THE FINE STRUCTURE OF BOVINE NASAL CARTILAGE

Extraction as a Technique to Study Proteoglycans and

Collagen in Cartilage Matrix

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ABSTRACT

Bovine nasal cartilage was studied by electron microscopy before and after extraction with 4 M guanidinium chloride or 1.9 M $CaCl_2$. These solvents removed matrix granules, basophilia, and 85% of the proteoglycan complex, measured as hexuronate. Simultaneously, many collagen fibrils were disaggregated into component microfibrils (approximately 40 A thick). In contrast to the above solvents, exhaustive extraction with 0.5 M guanidinium chloride removed 20% of the proteoglycan complex, and matrix granules were reduced in size but not in number. Extraction with 4 M $CaCl_2$ removed only 10% of the proteoglycan complex, did not remove matrix granules, and caused the normal banding pattern of collagen to disappear. The banding was restored by further treatment with trypsin. Trypsin, before or after 4 M $CaCl_2$, removed matrix granules and 90% of the proteoglycan complex.

We conclude that matrix granules are an electron microscopic representation of the proteoglycan complex, and consist of more than one proteoglycan macromolecule. It would appear that 4 M guanidinium chloride and 1.9 M $CaCl_2$, in addition to removing most of the proteoglycan complex, also disaggregate some of the collagen fibrils into their component microfibrils.

INTRODUCTION

The matrix of hyaline cartilage consists of two discrete macromolecular components: collagen, which constitutes the fibrillar matrix, and the interspersed proteoglycan complex (PGC).[1] Collagen and proteoglycan may each account for as much as 50% of the dry weight of hyaline cartilage, depending upon the type of cartilage analyzed (1).

Bovine nasal cartilage is the tissue from which PGC has been most extensively characterized by biochemical and physicochemical methods. The complex consists of noncollagenous protein cova-

[1] *Abbreviations used in this paper:* PGC, proteoglycan complex; PTA, phosphotungstic acid.

lently linked to chondroitin sulfate (2). From 65 to 85% of the chondroitin sulfate in cartilage can be extracted in the form of proteoglycans by disruption of the tissue in a rotating blade homogenizer operated at high speeds (3, 4). The linkage between protein and polysaccharide has been shown to consist of the neutral trisaccharide galactosylgalactosylxylose, which connects the terminal glucuronate residue of the chondroitin sulfate chain to the β-hydroxyl group of a serine residue in the polypeptide chain (5, 6, 7). The molecular weight of the proteoglycan was first estimated to be of the order of 4×10^6 by Mathews and Lozaityte (8). More recent work

has established that the proteoglycans are polydisperse, with molecular weights within the range 1.3×10^6 to 4.1×10^6 (9, 10). The molecules are highly hydrated and approximately spherical in shape (11). Although it has been known for some time that proteoglycans aggregate (8), it has only recently been realized that aggregation is mediated by a small amount of tenaciously bound glycoprotein (12). Aggregates have molecular weights of about 30×10^6 and contain about 13 subunit macromolecules (10). The biological significance of the aggregation phenomenon has not been established, but it has been suggested that aggregates are sterically constrained within the tissue, whereas subunits can freely diffuse (13). Aggregation of proteoglycans may therefore be necessary for maintenance of the chemical integrity of cartilage.

Electron microscopic studies of the matrix of a variety of cartilages (14–19) have revealed large numbers of small dense particles that have been termed matrix granules (19). These granules are sometimes associated with collagen fibrils and are usually linked together by delicate intergranular fibrils about 50 A wide. Matrix granules can be removed from cartilage matrix by digestion with either trypsin (19) or hyaluronidase (19, 20) and they thus appear to represent the proteoglycan complex. Matrix granules vary considerably in morphology, from spindle or stellate configurations (18) to more rounded shapes (19), depending on the type of cartilage examined. The maximum dimensions reported for these structures range from 100 to 700 A (14, 19).

This study utilizes electron and light microscopic techniques coupled with recently developed extraction procedures to study bovine nasal cartilage. Alterations of the structure of the matrix were observed when the tissue was extracted with solvents such as 4 M guanidinium chloride or 1.9 M CaCl$_2$, which have been found to extract efficiently and selectively from cartilage its proteoglycan complex (4). The results support the contention that matrix granules are the electron microscopic representation of the proteoglycan complex.

MATERIALS AND METHODS

Cartilage

Nasal septa was taken from 1 to 2 yr old cattle immediately after slaughter, cleaned of all non-cartilaginous tissue, including perichondrium, and sliced into uniform pieces about 0.5 mm thick (4). These slices were fixed either immediately or after extraction as described below.

Extraction

Slices were extracted at room temperature as summarized in Table I.

Guanidinium chloride and CaCl$_2$ solutions were buffered at pH 5.8 with 0.05 M N—morpholinoethane-sulfonic acid–NaOH. The trypsin used was a crystalline product from Worthington Biochemical Corp., Freehold, N. J., and was dissolved in 0.1 M NaCl–0.05 M Tris-HCl, pH 8.0. Guanidinium chloride was the Ultrapure grade from Mann Research Labs., New York.

Chemical Analysis

Proteoglycan complex was estimated as hexuronic acid. The Bitter and Muir modification (21) of the carbazole method of Dische (22) was used.

Light Microscopy

Cartilage slices were fixed in Lillie's acetic acid–ethanol-formalin solution (23) and stained with Lillie's trichrome stain (24).

Electron Microscopy

Cartilage slices were fixed for 1.5 hr at 0°C in 2.5% glutaraldehyde (Polysciences, Inc., Rydal, Pa.)

TABLE I

Extraction of Proteoglycan from Bovine Nasal Cartilage Slices

Solvents*	Time	Yield, per cent of total proteoglycan‡
0.5 M guanidinium chloride	24 hr	20
4 M guanidinium chloride	30 min	20
	1 hr	50
	24 hr	85
1.9 M CaCl$_2$	48 hr	85
4 M CaCl$_2$	24 hr	10
Trypsin, 0.2 mg/ml	24 hr	90

* 20 ml solvent was used per gram of cartilage slices.
‡ The extracted proteoglycan was estimated as hexuronic acid.
See the text for further details.

buffered at pH 7.0 with 0.2 M sodium cacodylate (25). The slices were then rinsed three times for 30 min with cacodylate buffer at 0°C, and cut into cubes 1 mm on a side. These fragments were postfixed at 0°C in Veronal acetate–buffered osmium tetroxide (26), dehydrated in graded alcohols and propylene oxide, and embedded in Epon 812.

Sections approximately 500 A thick were cut with Dupont diamond knives on an LKB Ultratome III. Sections were usually stained for 5 min with 3% aqueous uranyl acetate, then for 5 min with 5% lead citrate (27). In some cases the sections were stained for 30 min with 10% aqueous phosphotungstic acid (PTA).

Sections were examined with the Phillips EM-300 electron microscope.

Counting and Measuring of Matrix Granules

The size of the matrix granules in the intercellular matrix, and the number of these particles present per unit area, were measured in the following way. Five pieces of cartilage were processed for each measurement. One section of each piece of cartilage was examined in the electron microscope. An area of intercellular matrix in each section was photographed; intercellular matrix was easily recognized by the presence of very large collagen fibrils (see below). The five photographs representing each type of cartilage were divided into a total of 60 squares, 0.5 μ on a side. Squares in which matrix granules were counted and measured were selected by reference to a table of random numbers. The maximum dimension of the granules is reported as "size" and was determined with an optical comparator. The statistical significance of observed differences in the size and number of granules was analyzed with t and z tests.

RESULTS

Gross Alterations

Of the extractants used, only 4 M $CaCl_2$ caused any alterations of the dimensions of the unfixed cartilage slices. The slices treated with this solution shrank about 20% in linear dimensions and became quite brittle. Neither the glutaraldehyde nor the osmium fixative altered the linear dimensions of the slices. However, fresh or fixed cartilage shrinks about 20% during dehydration for Epon embedding. This change in linear dimensions corresponds to a decrease in volume of about 50%.

Light Microscopy

The unextracted tissue bound large amounts of the basic dye safranine O, especially in the pericellular (or territorial) matrix (Fig. 1). As the proteoglycan complex was extracted with 4 M guanidinium chloride (Figs. 2 and 3), safranine staining disappeared and uptake of fast green was noticed; both effects were most marked in the intercellular (or interterritorial) matrix. Fig. 3 represents an exhaustive extraction with 4 M guanidinium chloride; 15% of the original proteoglycan remains in the tissue (4). The residual basophilia is in the pericellular matrix, very closely associated with the cells.

Electron Microscopy

The chondrocytes appeared rounded or ellipsoidal (Fig. 4). The plasma membrane was folded into a moderate number of microvillous projec-

FIGURES 1–3 Bovine nasal cartilage slices fixed in acetic acid-ethanol-formalin and stained with safranine O, fast green, and hematoxylin according to Lillie (24). Magnification is 560 diameters in each case.

FIGURE 1 Untreated cartilage. Note the extreme avidity of the matrix for the basic dye safranine O. Basophilia is especially marked in the pericellular matrix. No uptake of fast green is evident.

FIGURE 2 Same cartilage after extraction for 1 hr with 4 M guanidinium chloride. Basophilia is now absent from the intercellular matrix and reduced in the pericellular matrix. Fast green is now taken up in the intercellular matrix, presumably by collagen. Hematoxylin staining is restricted to nuclei. Half of the total proteoglycan has been removed from the tissue. Fixation followed extraction with guanidinium chloride.

FIGURE 3 Same cartilage after extraction with 4 M guanidinium chloride for 24 hr. Most of the proteoglycan (85% of the total) has been removed; the extraction can be continued indefinitely without increase in yield. Basophilia is completely removed from both pericellular and intercellular matrix, but is still evident in the cells and in the margin of pericellular matrix closest to the cells. Hematoxylin uptake by nuclei is still evident. Fixation followed extraction.
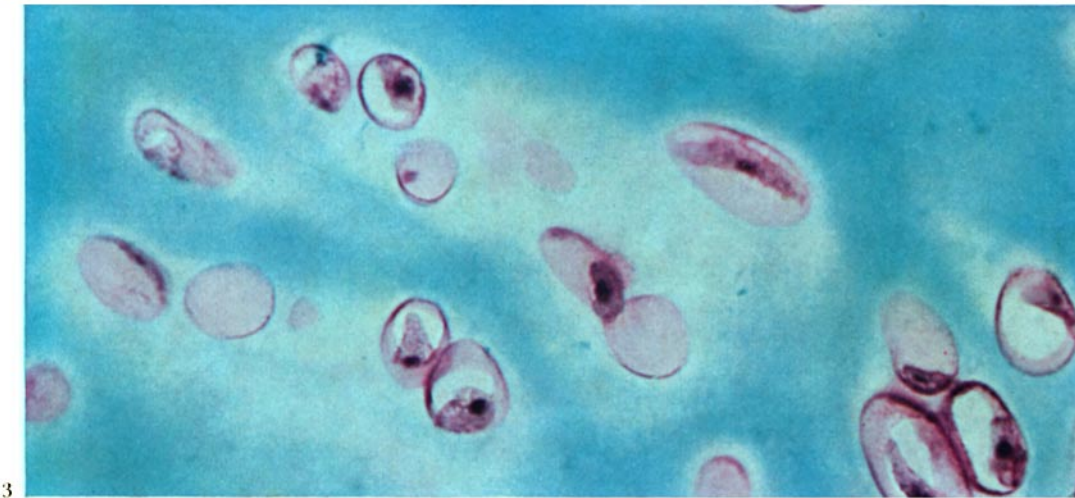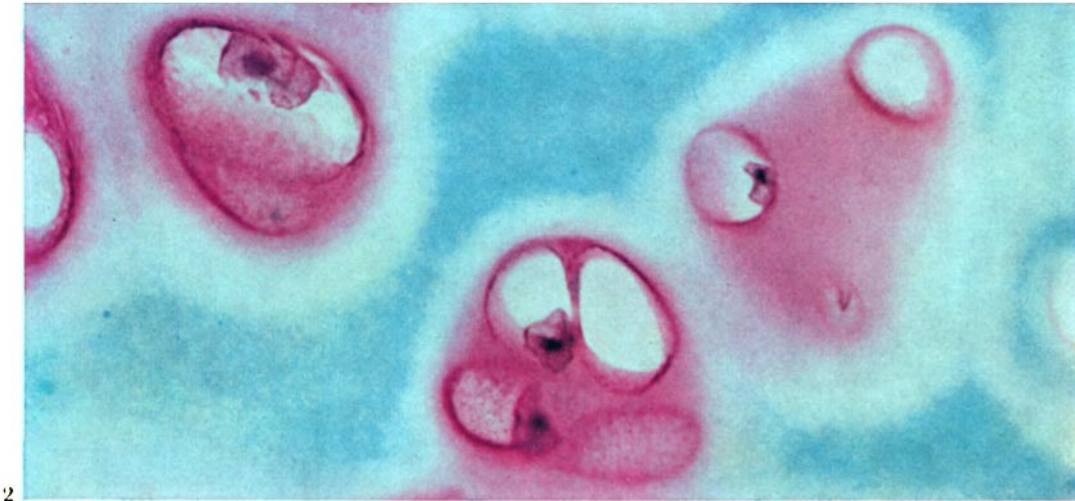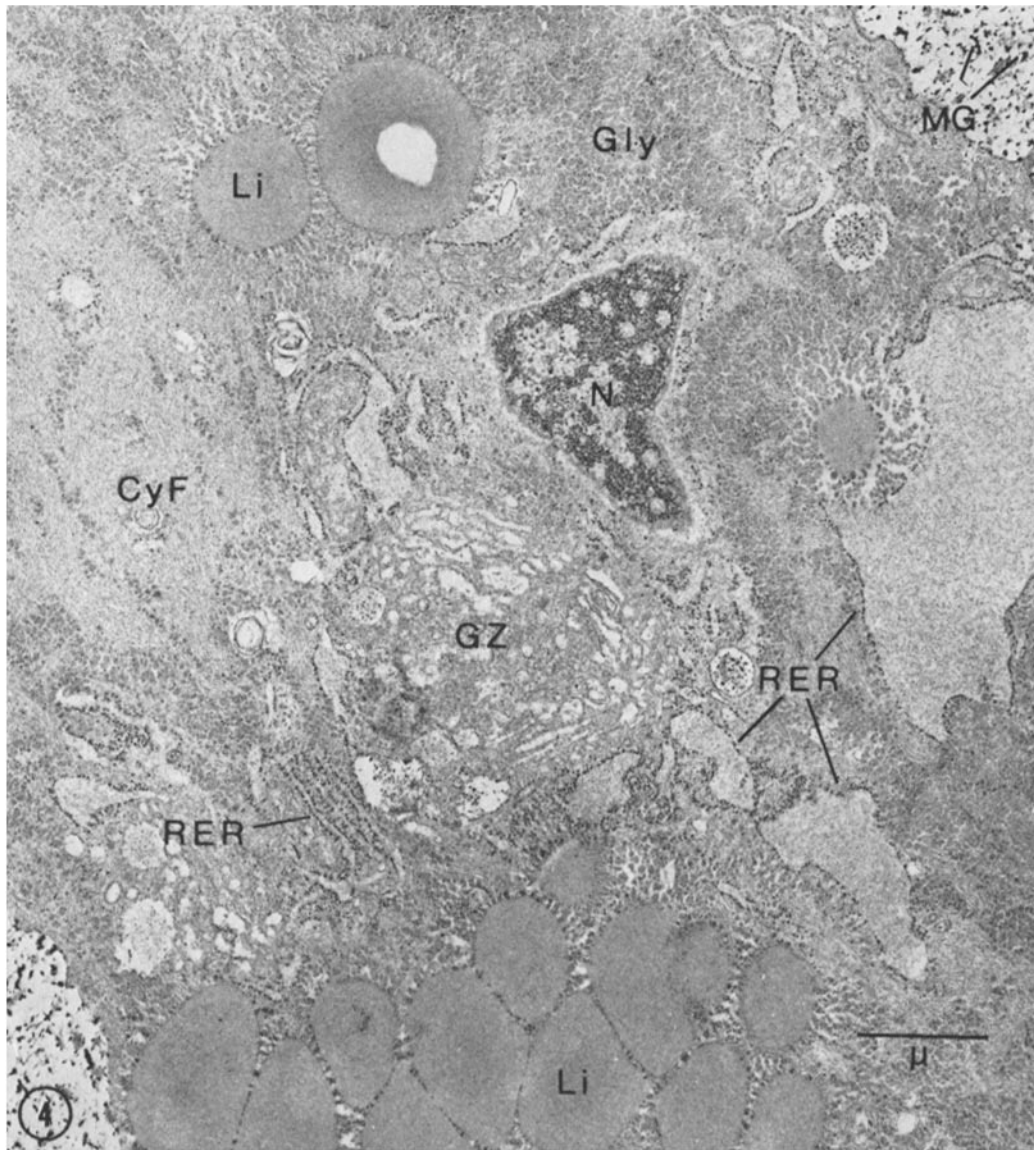
FIGURE 4 Electron micrograph of a portion of a chondrocyte in bovine nasal cartilage. The plasma membrane is seen at upper right and lower left. Note the pericellular matrix granules (*MG*). The cytoplasm contains a moderate amount of rough endoplasmic reticulum (*RER*) plus glycogen particles (*Gly*), lipid droplets (*Li*), and cytoplasmic filaments (*CyF*). A tangentially sectioned nucleus (*N*) is present with well-demonstrated nuclear pores. The Golgi zone (*GZ*) is prominent. × 17,100.

tions, and was often broken. Most conspicuous in the cytoplasm were numerous cytoplasmic filaments about 100 A wide and numerous coarse granules of glycogen (Fig. 5). The Golgi complex was prominent and frequently contained dilated vesicles enclosing small dense particles. A small amount of rough endoplasmic reticulum was present, and unattached ribosomes were not numerous. A few lipid droplets were noted in the cytoplasm. The nuclei were ovoid and usually contained a single large nucleolus.
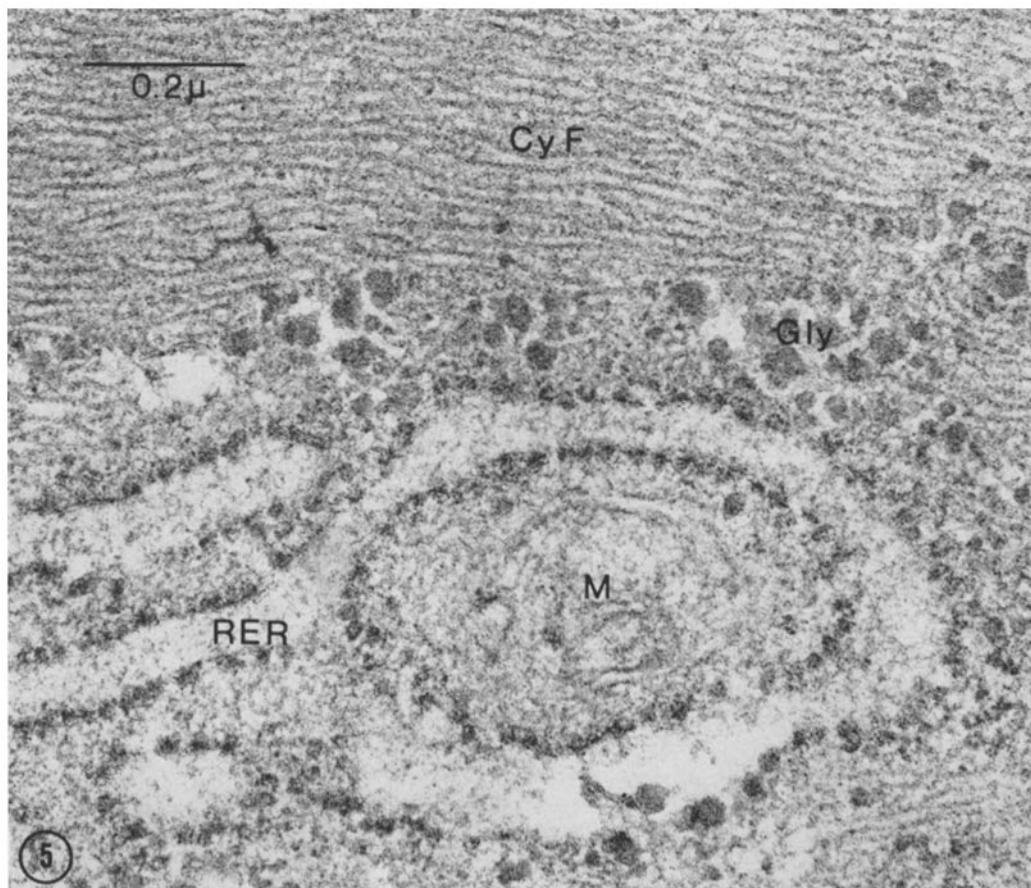
The matrix was composed mostly of collagen

FIGURE 5   Cytoplasm of bovine nasal chondrocyte illustrating in detail the characteristic cytoplasmic filaments (CyF) which measured approximately 100 A in diameter. Also shown is a portion of rough endoplasmic reticulum (RER) and a mitochondrion (M). Mitochondria were small and not very numerous in these cells. Gly, glycogen particles. × 108,000.

fibrils and matrix granules. Collagen fibrils were found throughout the matrix. In the intercellular matrix, collagen fibrils varied in width from 160 to 2900 A, whereas fibrils in the pericellular matrix were smaller and more uniform in width (150–250 A). The axial periodicity of collagen was easily demonstrated with PTA staining of the large fibrils of the intercellular matrix, but a characteristic staining pattern could not be distinguished in fibrils less than about 250 A wide. Matrix granules were abundant (Fig. 6). They ranged in maximum dimension from 50 to 1000 A, the larger sizes clearly representing two to three fused granules. The average granule size was 406 ± 185 A (Table II). They were usually rounded or ovoid, with one or more stellate projections. Fre-

quently granules appeared to be linked together by extremely fine intergranular fibrils, usually less than 50 A thick, which connected the projections of adjacent granules (Fig. 6).

Infrequently, clusters of matrix vesicles were observed between collagen fibrils of the matrix (28). These membrane-bounded extracellular particles varied from 400 to 3000 A in maximum dimension. Most vesicles contained a homogeneous substance of high electron opacity but were not associated with apatite crystals.

Effects of Extraction with 0.5 M Guanidinium Chloride

Extraction of bovine nasal cartilage slices with 0.5 M guanidinium chloride for 24 hr removes

Effects of Extraction on the Size and Number of Matrix Granules of Bovine Nasal Cartilage

Size of matrix granules, angstroms‡	Number of matrix granules per square micron§	Solvent	Probability limit for observed differences in number of matrix granules*			
			4 M CaCl₂	4 M guanidinium chloride		0.5 M guanidinium chloride
				60 min	30 min	
406 ± 187	144 ± 36	Unextracted	0.4-0.5	<0.01	0.2-0.3	0.02-0.05
306 ± 147	192 ± 88	0.5 M guanidinium chloride	0.01-0.02	<0.01	<0.01	
355 ± 150	128 ± 40	30 min 4 M guanidinium chloride	0.5-0.6	0.1-0.2‖		
360 ± 153	108 ± 36	60 min	0.02-0.05			
495 ± 251	136 ± 28	4 M CaCl₂				

* The values tabulated are the probabilities that the observed difference, or greater ones, could arise purely by chance.

‡ The values given are the mean and standard deviation. At least 500 granules were measured in each category.

§ The values given are the mean and standard deviation. A total of at least 500 granules in 15 different areas selected at random were counted in each category.

‖ For differences in the size of granules, in each case except this the probability that the observed difference is the result of chance variation is less than 0.01. In this case, the probability is 0.5-0.6.

20% of the proteoglycan, a value which does not increase if the extraction is continued for an additional 24 hr; in this regard the effect of 0.5 M guanidinium chloride is the same as that of other low ionic strength solvents such as 0.15 M KCl (29). Extraction with 0.5 M guanidinium chloride decreased the average size of the matrix granules to 306 ± 147 A but did not decrease the number of granules found per unit area (Figs. 6 and 7; Table II). There was in fact a significant increase in the number of granules from 144 ± 36 per μ^2 to 192 ± 88 per μ^2. This increase was probably a result of the fact that fewer clumps of granules were found after extraction (Figs. 6 and 7).

Effects of Extraction with 4 M Guanidinium Chloride and 1.9 M CaCl₂

Brief exposure of cartilage slices to 4 M guanidinium chloride reduced both the size and number of the matrix granules. After 30 min of extraction, which removed 20% of the hexuronate, the average size of the granules dropped to 355 ± 150 A, but after 1 hr of extraction, which removed 50% of the hexuronate, the size of the granules was not changed further (Table II). The number of granules decreased to 128 ± 40 per μ^2 after 30 min and to 108 ± 36 per μ^2 after 1 hr.

Extractions of the slices either with 4 M guanidinium chloride for 24 hr or with 1.9 M CaCl₂ for 48 hr removed 85% of the hexuronate and almost all of the matrix granules from the intercellular matrix (Figs. 8-10). The granules persisted to some extent in the pericellular matrix, a result which is in accord with the light microscopic finding (Fig. 3) that basophilia persists in the pericellular areas after extraction. Concomitant to the disappearance of matrix granules was the appearance of bundles of microfibrils about 40 A in diameter which were prominent in the intercellular matrix (Figs. 8-10). Continuity was often seen between the microfibrils and collagen fibrils (Figs. 8 and 9).

Effects of Extraction with 4 M CaCl₂

Extraction of cartilage slices with 4 M CaCl₂ for 24 hr removes less than 10% of the hexuronic acid, and most of the remaining proteoglycan complex cannot be removed by subsequent extraction with 4 M guanidinium chloride (13). In a sense, 4 M CaCl₂ acts as a fixative.

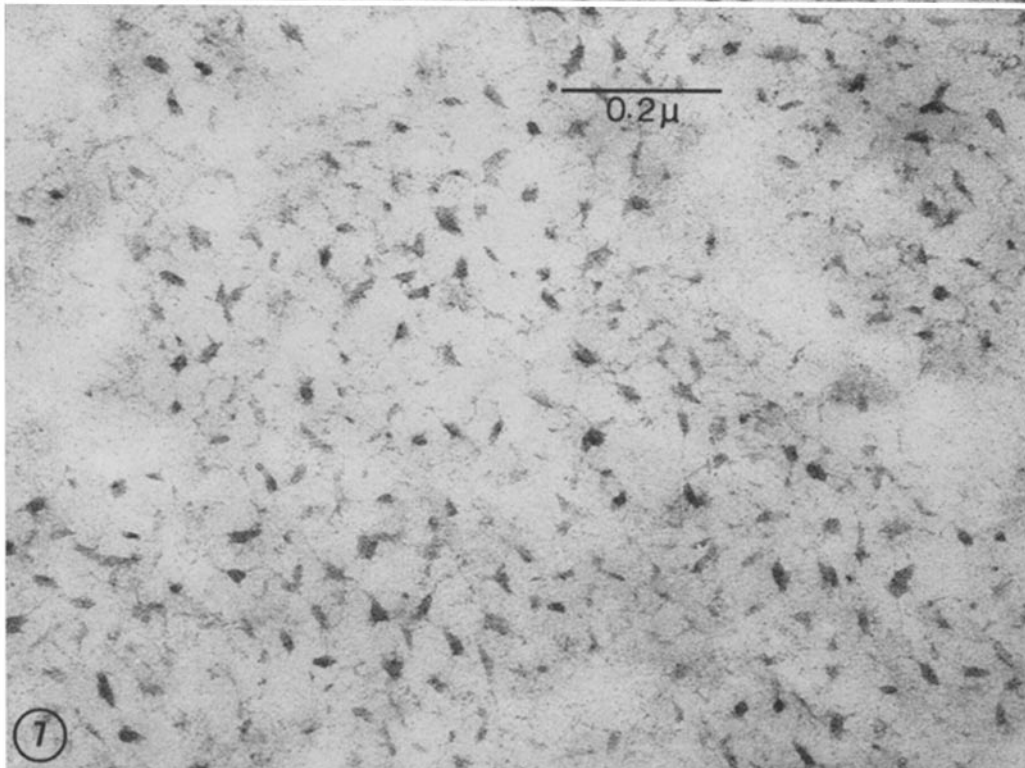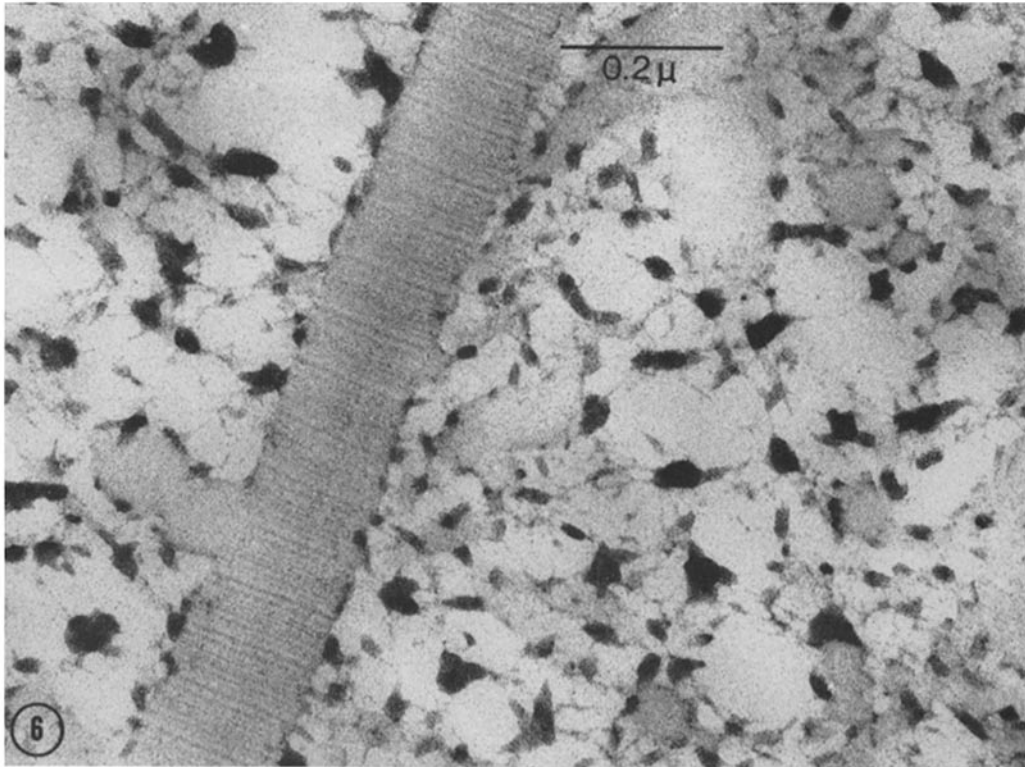Treatment with 4 M CaCl₂ alters both matrix

FIGURE 6 Intercellular matrix of untreated cartilage. Note the numerous, irregularly shaped, densely staining matrix granules. Many granules are linked together by delicate intergranular fibrils about 50 A in diameter. A collagen fibril with faint cross banding traverses the field at left. × 108,000.

FIGURE 7 Intercellular matrix after exposure to 0.5 M guanidinium chloride for 24 hr. (Compare to Fig. 6, which is reproduced at the same magnification.) Matrix granules are smaller and less dense than in Fig. 6, but not less numerous. × 108,000.

granules and collagen fibrils. The individual matrix granules increased in size to 495 ± 251 A after 4 M CaCl₂ treatment, and there appeared to be an increase in clumping of the granules. Although the dimensions of the granules increased, the number of granules per unit area remained essentially unchanged (Table II). Fig. 11 demonstrates the alterations of collagen fibrils brought about by 4 M CaCl₂. The axial periodicity characteristic of collagen is entirely absent even though the outlines of the fibrils can be recognized. Curiously enough, the axial periodicity of native collagen could be restored by digestion of the 4 M CaCl₂-treated tissue with trypsin (Fig. 12).

Effects of Trypsin

Trypsin removed 95% of the hexuronate and all the matrix granules, including those in the pericellular area. Unlike 4 M guanidinium chloride and 1.9 M CaCl₂ (see above), trypsin left no 40 A microfibrils in the tissue. The effect of trypsin was the same whether it was used alone or after 4 M CaCl₂.

DISCUSSION

Light Microscopic Results

The loss of basophilia from the matrix of cartilage extracted with 4 M guanidinium chloride reflects the loss from the tissue of 85% of its polyanions. The increased binding of fast green, on the other hand, is not as easily explained. A similar result was obtained in a previous study (30) in which sections of cartilage were digested with a proteolytic enzyme. Decreased avidity of the matrix for cationic dyes and simultaneous increase in acidophilia were noted. It was suggested (30) that the increased acidophilia was the

result of hydrolytic formation of free α-amino groups by the proteolytic enzyme. The present results suggest that the increased acidophilia was the result of loss from the tissue of polyanions which otherwise compete for the basic dye-binding sites in the tissue.

Composition of Matrix Granules

The results substantiate previous reports that matrix granules contain proteoglycan (19, 20). Agents which extract proteoglycans, such as trypsin, 4 M guanidinium chloride, or 1.9 M CaCl₂, also remove both basophilia and matrix granules. Little doubt can remain that these structures are an electron microscopic representation of the proteoglycan complex in the matrix of cartilage.

Matrix granules in a variety of cartilages contain a homogeneous granular material and are associated with very fine fibrils. The exact morphology of the granules (i.e., whether rounded, spindle shaped, or stellate) varies from tissue to tissue and may depend on the number of fibrils attached to each granule. The fact that the intergranular fibrils are removed by digestion with hyaluronidase (20) or trypsin suggests that they are similar in composition to the granules and does not support the suggestion (17) that they are immature collagen. It may be that these fibrils are the electron microscopic representation of the glycoprotein link (12), a minor constituent of the proteoglycan complex which is essential for the formation of aggregates of proteoglycans.

Although the matrix granule has been positively identified as the electron microscopic equivalent of the proteoglycan complex, the native state of the proteoglycans in cartilage has not yet been determined. Hydrodynamic measurements (9, 29) indicate that the aggregated proteoglycans

FIGURE 8 Intercellular matrix after exposure to 4 M guanidinium chloride for 24 hr. Matrix granules are no longer present. The collagen fibrils which traverse the field diagonally appear to disaggregate into component microfibrils. (A similar disaggregation was seen after extraction with 1.9 M CaCl₂.) × 108,000.

FIGURE 9 Intercellular matrix after extraction with 4 M guanidinium chloride for 24 hr. The field is similar to that seen in Fig. 8 but here stained with phosphotungstic acid (PTA) to enhance the banding of collagen fibrils. The loss of banding is apparent in the central portion of a large fibril traversing the field. This loss of banding probably reflects disaggregation. × 75,000.

FIGURE 10 Microfibrils of disaggregated collagen at high magnification. The microfibrils are about 40 A wide but are irregular in thickness and thus appear to be twisted or beaded. × 360,000.

Figure 8
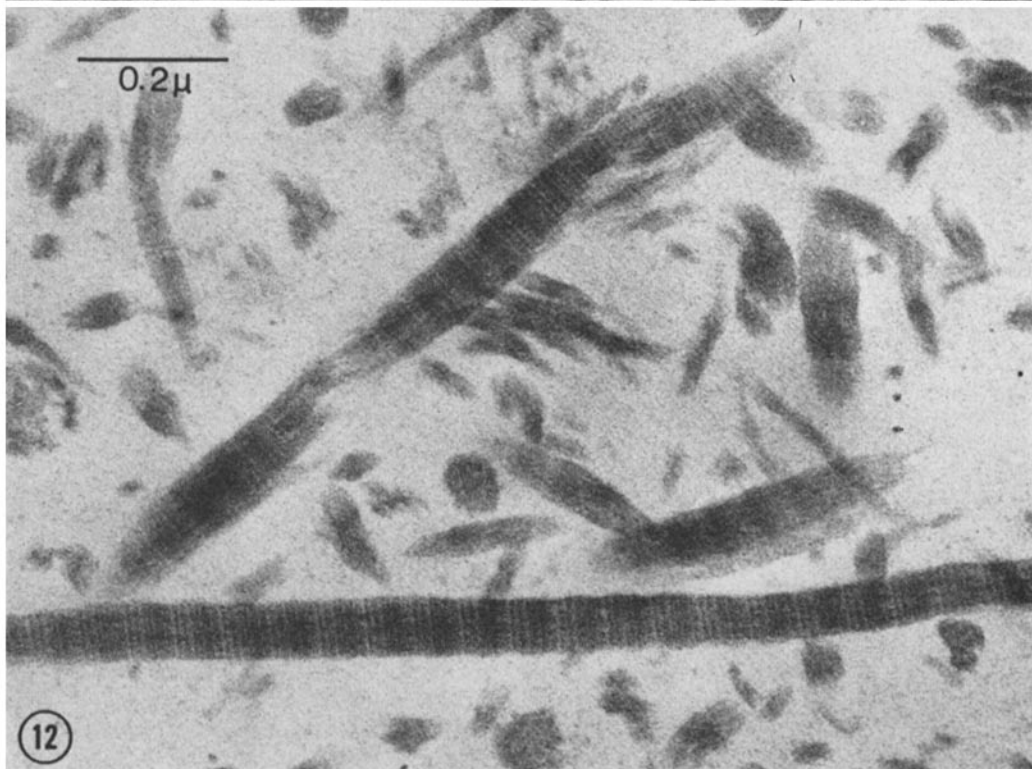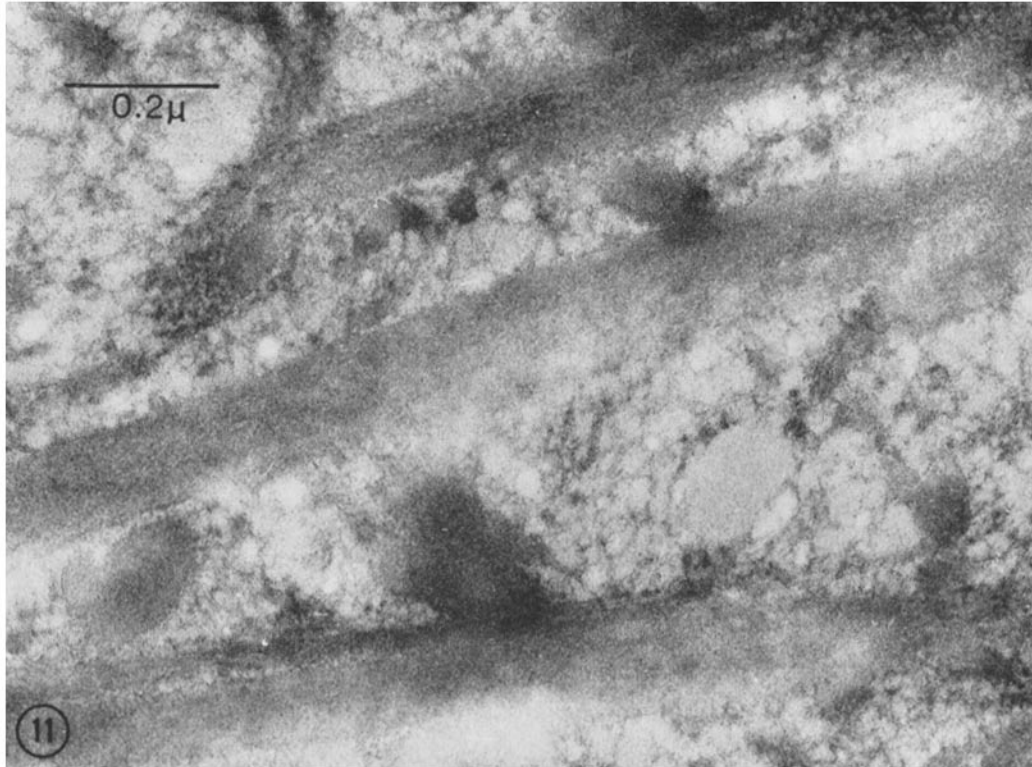
0.2μ

Figure 9

0.3μ

Figure 10

0.1μ
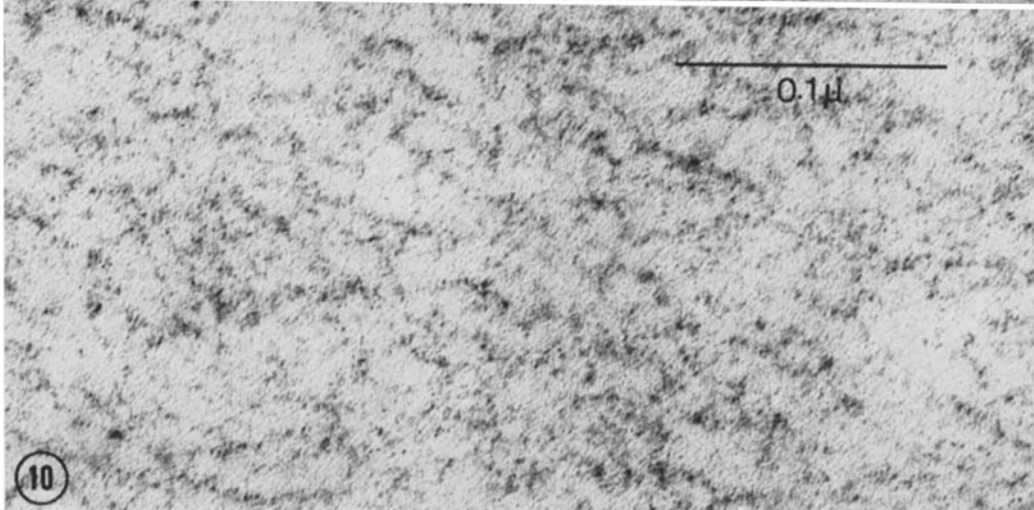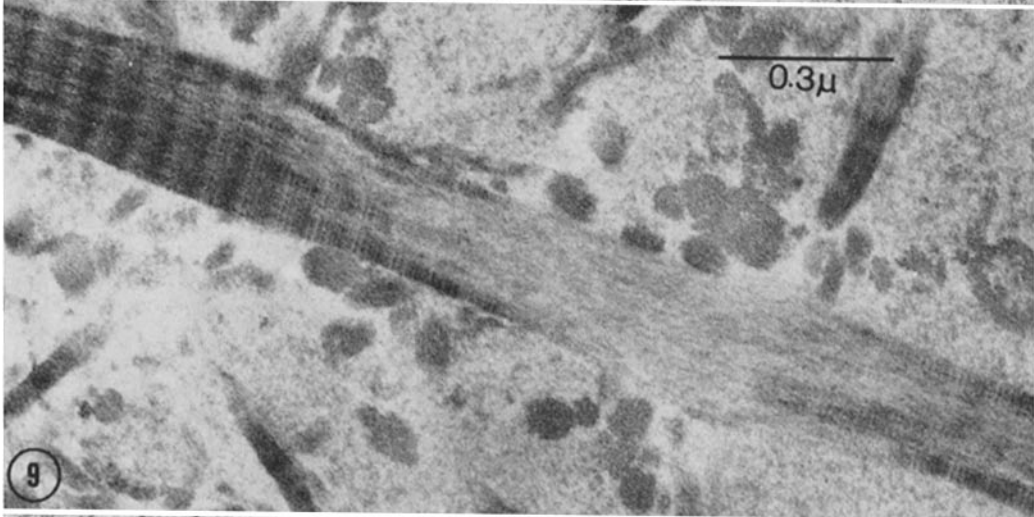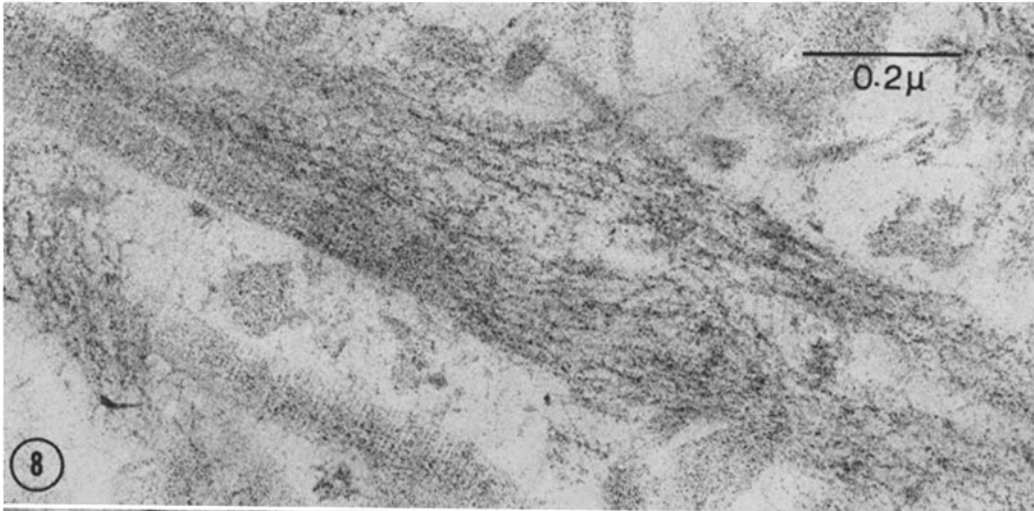
FIGURE 11 Intercellular matrix stained with PTA after extraction with 4 M CaCl₂. Collagen fibrils are homogeneous, smudged, and have lost their characteristic axial periodicity. × 100,300.

FIGURE 12 Intercellular matrix; comparable to Fig. 11 except that tissue slices were digested with trypsin after extraction with 4 M CaCl₂. Axial periodicity of the fibrils has been restored. × 100,300.

extracted from cartilage measured about 6000 A in length, a value which is considerably larger than the dimensions of a matrix granule. On the other hand, the same hydrodynamic measurements predict a volume for the proteoglycans in solution which is six times greater than the volume of the tissue from which it was extracted; the proteoglycans apparently swell considerably during extraction. Thus hydrodynamic techniques cannot be used to measure the dimensions of the proteoglycans as they are found in cartilage, although these techniques can at least in principle be used to obtain precise values of molecular weights. On the other hand, cartilage is reduced in volume by one-half during dehydration for embedding in electron microscopic studies (see above); this shrinkage very likely is a result of preferential loss of water from the hydrophilic proteoglycan complex. Thus electron microscopic studies yield a particle size which is probably smaller than that actually present in the untreated cartilage.

The fact that granules were reduced in size but not in number after extraction with 0.5 M guanidinium chloride implies that each matrix granule consists of more than one proteoglycan subunit. With this information in hand we can only conclude that the size of aggregates of PGC in cartilage lies between an upper limit suggested by hydrodynamic studies (6000 A) and a lower limit suggested by electron microscopy (406 A).

It is interesting that an exhaustive extraction with 0.5 M guanidinium chloride and a comparable limited extraction with 4 M guanidinium chloride produced different alterations in the matrix granules. In the former case there was a decrease in the size of granules without a decrease in their number, and in the latter case there were decreases in both the size and in the number of granules. These results can be explained with data obtained from a study of the centrifugal properties of proteoglycans in the extracts. The 0.5 M guanidinium chloride extract, when compared to a subsequent 4 M guanidinium chloride extract, contained fewer and smaller aggregates of proteoglycan (13). These results suggest that each matrix granule contains both aggregated and monomeric proteoglycans. The 0.5 M guanidinium chloride specifically extracts the monomers as well as some of the smaller aggregates, whereas 4 M guanidinium chloride extracts both aggregates and monomers. Thus the latter solvent can remove entire matrix granules and is not restricted to reducing the size of granules as is the case with 0.5 M guanidinium chloride.

Collagen Disaggregation

Microfibrils about 40 A wide were seen in cartilage matrix after extraction with 4 M guanidinium chloride or 1.9 M $CaCl_2$. Continuity was present between the microfibrils and collagen fibrils indicating that microfibril bundles probably represented collagen fibrils which had been partially disaggregated by the solvents. Similar microfibrils 30–50 A wide have been observed in lyophilized steer skin collagen treated with 0.2 M acetic acid (31). They appear to represent a basic structural unit of the collagen fibril.

Effects of 4 M $CaCl_2$

4 M $CaCl_2$ removes very little proteoglycan complex but causes the tissue to shrink, become brittle, and to be very easily liquified by digestion with papain at room temperature (13). We thus were led to suspect that at least one effect of 4 M $CaCl_2$ was to denature the collagen. This suspicion was substantiated by the observation that tissue treated with 4 M $CaCl_2$ had collagen fibrils without a discernible 640 A axial periodicity. Surprisingly, the native collagen banding could be restored to the tissue by treatment with trypsin, which implies that proteoglycans are necessary to maintain the changes in collagen induced by 4 M $CaCl_2$.

Unextractable Proteoglycan Complex

Exhaustive extraction with either 4 M guanidinium chloride or 1.9 M $CaCl_2$ leaves 15% of the proteoglycan in the tissue. This 15% can only be removed by more severe extraction, such as with trypsin or with alkaline solutions. The residual PGC appears to correspond to the pericellular basophilia seen in the light microscope and the pericellular matrix granules seen by electron microscopy after extraction with 4 M guanidinium chloride or 1.9 M $CaCl_2$. That residual PGC is equivalent to persistent pericellular basophilia is substantiated by our observation that trypsin removes the residual pericellular basophilia and matrix granules as well as most of the 15% of PGC remaining after extraction with 4 M guanidinium chloride.

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