Organization of Extracellular Matrix in Epiphyseal Growth Plate

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Three cations of varying size and charge density, egg-white lysozyme, protamine and ruthenium red, were used to stain the extracellular matrix of epiphyseal cartilage growth plate. With these stains, it was possible to distinguish three types of proteoglycans or materials associated with them, which may well have as their major differences the type of cross linking to the tissue. One type was stained by ruthenium red and protamine but not by lysozyme, was extractable with 3 M guanidinium chloride and was relatively uniformly dispersed throughout the matrix of the growth plate. The other two types were stained by all three cations, were not extractable with 3 M guanidinium chloride and were intimately associated with fibrils. One of these was found on the collagen fibrils, was relatively scanty in the resting zone near the articular surface, relatively restricted to the extralacunar area in the columnar zones and appeared to diminish in amount in the hypertrophic zone. This material often had a 640-Å periodic array on the surface of collagen fibrils. The third type also was stained by all three cations and was not extractable with 3 M guanidinium chloride. It was distinguished from the other class of lysozyme-reactive matrix components by the larger volume of distribution occupied by the stained material. It also had a different distribution in that it was widely dispersed in the resting zone, was restricted to the lacuna in the columnar zone and was absent in the hypertrophic zone. Thus, cartilage matrix as well as the chondrocytes undergo differentiation in the epiphyseal growth plate. (Am J Pathol 65:515–534, 1971)

AMONG THE CONNECTIVE TISSUES, cartilage has a low ratio of cells to extracellular materials, including those which are polysaccharide in nature. It has, therefore, been used by morphologists, histochemists, biochemists and biophysicists as the prototype tissue for the study of the so-called ground substance of connective tissue. Aside from the cytology of chondrocytes, electron microscopic studies of this tissue have often been disappointing, primarily because routine methods of fixation and embedding demonstrate only a small amount of extracellular polysaccharide-rich material. This problem arises for a number of reasons, including the fact that some polysaccharide is lost from the tissue during fixation.[1] More recently, a number of cationic materials

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have been used with variable success to precipitate the highly charged anionic glycoproteins and proteoglycans in the tissue.[2-4] The ruthenium red (RR) method adapted for electron microscopy by Luft[5] appears to be among the most promising of these. We have used larger cations such as the small proteins, lysozyme (LYS) and protamine (PR).[6,7] These proteins have certain disadvantages, including poor diffusibility into the tissue and denaturation by fixatives, which can be circumvented by using them as vital stains in short-term organ culture. This is a detailed report of our observations comparing the ultrastructure of the epiphyseal growth plate of mammalian cartilage as visualized using these three stains, RR, LYS and PR.

Because of the confusing nomenclature for polysaccharide-containing materials, the following terminology will be used in this communication:

Glycosaminoglycan: carbohydrate polymers composed of repeating dimers of monosaccharides, one of which contains an amino group (synonym: acid mucopolysaccharide).

Proteoglycan: an ordered complex between several glycosaminoglycan units and a protein backbone (synonyms: protein polysaccharide, mucoprotein).

Glycoprotein: a protein, other than a proteoglycan, to which a sugar chain is attached.

Materials and Methods

Tissues

Epiphyseal growth plate from two sources were used, the scapula of young puppies, 3–6 weeks old, and the femurs of 16-day mouse embryos or newborn mice. Puppy tissue was stained with RR or LYS or studied after routine fixation with glutaraldehyde. Some blocks of puppy scapula were stained with RR after 18 hours extraction in 3 M guanidinium chloride.[8] Mouse tissue was stained with RR, LYS, LYS altered by UV irradiation or PR, or studied after glutaraldehyde fixation.

Staining Procedures

Ruthenium Red. This was done according to the procedure of Luft.[5] The tissue was then dehydrated through alcohols and embedded in Epon 812. Thick (1-μ) sections were cut and stained with borax methylene blue. Ultrathin sections were then cut and stained with uranyl acetate and lead citrate before being examined in the electron microscope. The procedures for embedding and staining of sections were the same with all the staining technics.

Unstained Tissue. Small fragments of tissue were fixed for 4 hours in cold 5% cacodylate buffered glutaraldehyde, washed overnight in buffer and embedded. A similar schedule was used for all the subsequently described stains.

Protamine. Fragments of tissue were cultured overnight in tissue culture medium containing 1 mg/ml PR or fluorescein-labeled PR.[9] They were then fixed and embedded.

Lysozyme. Fragments of tissue were cultured overnight in 1 mg/ml crystalline hen's egg white LYS or fluorescein-labeled LYS, then fixed and embedded.[9] In a few experiments, LYS was first altered by ultraviolet irradiation before being added to the tissue culture medium.[9]

The Nature of the Staining Reactions

RR, LYS and PR all are cations known to form complexes with anionic polyelectrolytes of cartilage matrix, which are presumably glycoproteins and proteoglycans. RR stains tissues rich in proteoglycans or other polysaccharides [5] and is also occasionally capable of staining some intracellular materials.[10] We have applied chondroitin sulfates A, B and C, heparitin sulfate, keratan sulfate and hyaluronic acid to cellulose-acetate strips and stained them with RR. All these glycosaminoglycans were stained except hyaluronic acid. The use of fluorescein-labeled LYS and PR has clearly shown that the complexes seen in the electron microscope after vital staining contain these proteins which, as will be seen, do not bind at identical sites.[9] PR, unlike LYS, induces changes in the growth rate and histology of embryonic cartilage, which complicates interpretation of findings.[7] In order to function as a stain in this manner, the LYS molecule must be intact, since, as will be described later, UV irradiation results in considerable loss and alteration of its ability to complex with the matrix.[9] That LYS and PR complex with the anionic polysaccharides of cartilage is indicated by three lines of evidence. In the test tube, LYS is known to form dissociable complexes with glycosaminoglycans of cartilage and their parent proteoglycans.[11] Second, after incubation in media containing LYS or PR, cartilage matrix loses its metachromasia in the areas where fluorescein-labeled LYS or PR are bound.[9,12] Third, an experiment was done in which 18-day mouse embryonic femur cartilage was digested with hyaluronidase, papain or collagenase for 4 hours and then cultured for 8 hours in medium containing 1 mg/ml of fluorescein-labeled LYS. Tissue digested with hyaluronidase or papain, but not collagenase, lost its ability to bind the labeled LYS. Interpreting LYS reactivity with matrix components is rendered somewhat more difficult by the fact that complexes with these compounds have been found, at least in amounts sufficient to be histologically demonstrable by routine stains, only in those types of cartilage containing an epiphyseal growth plate; the reasons for this are thought to be related to the varying nature of the matrix in the different sites.[9,12] However, the use of fluorescein-labeled LYS, which is a much more sensitive technic, demonstrates that complexes with LYS do form in the tissues where such complexes are not otherwise demonstrable.

Results

The morphology of cartilage from puppy scapula and mouse femur was essentially similar and so the tissues will be described together.

Matrix and Cell-Matrix Relationships

Glutaraldehyde-Fixed Tissue Unstained with PR, LYS or RR

Tissue processed in this way appears essentially as others have described it.[13-16] Optically, the matrix was relatively empty (Fig 1). Small, electron-dense droplets were scattered in the matrix, often on the surface of collagen fibrils which were prominent only outside the lacuna. These droplets, which apparently contained proteoglycans, were leaf-

like in configuration, as described by others.[14] The collagen fibrils were narrow, randomly arranged and lacked a 640-Å axial period. They were wider in the puppy than in the mouse. Beginning in the lower columnar zone and extending into the hypertrophic zone, the collagen fibrils became more crowded and began to be aligned in a more parallel arrangement to each other. Calcification began in the perpendicular connective tissue septa between columns of cells, never involved the septa separating cells within a given column, was often associated with small cytoplasm-derived vesicles [17,18] and appeared unrelated to collagen fibrils.

Lysozyme

In the columnar zone, the chondrocyte lacuna, which had seemed relatively empty in glutaraldehyde-fixed routinely embedded tissue, was largely occupied by dense material (Fig 3). This material, which is considered to represent a LYS-matrix complex, graded off toward the lateral edge of the lacuna. The lateral edge of this material was further from the chondrocyte plasma membrane in the resting zone than lower (closer to bone) in the growth plate where it had a more abrupt margin (Fig 2). Indeed, in the upper (closer to the articular surface) resting zone in contrast with the columnar area, there was little morphologic distinction between the lacunar and extralacunar spaces (Fig 2).

In the matrix outside the lacuna of the columnar zone, which was richer in collagen than the lacunar area, dense deposits were restricted to the surface of the collagen fibrils. In the mouse, these deposits were rather irregularly arranged but occasional short stretches were found in which they were 640 Å apart. In the puppy, there was a very regular arrangement of these deposits at 640-Å intervals, with the deposits measuring about 200 Å in width. They could be seen coating the filaments which had aggregated laterally to form the fibril, and protruded into the surrounding matrix (Fig 4). Except for the collagen and its associated droplets, the remainder of the extracellular space appeared virtually empty (Fig 4). LYS did not stain the lacuna of the hypertrophic zone.

UV-Irradiated LYS

There were only two places where electron-dense material not seen in routinely prepared material was noted. One was in the chondrocyte lacuna at the plasma membrane, where they formed projections at irregular intervals from the chondrocyte surface. The other was within

the chondrocyte, where material of similar density was sequestered within vacuoles which appeared to be lysosomes (Fig 5). Alteration of the LYS molecule thus changed its function as a "stain," since altered LYS seemed to be ingested by cells and combined less with the matrix than the unaltered molecule.

Protamine

This procedure was used only on femurs of mouse embryos, and a report of the findings has been published.[7] Unlike the response to LYS, short-term culture in the presence of PR resulted in a virtual arrest in growth of the cartilage, apparently due to an arrest of matrix synthesis.[7] In general, the tissue morphology was similar to that seen with LYS staining, with four major differences. (1) The cells were more closely crowded together. (2) The hypertrophic zone was stained PR. (3) The area stained in the lacuna had a more complex arrangement of deposits in that two types of morphologic pattern were seen. One was a coat on an underlying fibril, presumably collagen. The other was in the form of small crowded droplets (Fig 6). (4) Also, where the PR-matrix complex covered fibrils in the extralacunar area, it formed a uniform coat of dense material, in contrast to the periodic array of dense material seen when LYS was used (Fig 7).

Ruthenium Red

Since RR does not uniformly stain deeper parts of tissue blocks, only superficial portions were cut. The lacuna of the columnar zone in RR-stained cartilage, as with LYS and PR staining, was largely filled with electron-dense material. As with LYS staining, the differentiation between lacunar and extralacunar spaces was less marked in the resting zone (Fig 8–10). The RR-stained extracellular material had a different appearance from that seen in LYS-treated cartilage in the lacunar area, but was similar to that seen in PR-stained tissue in that the stained material in the lacuna had two forms. One form was arranged in a narrow linear array extending outward toward the edge of the lacuna, as though it had precipitated around a central rigid structure. A second form consisted of small punctate granules found between the linear arrays. In the resting zone, the fibril-like material and the granular punctate deposits were distributed fairly homogeneously (Fig 9–11) in contrast to the columnar zone where the linear deposits were virtually restricted to the lacuna. RR also stained the hypertrophic zone. Here, particularly close to the zone of calcification the stainable material, because it lacked the lacunar linear material, was much more uniform

than that in the columnar zone (Fig 11). Outside the lacuna of the columnar and hypertrophic zones, as with LYS, droplets staining intensely with RR were arranged along the surface of collagen fibrils, with the intervening matrix containing the smaller punctate granules. Deposits on the collagen were often regularly arranged with about a 640-Å periodicity in puppy scapula but with a much less regular arrangement in the mouse femur.

Puppy scapula extracted with guanidinium chloride (GuCl) and stained with RR showed surprising persistence of matrix morphology. The small, punctate granular deposits were everywhere absent. Densely stained material persisted in the lacunar and extralacunar areas of the resting zone, and the lacunae of the columnar zone (Fig 12 and 13) but the lacunae of the hypertrophic zone were emptier (Fig 15). There was much more of the persisting material in the resting zone, where it virtually filled the lacuna and was scattered in small foci in extralacunar areas. In the columnar zone, it was restricted to the lacuna (Fig 14). The persisting lacunar material was no longer arranged in a linear pattern but as a dense agglomerate perhaps because an underlying fibril had been altered by GuCl. Outside the lacuna, collagen-associated deposits in periodic array along collagen fibrils remained (Fig 15). These deposits, however, seemed more frequent in the columnar than the hypertrophic zone and were virtually absent in the resting zone (Fig 16).

Discussion

Proteoglycans

Current concepts of the molecular composition of cartilage-matrix acid polysaccharides are that they are in the form of proteoglycans in which glycosaminoglycan chains are attached to a central or core protein by glycosylserine linkages [19] and extend laterally as nonrigid, unbranched chains from the central polypeptide chain.[20] The glycosaminoglycan chains are not believed to form lateral chemical bonds with each other and more than one type of glycosaminoglycan may attach to a single protein.[20–25] The classification of these proteoglycans is largely based on analyses done on tissues extracted by two methods. One, the so-called disruptive method, employs aqueous extraction and has yielded one heavy fraction termed PPH (for protein polysaccharide, heavy) and six lighter fractions termed PPL (for protein polysaccharide, light) 1 through 6.[20–24] Of these, PPL3 and 5 are by far the most abundant, at least in nasal septum.[20–24] These fractions are

not all chemically distinct since the two major ones are considered to be separable partly on the basis of their degree of molecular aggregation.[20-24] More recently, Hascall and Sajdera have extracted cartilage from nasal septum with high-molarity salt solutions, especially GuCl, which are termed dissociative solvents.[8,25] Using this technic, they extract a single species of proteoglycan, termed proteoglycan subunit or PGS, and several glycoproteins. One of these glycoproteins, termed link glycoprotein, is considered to cross-link proteoglycans to form larger aggregates, termed protein-polysaccharide complex, or PPC.[20,25] Very recently, Rosenberg *et al*, on the basis of chemical, physical and electron microscopic evidence,[20,21] have attempted to reconcile these two views. They believe PPL3 of the older terminology, represents monomers and dimers of a fundamental proteoglycan unit, while PPL5 is considered to be a larger aggregate formed by the attachment of similar molecules to a central-link glycoprotein.

To properly correlate these biochemical concepts with the morphologic observations presented here, it is important to recall the following: data on GuCl-extracted cartilage has thus far been published only for nasal septum; in that tissue only about 85% of the mucoproteins are extractable with this procedure; and these concepts are based on analyses of the extracted material. Such a correlation must also consider the nature of the staining reactions used. Although RR complexes with materials other than proteoglycans, including some glycoproteins,[5,10] we conclude from evidence presented earlier that the bulk of the RR-stained material is indeed glycosaminoglycan moieties of proteoglycan. This conclusion is supported by a simple experiment in which solutions of proteoglycan subunits (PGS of Hascall and Sajdera), protein-polysaccharide complex (PPC, *ie*, PGS aggregated by link glycoprotein) and mixed glycoprotein fractions of Hascall and Sajdera [25] were each mixed with the staining solution of RR. Only those solutions containing proteoglycan (PGS and PPC) formed visible precipitates. In addition, metachromatic stains of sections of paraffin-embedded cartilage that had been extracted with GuCl showed persistent metachromasia only in areas where RR staining was seen in the electron microscope.

It is possible to distinguish three morphologic patterns of material stained by RR. One is present as droplets on the surface of collagen fibers in periodic array. A second is present as punctate granules and is not obviously associated with collagen and has a punctate, granular form. A third morphologic variant is found as a dense coat on narrower

fibrils, which are presumably also collagen, but with a lesser degree of lateral aggregation. These three types of materials have different distributions in the three zones of cartilage. The punctate, noncollagen-associated material is rather uniformly distributed in all three zones. However, the two types of fibril-associated material are arranged differently in the three zones. The one associated with small fibrils on which it forms a relatively uniform coat is diffusely scattered throughout the resting zone, concentrated in the lacunae of the columnar zone and lost in the hypertrophic zone. The one associated with larger collagen fibrils and arranged in periodic array is scant in the resting zone, and is extralacunar in location in the columnar and lower zones. Extraction with 3 M GuCl for 18 hours results in removal of the punctate material not associated with fibrils, at least as assessed morphologically. The punctate granular material would therefore seem to correspond to the GuCl-extractable material characterized by Hascall and Sajdera, while the remaining two materials stained by RR represent materials more tightly complexed to the tissue and as yet uncharacterized. It is unlikely that the altered staining after extraction can be explained by GuCl changing materials so that they can no longer react with RR, since the GuCl-treated tissue was thoroughly washed before it was stained. The data of Hascall and Sajdera [8,25,26] on GuCl extracts of nasal septal cartilage strongly suggest that the denaturing effects of this procedure on extractable glycoproteins and proteoglycans are completely reversible when the GuCl is dialyzed out. Even if significant irreversible denaturation of the nonextracted proteins occurred, there is no reason to suspect that translocation or alteration of carbohydrate moieties, which are those apparently responsible for RR reactivity, occurred during extraction.

A comparative biochemical study of the glycosaminoglycan moieties of proteoglycans extractable or nonextractable with GuCl, from each of the cartilaginous zones, has recently been completed in this laboratory, using the methods of Antonopoulos *et al.*[27,28] In these methods, papain digestion of the tissue, which releases glycosaminoglycans from proteoglycans, is done. The glycosaminoglycans are then precipitated with cetylpyridinium chloride on a cellulose column and the molecular species of chondroitin sulfates are then eluted with increasing salt concentrations. Our data indicate that the types of glycosaminoglycans extractable and nonextractable with GuCl that are obtained from epiphyseal cartilage are somewhat different and that, unlike nasal cartilage, only about 50% of the glycosaminoglycans of epiphyseal cartilage are extracted with 3 M GuCl in 24 hours.[28] It is these unex-

tracted glycosaminoglycans which presumably stain with RR in tissue extracted with GuCl. Still unresolved are the problem of whether these glycosaminoglycan units are attached to a protein moiety identical to that of the extractable ones and also the nature of the linkage to other matrix components.

The LYS-reactive materials are of interest not only for comparison with those reacting with RR and PR, but because LYS is a normal constituent of cartilage matrix and its sites of binding to the matrix may be a reflection of its physiologic function. In this context, it may be important that, at least in chick embryo cartilage, LYS is most concentrated in the lacunar area, the site of densest staining by exogenous LYS.[29] When used as an cationic stain on mammalian epiphyseal cartilage, LYS did not complex with the material extractable with GuCl since it formed complexes only with material in the lacuna of the resting and columnar zones and on the surface of collagen, areas where RR staining persisted after extraction. Since GuCl extracted almost all the RR-reactive material in the lacuna of the hypertrophic zone, this would explain why LYS did not stain the lacuna in this area.

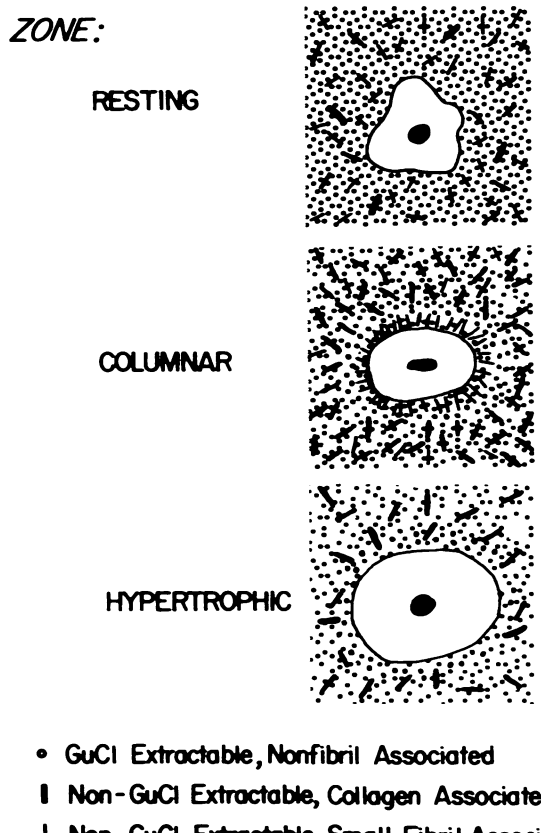
To confirm this morphologic interpretation, epiphyseal cartilage was extracted for 24 hours with 15 volumes of 3 M GuCl. The extract was dialyzed against water and aliquots of the dialysate incubated with equal volumes of RR, LYS and PR dissolved in solutions identical to those used for tissue staining. After 2 hours, obvious precipitates had formed with RR- and PR-containing solutions, while the LYS-containing solution had a barely perceptible precipitate. Thus, LYS was less effective in precipitating the GuCl-extractable matrix material than RR or PR. Electron microscopy of GuCl-extracted cartilage stained with LYS was not done. However, some tissue blocks from columnar zone were stained with fluorescein-conjugated LYS [9] and examined by fluorescence microscopy. Fluorescent material was easily seen in the lacunar area, further evidence that this type of LYS-reactive material was related to the RR-reactive material not extracted by GuCl. There are two reasonable interpretations of why the distribution of LYS staining corresponds to that of the material stained by RR but resistant to GuCl extraction. Either they are the same materials or different ones that have in common their location and resistance to GuCl extraction. The choice between these two alternatives depends on the nature of the reaction between LYS and RR and cartilage matrix. There are only two known candidate groups of polyanionic compounds present in this tissue which could complex with LYS, proteoglycans and glycoproteins. The evidence that RR stains proteoglycans has already been alluded

to. The loss of LYS reactivity after digestion with hyaluronidase and papain, but not with collagenase, as well as the known ability of LYS to precipitate proteoglycans [11] argues that it is with them that LYS has also complexed. However, these enzymatic digestions may well result in loss of other materials from the tissue, and the complex between LYS and proteoglycans is apparently formed by a salt linkage.[11] A rigorous argument cannot, therefore, be made for either alternative. The evidence, however, is strong that LYS functions as a stain by complexing with proteoglycan or some intimately associated compound such as a glycoprotein. The distribution and resistance to GuCl extraction strongly suggests that they are identical to or intimately associated with the matrix components stained by RR and resistant to GuCl extraction.

PR, because it alters the tissue, is less valuable as a stain. Despite this drawback, however, it does demonstrate three types of anionic materials, with a distribution and morphology approximating those seen with the other stains and therefore presumably identical or closely related. As with RR staining, one type was granular, another arranged as fine fibrils and the third coated collagen fibrils. The nature of the reactions of PR with tissue polyanions is not well known. However, its ability to combine with at least one glycosaminoglycan, heparin, is so widely known that it is used clinically in the treatment of overdosage with this anticoagulant.

The morphologic data presented here together with available biochemical data [28] argue for the structural organization of cartilage ground substance being composed of proteoglycans with at least two types of cross linking. The first is between proteoglycan subunits joined by the link glycoprotein of Hascall and Sajdera. Material aggregated in this manner would correspond to the punctate material stained by RR and PR, but not LYS, and extractable in 3 M GuCl. The second is between proteoglycans and collagen. Here the linkage is stronger, perhaps covalent since it is not disrupted by 3 M GuCl and this reagent does not disrupt covalent bonds. This would correspond to the material periodically arrayed on the surface of collagen stained by all three cations and not extracted by GuCl. The third material is also stained by all three cations and resistant to GuCl extraction. It has a fibrillar appearance when stained by RR or PR, perhaps because it forms a coat on an underlying fibril. But the available data are inadequate to determine if its binding to the tissue, although strong enough to resist extraction, is different from that of the second. The distribution of these three materials varies in each of the zones of the growth plate as illus-

trated in Text-fig 1. Thus, the extracellular matrix as well as the cells of the epiphyseal growth plate undergo progressive differentiation.



TEXT-FIG 1—This diagram illustrates the overall organization of proteoglycans in cartilage growth plate, as deduced from the data presented. The GuCl-extractable material is fairly uniform in distribution. The collagen-associated material not extractable with GuCl is scanty in the resting zone and extralacunar in columnar and hypertrophic zone. The other material not extractable with GuCl, which is arranged in a fibrillar pattern and hence presumably bound to an underlying fibril, is scattered in the resting zone, restricted to the lacuna of the columnar zone and seems to be absent in the hypertrophic zone. In the diagram, the central dark area represents the nucleus of a chondrocyte; the clear zone surrounding it, chondrocyte cytoplasm and the surrounding area, intercellular matrix.

Among other things, these data suggest the loss of a specific proteoglycan in the hypertrophic zone. Support for this is provided by immunocytochemical and chemical observations, which have yielded similar conclusions.[30–32] Also in keeping with this are data indicating a lower anionic charge density near the cartilage-bone junction of epiphyseal growth plate.[33]

Collagen

In areas other than the resting zone, the extralacunar matrix seemed different from the intralacunar matrix with regard to both of its major macromolecular components, collagen and proteoglycans, and their interrelationships. Well-formed collagen fibers were much more abundant outside the lacuna, suggesting that their aggregation is completed outside the lacuna. It may be that since collagen and polysaccharide synthesis are dissociable,[34] specific enzymes are concentrated outside the lacuna. It seems more likely that environmental conditions outside the lacuna are more conducive to the lateral aggregation of collagen molecules to form a fibril. A possible explanation for the lack of 640-Å axial periodicity in cartilage collagen fibrils is that they are simply too narrow to show this period. The lack of 640-Å periodicity of collagen-associated polysaccharide in mouse cartilage may have been related to a narrower width of collagen fibrils in this species. Whether this difference is age or species related cannot be determined from our data. The question of whether the reason for the periodic arrangement of polysaccharide on collagen is related to a specific molecule-to-molecule relationship between collagen and acid polysaccharide or to a uniformly stretched coat of proteoglycan on the collagen surface which ravels up around a specific site in collagen cannot be answered with certainty. There is both morphologic and biophysical evidence for a collagen-proteoglycan relationship in many connective tissues.[35–38] We suspect that the first of these two possibilities is correct, since, in PR-stained collagen, a uniform coat of stainable material was found on the surface of the collagen, suggesting that a particular species of LYS-reactive polysaccharide was arranged in this precise manner. Unfortunately, PR also induces other effects on cartilage besides simply complexing with proteoglycans [7] and the nature of the reactants in the tissue may have changed considerably.

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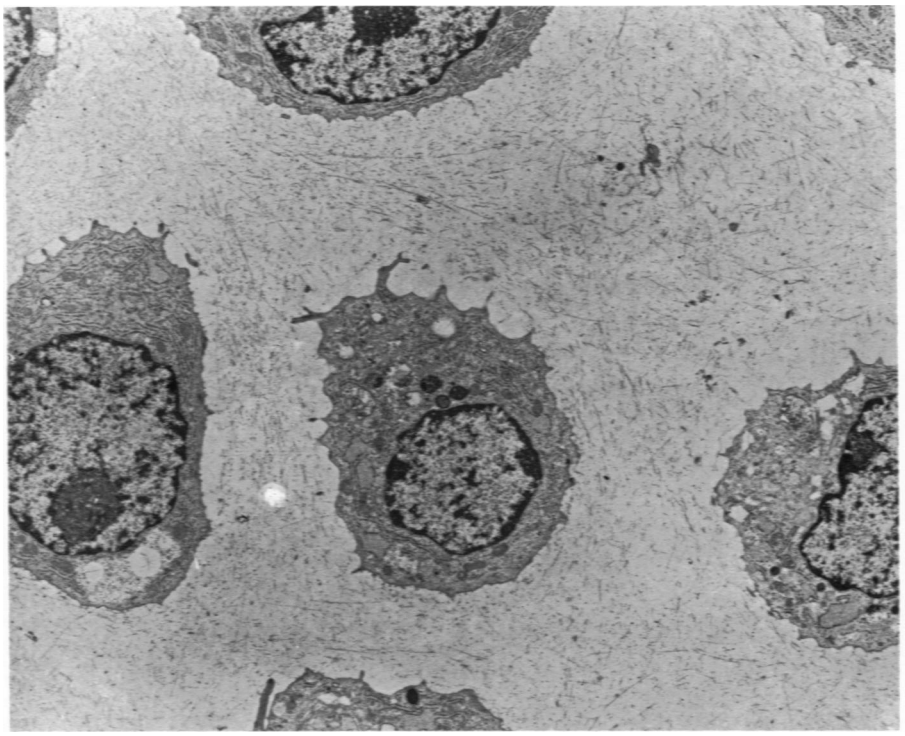
Legends for Figures

Fig 1—Resting zone, mouse. Routine glutaraldehyde fixation. Chondrocytes lie randomly in an extracellular matrix which is largely empty except for collagen fibrils. *Mark* = 1 μ (× 4000).

Fig 2—Resting zone, mouse, LYS stain. Dense complexes between LYS and anionic polyelectrolytes are rather uniformly spread throughout the extracellular matrix. *Mark* = 1 μ (× 6500).



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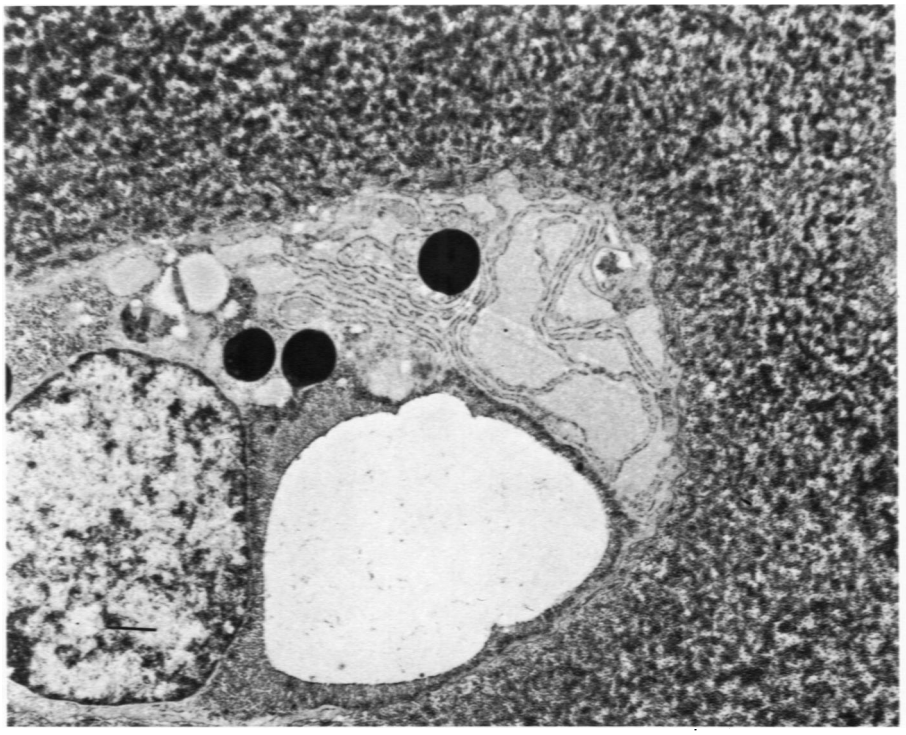

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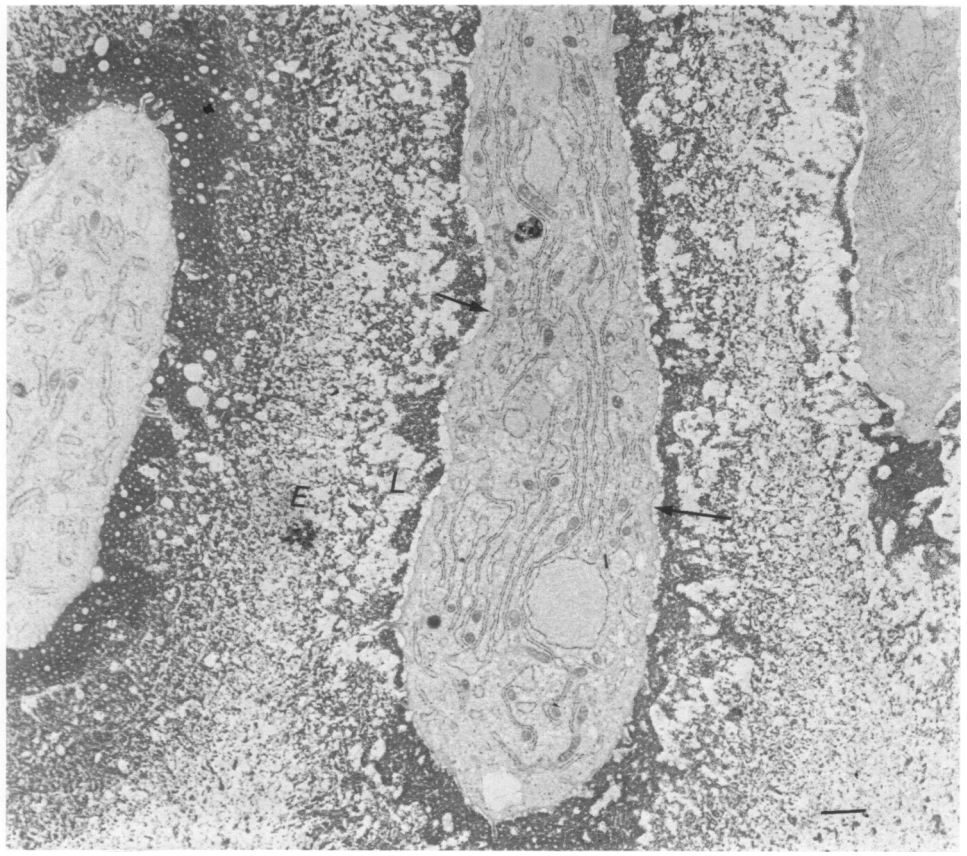

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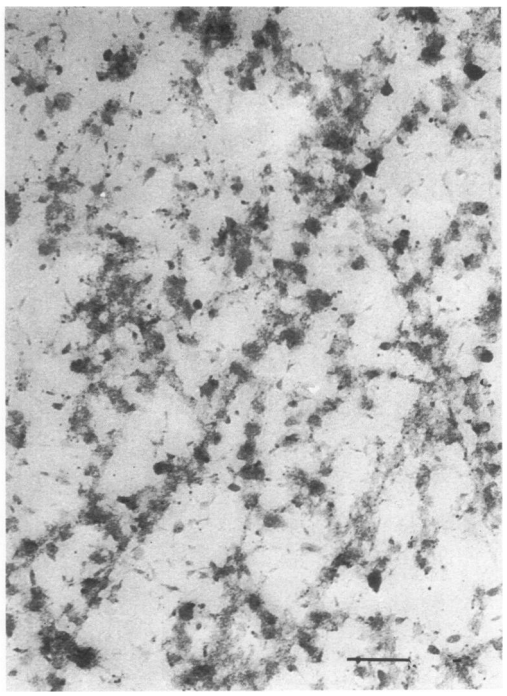

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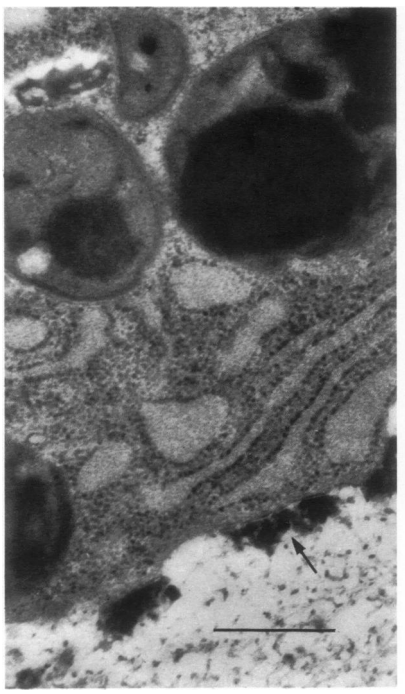

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Fig 3—Columnar zone, puppy, LYS stain. In contrast with the resting zone, LYS-reactive material is concentrated in the lacuna (*L*), where it is rather densely stained. The three cells illustrated are cut in different planes, illustrating some variability in the distribution of staining, but in each, the concentration of LYS-reactive material within the lacuna is striking. An unstained rim of variable width (*arrows*) surrounds chondrocytes. Outside the lacuna (*E*), the LYS-reactive material is dispersed and punctuate. *Mark*=1 μ (× 5000). **Fig 4**—Columnar zone, puppy, LYS stain, extralacunar area. Dense deposits are arrayed on collagen fibrils with a mean interval of 640 Å. The intervening material is not stained. *Mark*=0.2 μ (× 37,000). **Fig 5**—Columnar zone, puppy, UV-irradiated, LYS-treated. Unlike intact LYS-stained tissue, material is seen almost exclusively as scattered deposits on the surface of the chondrocyte (*arrow*). Material of similar density, apparently phagocytosed material, is present within membrane-limited organelles, apparently lysosomes. *Mark*=0.5 μ (approx × 30,000).

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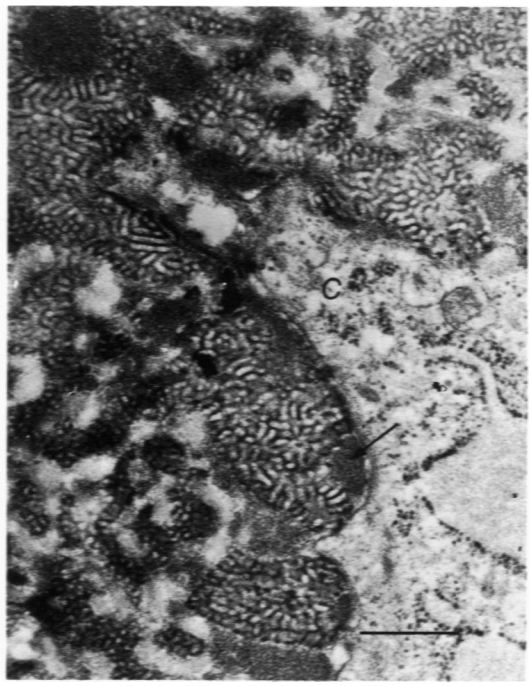

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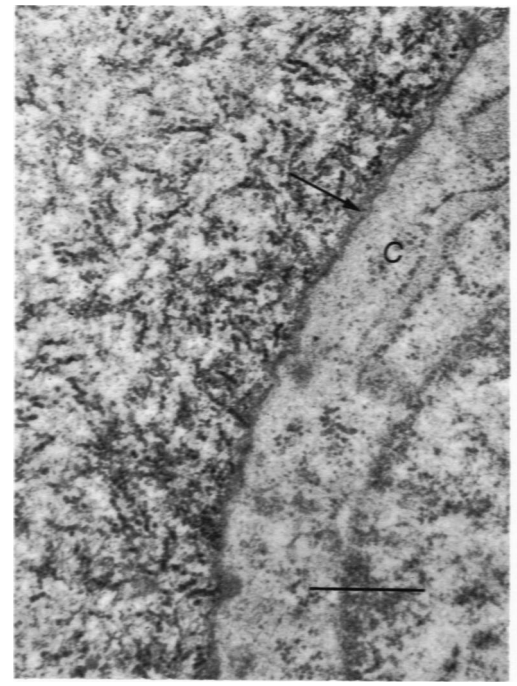

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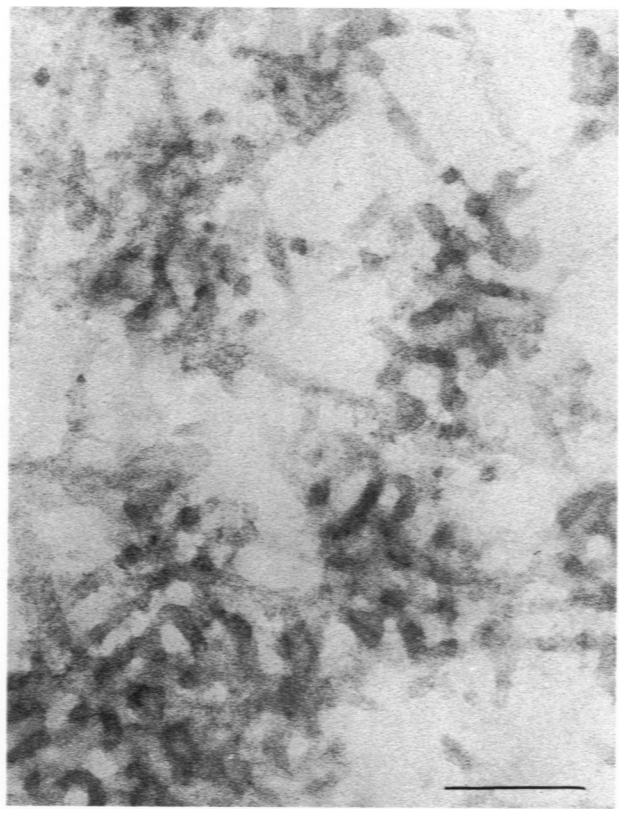

Fig 6—Columnar zone, mouse, PR stain. Two types of matrix densities are seen. One is in the form of punctate, crowded droplets (*arrow*), the other in a linear pattern. *C* indicates chondrocyte. *Mark*=0.5 μ (approx. × 25,000). **Fig 7**—Columnar zone, mouse, PR stain. This higher magnification reveals that the PR-reactive material associated with fibrils, where it is present, forms a uniform coat. *Mark*=0.2 μ (approx 90,000). **Fig 8**—Lower resting zone, mouse, RR stain. A layer of homogeneously stained material is closely applied to the plasma membrane (*arrows*). In the extracellular space, two types of stainable material are present. One has a fibrillar arrangement; the other is in a punctate form. *C* indicates chondrocyte. *Mark*=0.5 μ (× 30,000).

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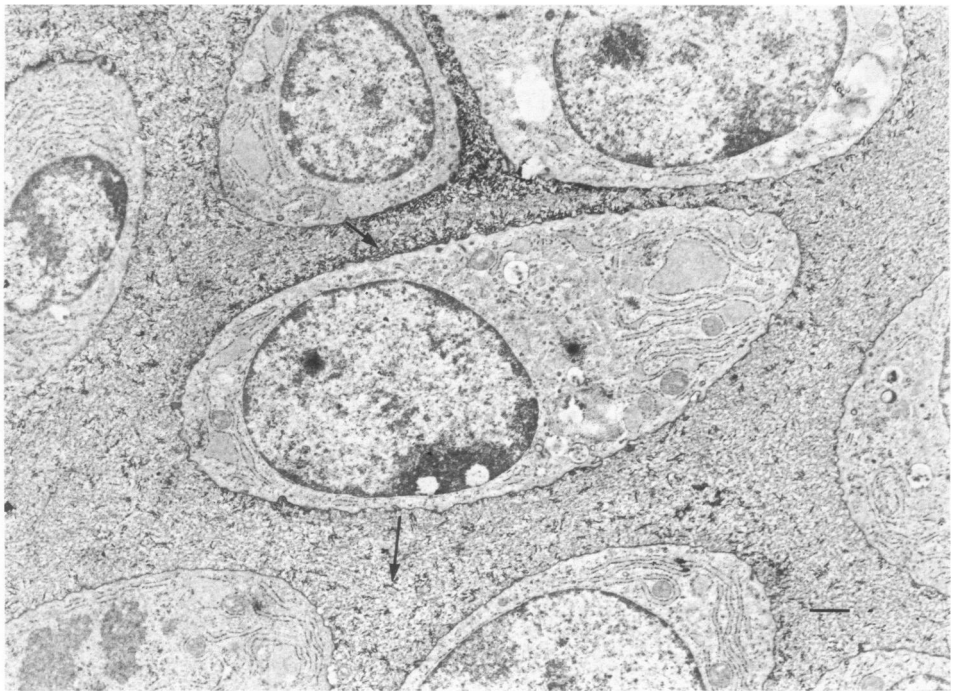

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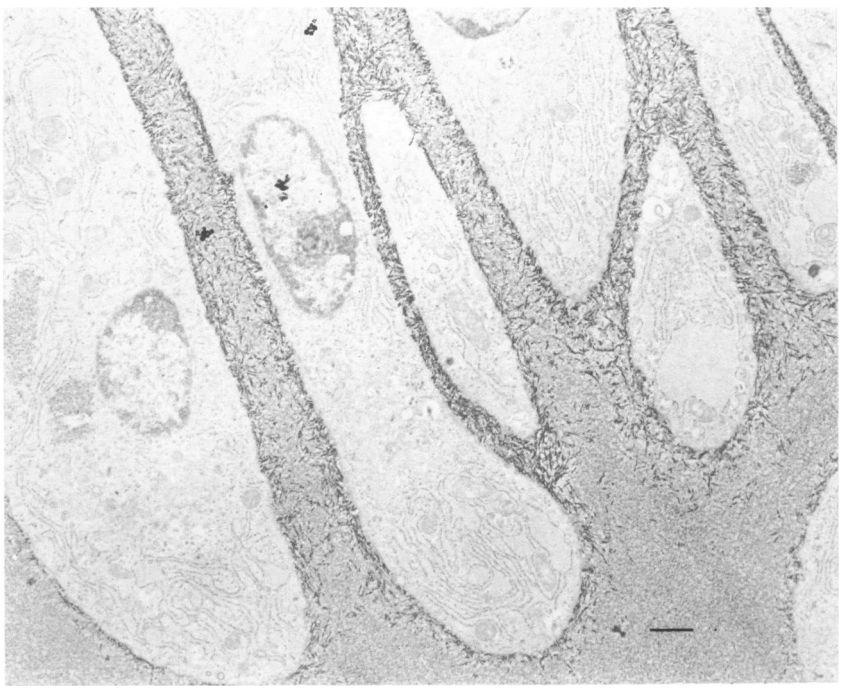
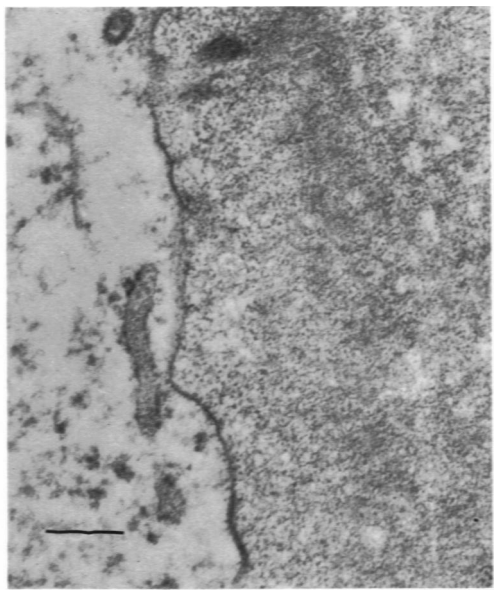


Fig 9—Resting zone, mouse, RR stain. The two forms of RR-stained material, fibrillar (*arrows*) and nonfibrillar, are more uniformly spread throughout the extracellular matrix than in the columnar zone. *Mark*=1 μ (× 5200). **Fig 10**—Columnar zone, mouse, RR stain. The extracellular matrix is now differentiated in that the fibrillar material is largely restricted to the lacunar area (× 5000).



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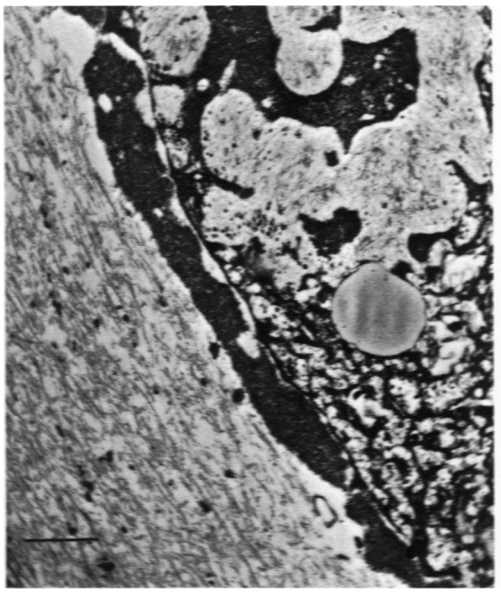

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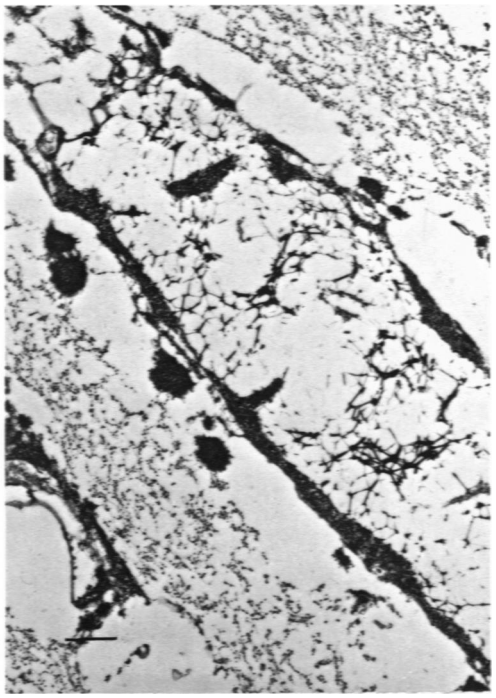

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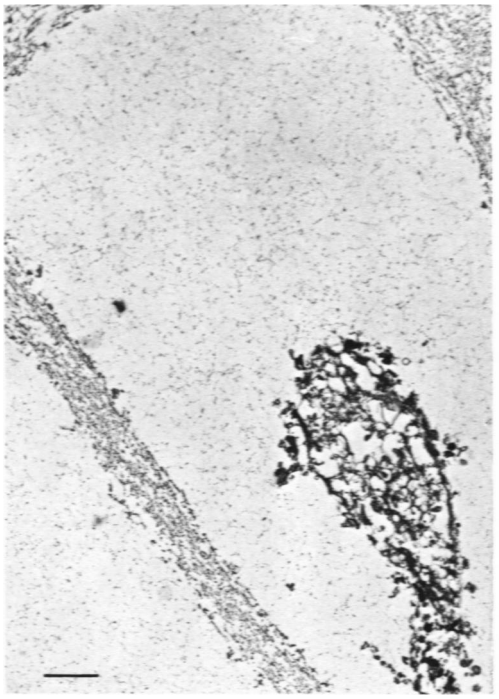

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Fig 11—Hypertrophic zone, mouse, RR stain. Here only one type of extracellular material is evident. The fibrillar material appears to be absent. *Mark*=0.25 μ (× 34,500). **Fig 12**—Puppy, resting zone, RR stain on GuCl-extracted tissue. Densely stained material almost fills the lacuna. In the extralacunar area, similar, but smaller masses of material are scattered between collagen fibrils. *Mark*=1 μ (approx × 8500). **Fig 13**—Puppy, columnar zone, RR stain on GuCl-extracted tissue. The dense material in the lacuna is present only in discrete loci. In the extralacunar area, fine dense deposits are arrayed on collagen. *Mark*=1 μ (approx × 6500). **Fig 14**—Puppy, hypertrophic zone, RR stain on GuCl-extracted tissue. There is almost no RR-reactive material left in the lacuna. *Mark*=1 μ (× 5200).

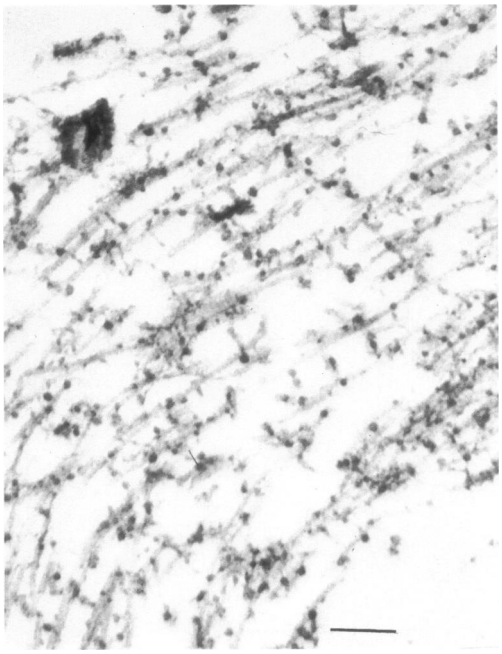

Fig 15—Puppy, hypertrophic zone, extralacunar area, RR stain on GuCl-extracted tissue. Collagen-associated material reactive with RR is present, but widely spaced. *Mark*=0.2 μ (approx × 40,000).

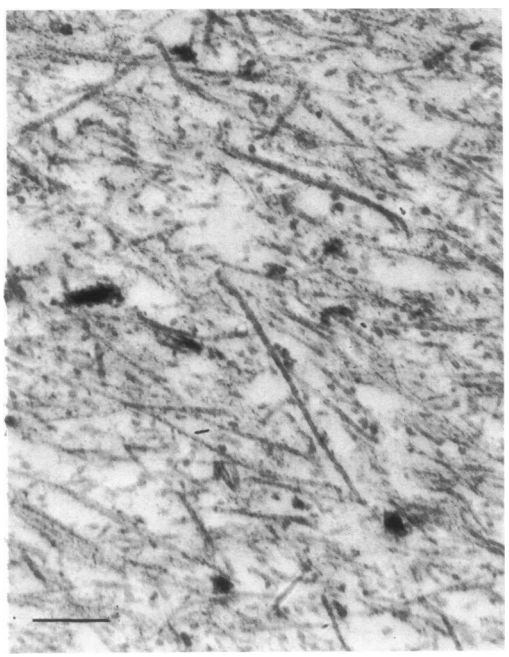

Fig 16—Resting zone, puppy, RR stain on GuCl-extracted tissue, extralacunar area. Collagen fibrils are free of the stainable deposits seen in other zones. However, larger dense deposits, similar to those seen within the lacuna, are present between the fibrils. *Mark*=0.2 μ (× 40,000).