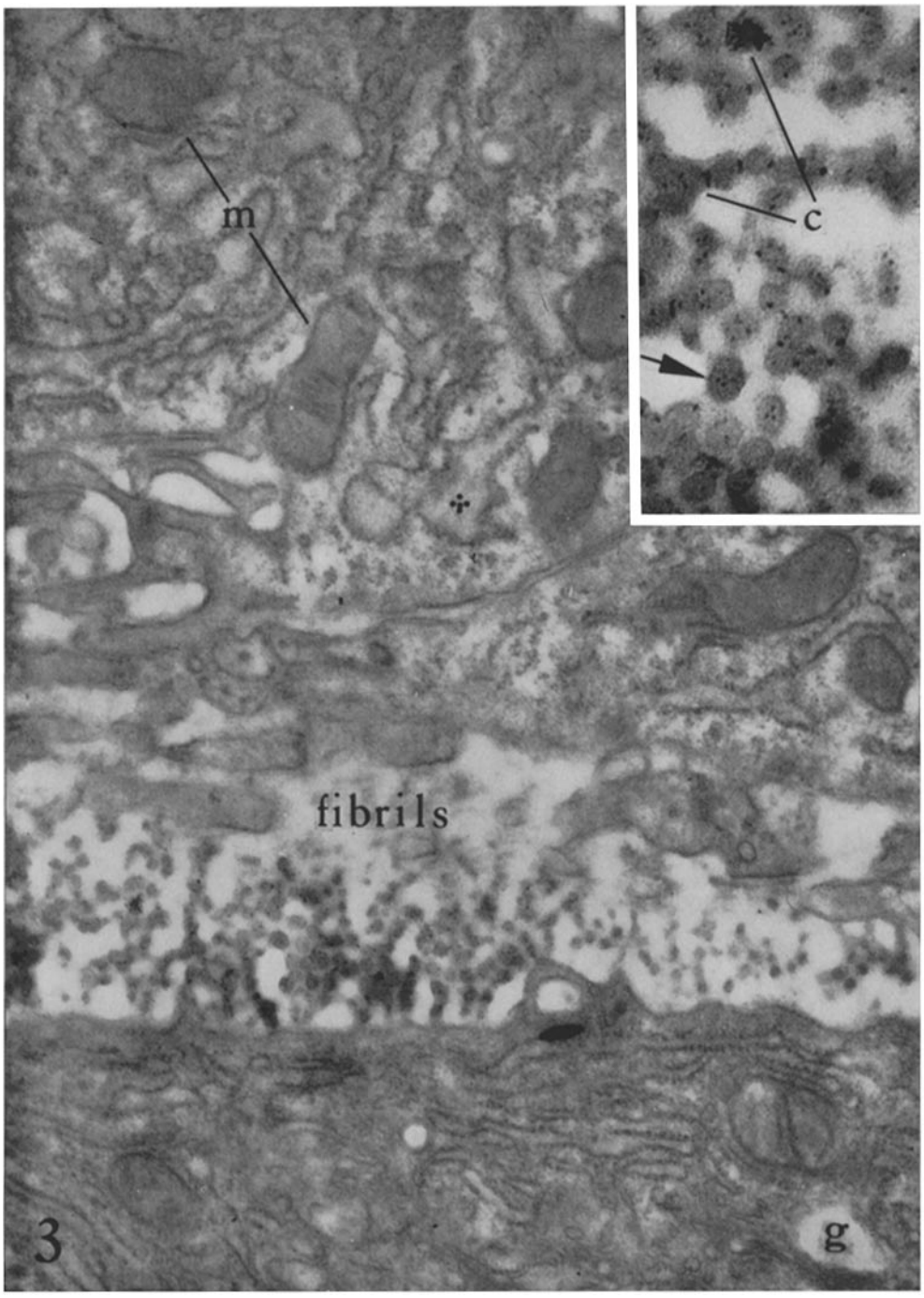


(Sheldon and Robinson: Crystal-collagen relationships in bone)

PLATE 312

FIG. 3. Electron micrograph of portions of two osteoblasts with collagen fibrils in the space between the two cells. The calcified matrix if it were shown would be below the bottom cell. Mitochondria (*m*) and Golgi membranes (*g*) may be seen within the osteoblast cytoplasm surrounded by the cytoplasmic membrane systems. In the upper cell a moderately dense, amorphous substance lies within the "cisternae" of the granular-surfaced membranes (*). Magnification, 37,000.

The inset shows a higher magnification of an area between the cells where the collagen fibrils are transversely sectioned and "vested" with the opaque coating. An aggregate of crystals is in the upper left corner (*c*). Three crystals are seen within a single transversely sectioned collagen fibril (arrows). Magnification, 80,000.



(Sheldon and Robinson: Crystal-collagen relationships in bone)