

EVIDENCE FOR CHANGES IN PROTEIN POLYSACCHARIDE ASSOCIATED WITH THE ONSET OF CALCIFICATION IN CARTILAGE

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ABSTRACT

Past work has suggested that protein polysaccharide may play a role in the calcification of cartilage. Recent electron microscopic studies on noncalcified cartilage have indicated that protein polysaccharide in cartilage matrix is represented by granules associated with collagen fibers. The present work has been designed for comparison of the matrix of noncalcified cartilage to that of calcified cartilage, with particular reference to these granules. Small blocks of tibia from 16-day embryos were fixed in cacodylate-buffered glutaraldehyde and postfixed in either phosphate- or Veronal-buffered osmium tetroxide. Special care was taken to maintain the pH above 7.0 at all times. For electron microscopy the tissues were dehydrated, embedded in Epon 812, sectioned, and stained with uranyl acetate or lead citrate. A marked decrease in the size of granules in the matrix of calcified cartilage compared to noncalcified cartilage was noted. Associated with the decrease in the size of granules was a condensation of matrix components and the presence of an amorphous electron-opaque material that was not seen in noncalcified areas. These results are interpreted to represent either a drop in concentration or a change in state of protein polysaccharide with the onset of calcification in cartilage.

INTRODUCTION

At the time that previous electron microscopic studies pertaining to the calcification of cartilage were conducted (1-3, 17-20), the nature of the various ultrastructural components of cartilage matrix was not known, and therefore changes in matrix composition associated with calcification could not be interpreted with certainty. Recent electron microscopic investigations (11), however, have indicated that dense granules associated with fibrils in cartilage matrix represent protein polysaccharide. Since several reports in the litera-

ture implicate protein polysaccharide with calcification in cartilage (5, 7, 8), the present study was designed as an investigation of the changes in the pattern of cartilage matrix components associated with the onset of calcification, with particular reference to the distribution of matrix granules in mineralized and nonmineralized cartilage.

MATERIALS AND METHODS

A preliminary study was conducted for determination of the time of the first morphological evidence of cal-

cification in the tibial cartilage of the chick embryo. Five embryos each were sacrificed at intervals of 14, 16, 18, and 20 days. The right tibia of each embryo was fixed in neutral (pH 7.4) buffered formalin, dehydrated, embedded in paraffin, and sectioned at $7\ \mu$ according to routine histological techniques. The sections were then stained with hematoxylin and eosin and by the von Kossa method (14).

Calcification was first noted at 16 days in the lateral areas of the hypertrophic zone next to the bone (Figs. 1 and 2). On the basis of this observation, five 16-day chick embryos were sacrificed, and the right tibias were dissected free of soft tissues. Transverse blocks (1 mm thick) were cut with a razor blade through the zone of hypertrophic cells and placed immediately in cold cacodylate-buffered (pH 7.4) glutaraldehyde (15). All blocks were postfixed in either phosphate- (12) or Veronal-buffered osmium tetroxide (4, 13). The tissues were dehydrated in increasing concentrations of alcohol (10) and embedded either

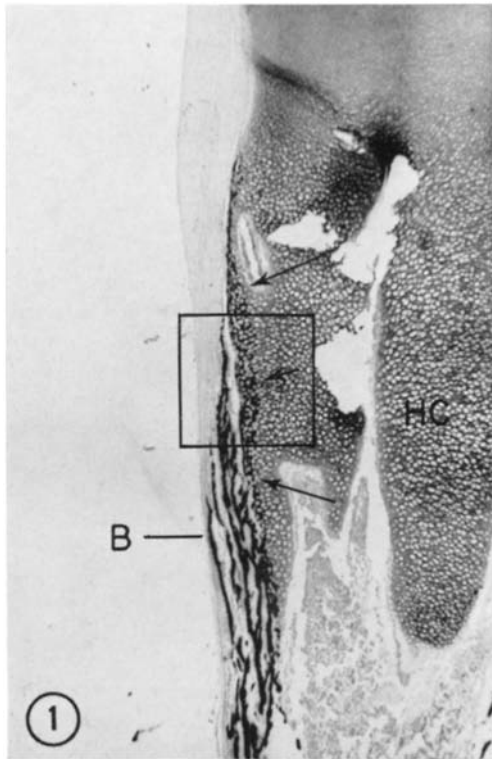


FIGURE 1 Light microscopic view of the distal end of the tibia of an 18 day embryo stained by the von Kossa method. Note the small areas of calcified cartilage (arrows) at the periphery of the zone of hypertrophic cells (HC) immediately adjacent to bone (B). Small islands of calcified cartilage first were seen here at 16 days. $\times \sim 100$.

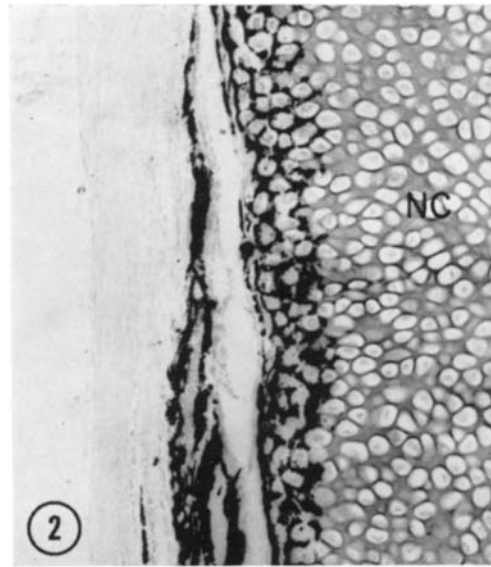


FIGURE 2 Higher magnification of the enclosed portion of Fig. 1. The approximate areas of calcified and noncalcified (NC) cartilage from the zone of hypertrophic cells that was examined by electron microscopy are indicated. $\times \sim 400$.

in pure Epon 812 or in a mixture of Araldite and Epon 812.

$1\ \mu$ thick sections were stained with 0.05% toluidine blue in 1% borax and examined by light microscopy for orientation purposes. Areas of calcified cartilage at the periphery of the hypertrophic zone were noted and trimmed for thin sectioning for electron microscopy. Comparable sections were taken from noncalcified areas of the hypertrophic zone (see Fig. 2 for approximate areas).

All sections were mounted on Formvar-carbon grids and stained with uranyl acetate (21) or lead citrate (16) for examination in an RCA EMU 3-H electron microscope.

RESULTS

Calcification of cartilage matrix was seen first in the zone of hypertrophic cells in proximity to bone. The crystallites appeared both immediately adjacent to the bone surface and as clusters within the matrix (Fig. 3), but no obvious preferential orientation to fibers could be noted.

Marked changes were seen in the matrix of mineralized areas of cartilage next to bone when compared to nonmineralized areas from the hypertrophic zone. The appearance of the matrix in the nonmineralized areas was similar to that described in previous work (11); in these areas the

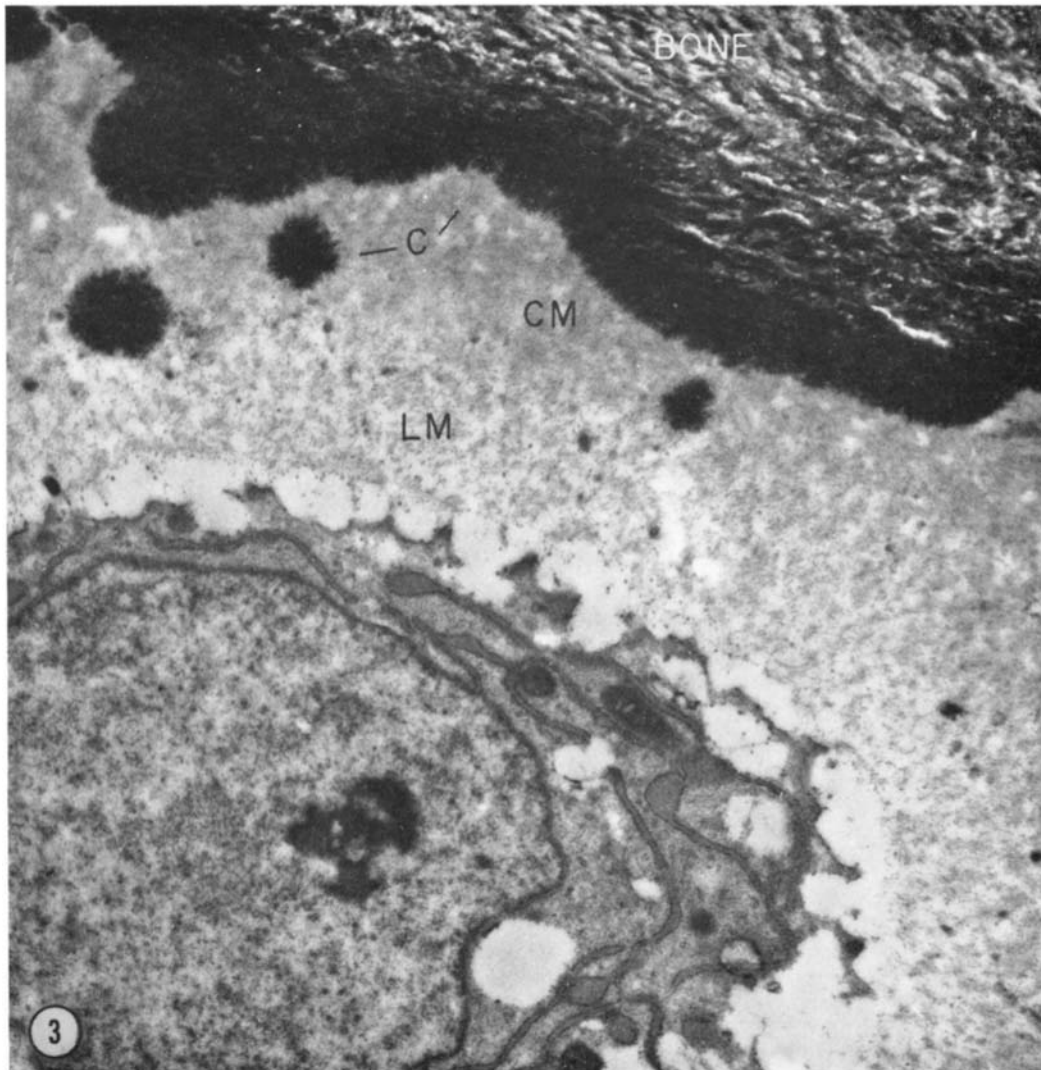


FIGURE 3 Electron micrograph of calcifying cartilage. Calcification (*C*) can be seen immediately adjacent to bone and as individual crystal clusters within the matrix. The cell is a hypertrophic chondrocyte. A demarcation can be noted between condensed areas of matrix (*CM*) and more loosely arranged matrix immediately surrounding the cell (*LM*). $\times 11,000$.

granules were prominent and the fibers were loosely arranged (Figs. 4 and 6). In contrast, in the mineralized areas the granules were markedly reduced in size. In addition, large portions of these mineralized areas showed marked condensation of matrix components and, in some cases, the presence of an amorphous, electron-opaque background material (Figs. 5 and 7). Islands of matrix, in which there were a marked reduction in granule size and a condensation of matrix

components put no obvious crystal deposits, were also often noted in the hypertrophic zone immediately adjacent to mineralized areas. The condensed areas were usually found at points in the matrix which were not adjacent to the lacunae of the hypertrophic chondrocytes (Fig. 5). Another consistent finding was the presence of apparently membrane-bounded dense bodies around crystal clusters and in other condensed areas of matrix (Fig. 7).

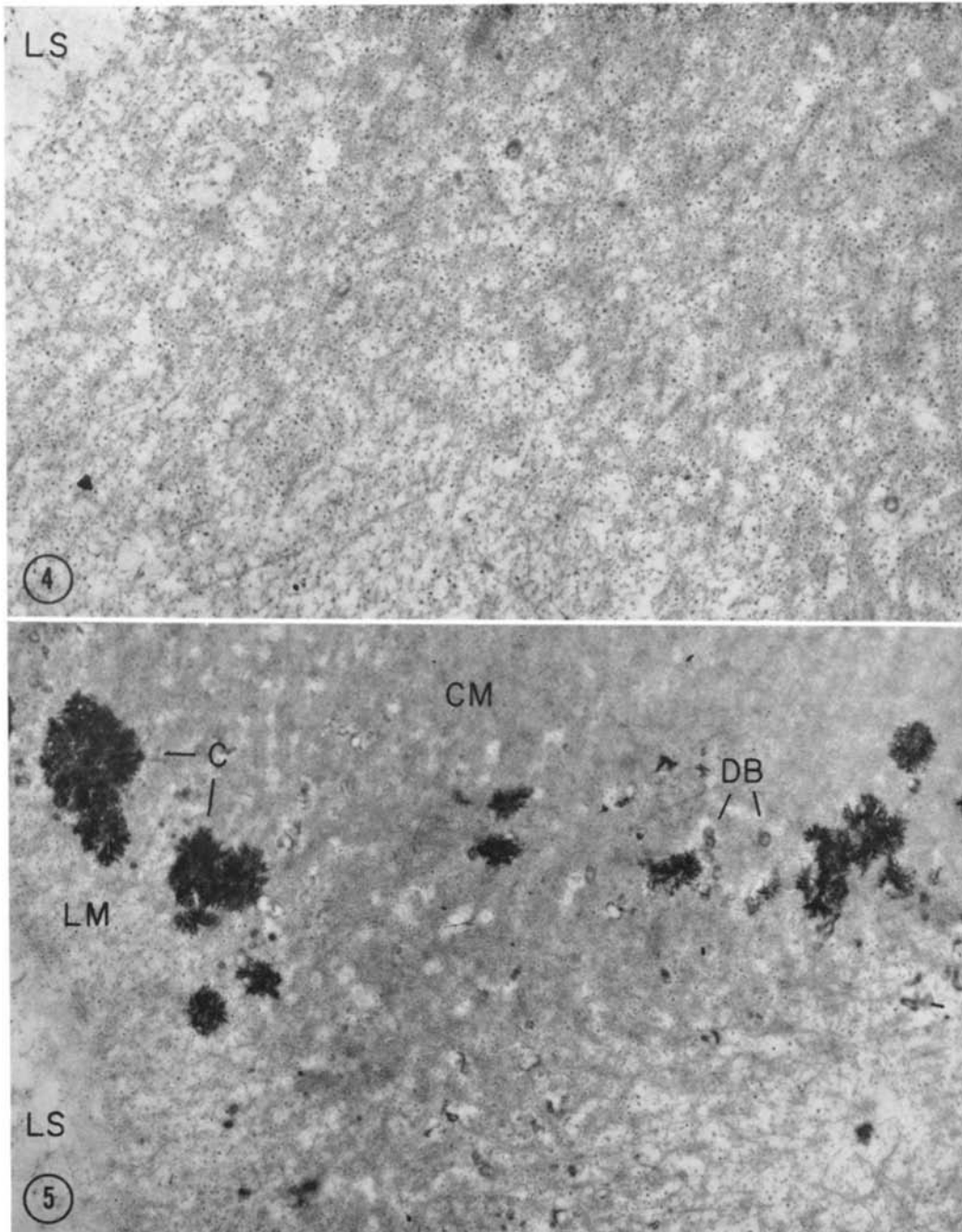


FIGURE 4 Noncalcified matrix from the zone of hypertrophic cells. The matrix is homogeneously distributed throughout the zone and is composed of dense granules and fibers. *LS*, lacunar space. $\times 12,000$.

FIGURE 5 Calcifying matrix from the zone of hypertrophic cells. In contrast to the matrix as seen in Fig. 4, the matrix here is characterized by an over-all decrease in the size of the granules. The matrix can further be divided into condensed (*CM*) and loosely arranged (*LM*) areas. The crystal clusters (*C*) can be noted at the junction of the two types of matrix and in the condensed areas. This condensed area is located between the lacunar spaces (*LS*) of two hypertrophic cells. Dense bodies (*DB*) can be seen within condensed areas. $\times 12,000$.

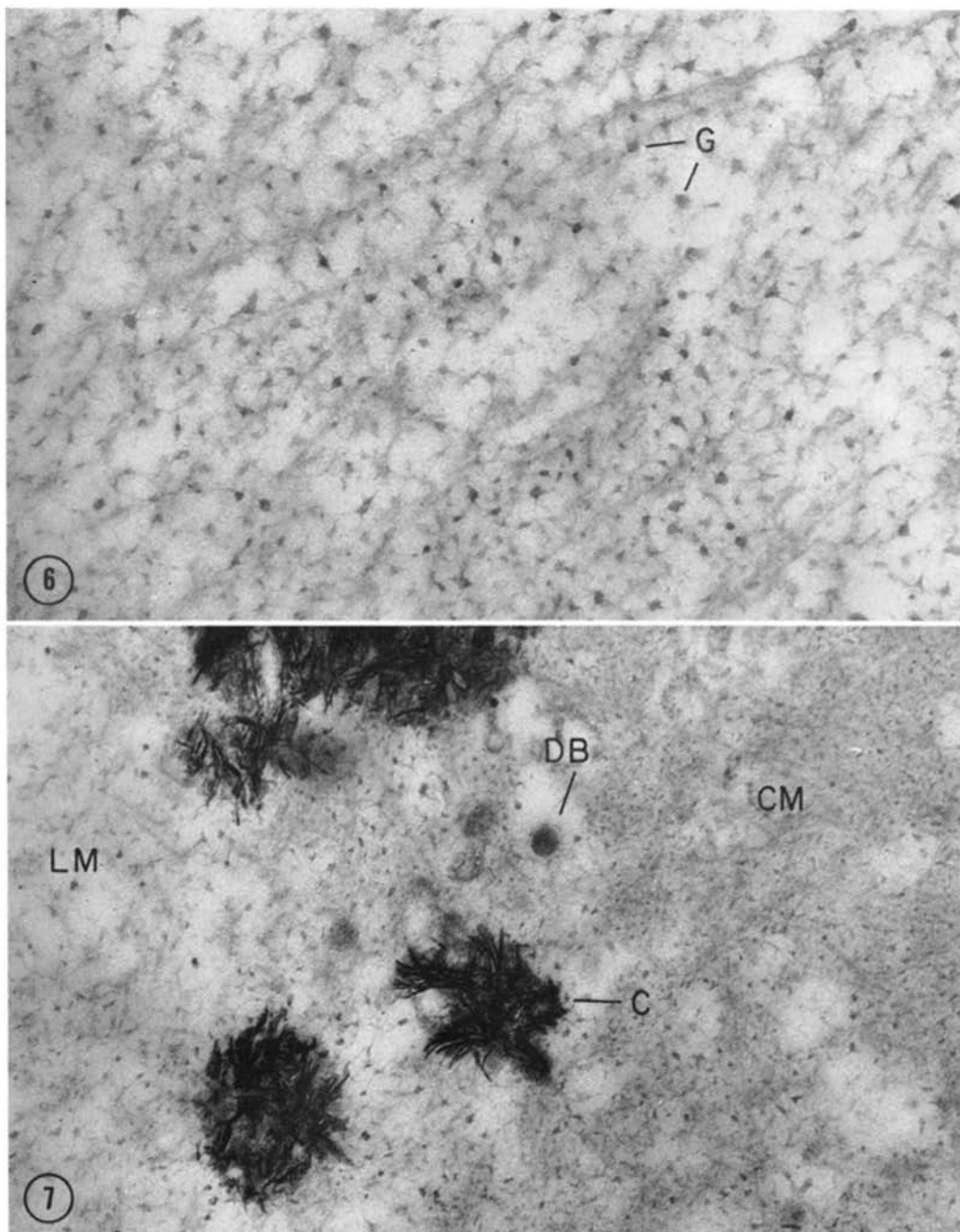


FIGURE 6 Higher magnification of noncalcified matrix. Note the prominence of the matrix granules (*G*) and their relationship to fibers. $\times 52,000$.

FIGURE 7 Higher magnification of an area of calcification. There is a distinct demarcation between loosely arranged matrix (*LM*) and the condensed matrix (*CM*). In contrast to Fig. 6, there is a decrease in granule size and the presence of an amorphous, electron-opaque background material in the condensed areas. Note also the presence of dense bodies (*DB*) at the junction of the two areas of matrix. *C*, crystallites. $\times 52,000$.

DISCUSSION

Our observations indicate that the onset of calcification is marked primarily by a reduction in size of matrix granules and the appearance of condensed matrix. Since there is evidence that these granules represent protein polysaccharide within the matrix (11), these studies indicate that a change in the character of this component is associated with calcification. Whether the change in the granules reflects an actual decrease in the concentration or a change in the state of protein polysaccharide or the appearance of a qualitatively different compound could not be determined.

Studies by other investigators have also suggested that calcification in cartilage is associated with a change in the state of the ground substance (5, 7, 8). Hirshman and Dziewiatkowski (7) have reported that immediately prior to calcification there is a loss of staining for protein polysaccharide by immunofluorescence techniques. The reduction in granule size observed in the present study may then be a morphological manifestation of this loss in staining.

The release or activation of hydrolytic enzymes could theoretically explain the changes observed in this study. Hydrolytic enzymes have been demonstrated in cartilage (9), and recently an enzyme has been isolated from cartilage that is capable of degrading protein polysaccharide (5). This enzyme has an optimum pH of 4.0, which may suggest that it is lysosomal in origin. The presence of a lysosomal enzyme (acid phosphatase)

in premineralizing and mineralized areas of hypertrophic cartilage has also been demonstrated by histochemical techniques (6). This lysosomal enzyme was not found in other areas of cartilage.

The nature of the dense bodies seen in the present study is unknown, but similar inclusions in the matrix of calcifying cartilage have been noted by both Anderson (2) and Schenk.¹ The suggestion has been made that they represent membrane-bounded fragments of hypertrophic chondrocytes (2). Since these bodies were seen in large numbers in condensed areas of matrix undergoing mineralization, it is tempting to speculate that they may contribute to the observed matrix changes by containing the previously described hydrolytic enzymes (5, 9).

No clear explanation is available for the condensation of matrix components or for the nature of the amorphous background material, but studies designed to elucidate these changes are now being conducted.

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¹ Schenk, R. 1968. Personal communication.