ELECTRON MICROSCOPIC STUDIES OF CARTILAGE MATRIX USING LYSOZYME AS A VITAL STAIN

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During the course of a series of studies which had as their aim the elucidation of the role of lysozyme in cartilage metabolism (1–4), it was found that, when egg white lysozyme (LYS) was added to organ cultures of ossifying cartilage (cartilage which contains an epiphyseal growth plate) but not nonossifying cartilage (cartilage which does not contain a growth plate), the lysozyme bound to cartilage matrix in specific anatomic sites. In recent years a number of techniques, including staining with lanthanum and bismuth salts (5, 6), ruthenium red (7), and colloidal iron (8), as well as the addition of cetylpyridinium chloride to fixatives (9), have been used for the visualization of mucopolysaccharides for electron microscopy. The rationale of most of these methods, where they are not completely empirical, involves the use of a relatively small cation to precipitate the anionic mucopolysaccharides *in situ*, thus preventing their solubilization in aqueous fixatives. The precipitant may also act as a stain, or metallic stains may then be used to increase contrast. One difficulty with this approach, at least on a theoretical level, is that the naturally occurring connective tissue polysaccharides are unbranched, nonrigid polymers. Precipitation of the large polysaccharides molecules with small cations should result in considerable ravelling up the chains, thus giving a false impression of their conformation and extent. A possible approach to this problem could be the use of larger cations which would at least fix portions of the polysaccharide chains in an extended state. On the basis of previous studies (1–4), LYS appeared to

be a good candidate for such a cation. It is a well-characterized, commercially available protein with an isoelectric point of 11.2. LYS is a roughly ellipsoidal molecule, measuring 45 × 30 × 30 A, with a molecular weight of 14,600 (10). It is known to precipitate cartilage polysaccharides and their parent mucoproteins under physiological conditions (11) and to complex with these compounds when added to organ cultures of preosseous cartilage (2–4). It does not degrade mammalian polysaccharides (12) and does not significantly affect the growth or calcification of epiphyseal cartilage in organ culture (2–4). The technique of short-term organ culture in the presence of added LYS also appeared to offer the advantage that the problem of diffusion of the molecule into the tissue during the process of fixation, which occurs with some other techniques, might be considerably mitigated.

MATERIALS AND METHODS

Organ cultures of puppy scapular cartilage-bone junctions were done by techniques previously described (2). LYS (1 mg/ml) was added to the culture medium, and explants treated in this manner were compared with others treated identically except that LYS was not added. After 18 hr, tissues were fixed for 2 hr in 5% cacodylate-buffered glutaraldehyde, washed overnight in buffer, postfixed in osmium tetroxide, and embedded in Epon. Thin sections from the columnar and hypertrophic zones were then examined in an electron microscope, either unstained or after staining with lead citrate or uranyl acetate.

RESULTS

Specimens cultured in medium free of LYS had an appearance similar to that described by others (9). The chondrocytes lay in a relatively empty matrix containing scattered collagen fibrils, which lacked a 640 A axial periodicity, and scattered electron-opaque droplets of various sizes (Fig. 1). Specimens cultured in the presence of LYS showed a matrix with a strikingly different appearance. The chondrocyte lacunae were largely filled with electron-opaque material the concentration of which decreased toward the interlacunar matrix (Fig. 2). This material approached close to the cell membrane but only rarely touched it. At higher resolution, the dense material was seen to be made up of closely packed particles

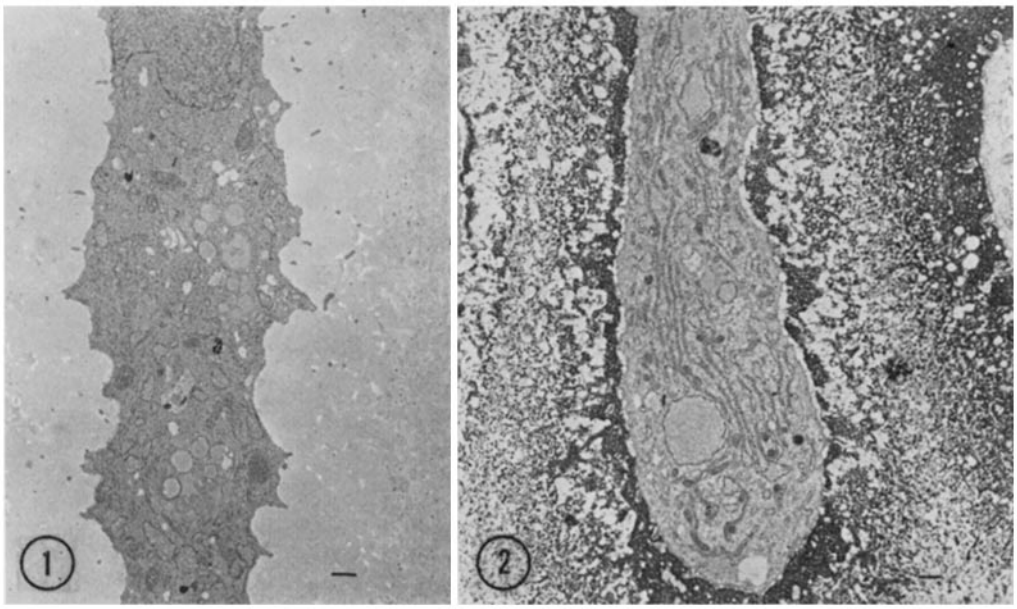
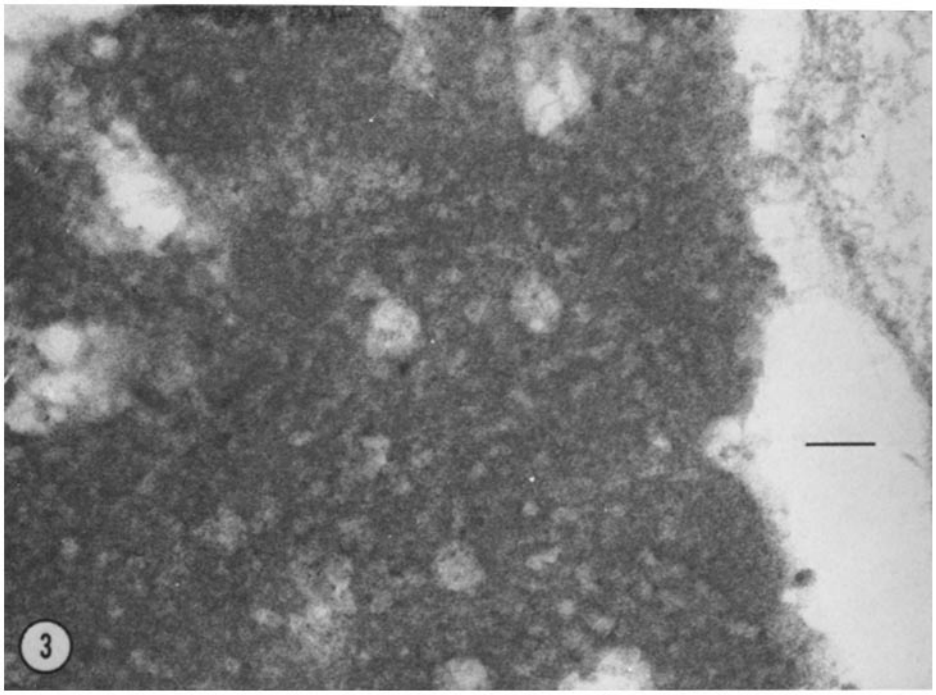


FIGURE 1 Columnar zone, control specimen. A chondrocyte lies in an empty-appearing matrix. Marker: 1 μ. × 3000.

FIGURE 2 Columnar zone, LYS-treated specimen. Here, dense material fills much of the chondrocyte lacuna, apparently decreasing in concentration toward the lacunar edge. The matrix outside the lacuna contains similar material, but less densely packed and in a more punctate pattern. Marker: 1 μ. × 3000.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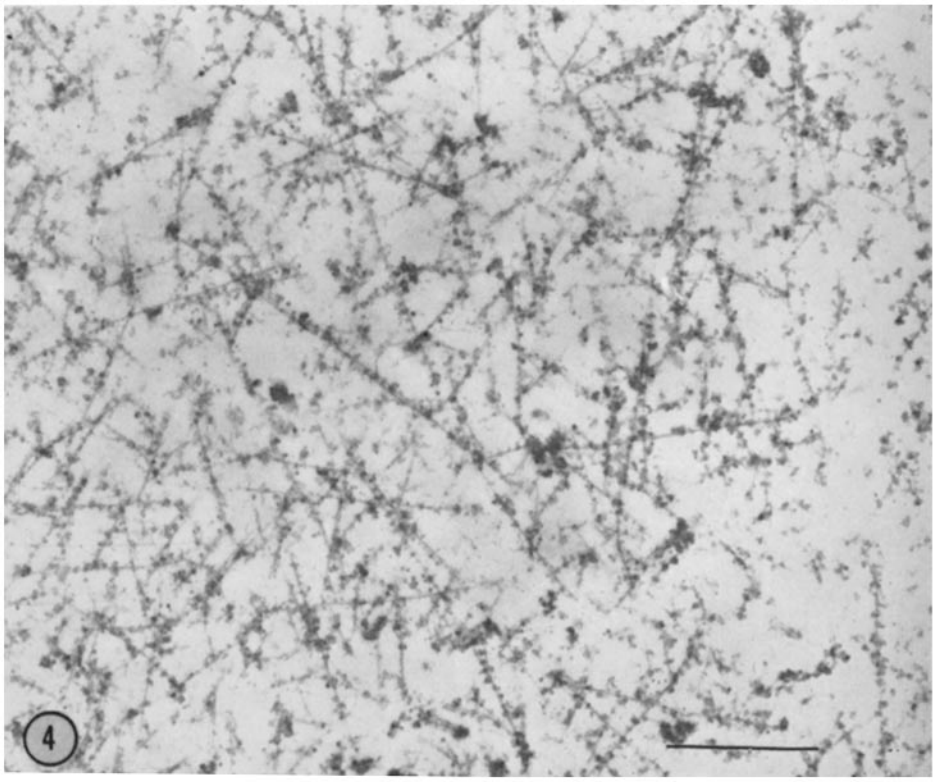
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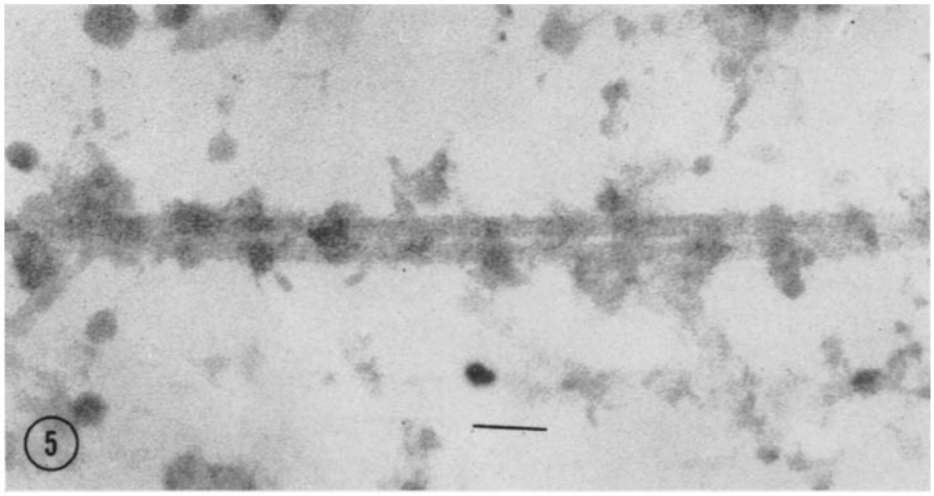

FIGURE 5 A higher magnification of collagen fibrils from Fig. 4. The dense deposits coat the filaments within the fibril, extending into the surrounding matrix at the lateral edge of the fibril. Marker: 0.1 μ. × 98,000.

(Fig. 3). In the matrix outside the chondrocyte lacuna, these deposits were more dispersed, punctate, and, for the most part, arrayed on the surface of collagen fibrils (Fig. 4). This array was quite regular, the mean distance between the deposits measuring 642 A (SD, 61 A). At higher resolution, dense material was found stretched along the filaments which made up the collagen fibril with the larger lateral projections on the fibril surface (Fig. 5).

The hypertrophic zone, in contrast to the remainder of cartilage, did not appear to be altered by the added LYS. In unstained specimens, similar tissue patterns were seen, but with greatly diminished contrast.

DISCUSSION

LYS as a vital stain for cartilage matrix thus appears to have usefulness in electron microscopic studies. The use of fluorescein-labeled LYS has shown that LYS is bound in precisely the areas where dense material is seen with the electron microscope (3). The micrographs show that cartilage matrix is heterogeneous, at least in its reactivity with LYS, there being two locations with particularly great affinity for LYS, the chondrocyte lacuna and certain areas on the surface of collagen fibrils. Sajdera has shown that the polysaccharides in the lacuna are particularly resistant to guanidinium chloride extraction, another indication that the polysaccharide composition of this site is different from that elsewhere in cartilage matrix (13). Materials other than protein-polysaccharide, which may also be heterogeneously distributed in cartilage, may also have complexed with LYS in this area. If this is so, their contribution to the density is probably minor, since by far the greatest amount of anionic material in cartilage is protein-polysaccharide. The observation of an ordered array of polysaccharide on the surface of collagen fibrils has been made in other tissues where an intrinsic 640 A axial periodicity of col-

FIGURE 3 Lacuna, LYS-treated specimen. The dense material, representing LYS-polysaccharide complexes, is seen to consist of small, closely packed, dense particles. Marker: 0.1 μ. × 97,000.

FIGURE 4 Interlacunar matrix, LYS-treated specimen. The dense LYS-polysaccharide complexes are virtually restricted to collagen fibrils, from which they protrude laterally. They are in an orderly array, the mean distance between them being 642 A. Marker: 0.1 μ. × 22,000.

lagen is demonstrable (6, 14, 15), and an intimate association between collagen and polysaccharide has been deduced from biophysical data (16). This is, however, apparently the first demonstration of a 640 A period associated with intact preosseous cartilage collagen outside the articular areas and the zones immediately adjacent to bone. There are a number of possible explanations for this periodically arranged LYS-reactive material. Polysaccharides, or a species of polysaccharide with an affinity for LYS, may actually be arranged in this manner. The polysaccharides may be uniformly stretched on the surface of collagen, but arranged in 640 A segments which ravel up to leave areas bare of polysaccharide as they precipitate with LYS. Or, perhaps more likely, the LYS-polysaccharide complex ravels up around an insertion into an underlying regularity in the adjacent collagen. At present, there does not appear to be adequate information to choose among these or other possible interpretations.

The inability of exogenous LYS to form visible complexes in the hypertrophic zone, precisely the area where LYS is normally present in highest concentration (1), suggests that, as seems most likely, those sites which are potentially reactive with LYS are already occupied, or that LYS-polysaccharide complexes cannot form either because the polysaccharides are different in this area or because local conditions will not permit the complex to form. The amount of LYS normally present, even in the hypertrophic zone, is relatively small when compared to the amount added to the culture medium (1), so that densities formed by LYS-polysaccharide complexes in the native tissue would be difficult to differentiate from other matrix densities.

Finally, it should be pointed out that since lysozyme is a normal constituent of cartilage, the pattern in which it complexes with cartilage matrix may be a reflection of what its role in cartilage metabolism may be, particularly since it forms visible complexes with ossifying but not with nonossifying cartilage and because its sites of binding are anatomically so precise.

SUMMARY

Egg white LYS forms complexes with at least some cartilage polysaccharides which are visible by electron microscopy. These complexes are heterogeneously distributed in the tissue in patterns fitting some of the available chemical data, thus suggesting that LYS may be a useful histochemical tool for the study of cartilage ultrastructure.

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