

# Cartilage Ultrastructure after High Pressure Freezing, Freeze Substitution, and Low Temperature Embedding.

## II. Intercellular Matrix Ultrastructure - Preservation of Proteoglycans in Their Native State

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**ABSTRACT** The extracellular matrix of epiphyseal cartilage tissue was preserved in a state believed to resemble closely that of native tissue following processing by high pressure freezing, freeze substitution, and low temperature embedding (HPF/FS). Proteoglycans (PG) were preserved in an extended state and were apparent as a reticulum of fine filamentous threads throughout the matrix. Within this network, two morphologically discrete components were discernible and identified with the carbohydrate and protein components of PG molecules.

Numerous points of contact were clearly visible between components of the PG network and cross-sectioned collagen fibrils and also between PG components and chondrocytic plasmalemmata. These observations provide direct morphological indication that such relationships may exist in native epiphyseal cartilage tissue.

The intercellular matrix of epiphyseal cartilage tissue consists of a proteoglycan (PG)<sup>1</sup> phase trapped within a network of collagen fibrils (24, 28). It is by virtue of their presence in high concentration (7, 11, 24) that the PG are able to exert a considerable pressure upon the collagen network and, as a consequence of this, the matrix is rendered resilient to compression from external forces. The collagen fibrils themselves confer upon cartilage tissue its shape and high tensile strength (11, 24, 26, 28).

The structure of cartilage PG-subunits and the nature of the interaction that exists between these molecules and hyaluronic acid residues to form PG aggregates have been elucidated by biochemical analysis of isolated matrix components (7, 11, 26). However, due to the poor state in which the matrix is preserved under standard chemical fixation (SCF) conditions, it has been impossible to determine with precision (i.e., within the range of a few nm) the structural organization of

PG aggregates and the relationship that these macromolecules have to collagen fibrils in the native state.

One of the main sources of difficulty is that PG are highly water-soluble and thus are extracted from the extracellular matrix compartment during incubation of cartilage tissue slices in aqueous fixation media (5, 44). Although the problem of PG solubilization has been overcome by the use of cationic dyes, the mechanism by which these compounds act is such that PG are precipitated within the matrix in the form of condensed 'granules' (10, 22, 29, 37, 38, 40, 41, 43). These dyes thus specifically induce structural alterations within the matrix and hence destroy its fine ultrastructure (10, 38, 39).

In addition, rapid swelling of tissue slices occurs (within ~30 min) on exposure to aqueous fixation media as a consequence of the under-hydrated state in which PG exist in the native state (11, 15, 23, 24). Considerable distortion of matrix components takes place during this process and the effect is accentuated during the dehydration and embedding procedures when retraction of the slices occurs (35, 37, 46).

In the current investigation, we describe a novel method for the preservation of cartilage tissue by which the fine ultrastructure of the extracellular matrix is preserved. The procedure combines rapid high pressure freezing with freeze substitution and low temperature embedding (HFP/FS).

<sup>1</sup> *Abbreviations used in this paper:* F (strands), fine, faintly staining strands; HPF/FS, high pressure freezing, freeze substitution, and low temperature embedding; PG, proteoglycan(s); RHT, ruthenium hexammine trichloride; RHT-CF, RHT-chemical fixation; SCF, standard chemical fixation; T (strands), thick, darkly staining strands.

## MATERIALS AND METHODS

**Standard Chemical Fixation (SCF) (14):** Freshly excised tissue blocks from rat epiphyseal cartilage were incubated in a primary fixation medium consisting of 2% (vol/vol) glutaraldehyde in 0.05 M sodium cacodylate solution (the final solution having a pH of 7.3) for 2.5 h at ambient temperature. Postfixation was conducted in 0.1 M sodium cacodylate buffer containing 1% (wt/vol) osmium tetroxide (the final solution having a pH of 7.3), also for a period of 2.5 h at ambient temperature. Dehydration and embedding (in Epon 812) were carried out at ambient temperature, and resin polymerization at 60°C.

**Ruthenium Hexammine Trichloride (RHT)-Chemical Fixation (RHT-CF) (13):** The procedure adopted was identical to that described for SCF above except that both primary- and postfixation media contained, in addition to the components cited, 0.7% (wt/vol) RHT.

**HPF/FS:** The procedure, which is summarized below, is identical to that described in detail in the accompanying paper. Freshly excised tissue disks from rat epiphyseal cartilage were rapidly frozen (within 40 ms) to -196°C while being maintained under a hydrostatic pressure of  $2.1 \times 10^8$  Pa (2,100 bar).

Frozen tissue water was then substituted with methanol in three stages: 17 h at -90°C, 13 h at -60°C and 12 h at -35°C, with one change of medium being made during the 13-h period at -60°C. Substitution media contained, in addition to methanol, 2% (vol/vol) glutaraldehyde and 0.5% (wt/vol) uranyl acetate. Tissue disks were then incubated in methanol alone for 3 h at -35°C. Resin-infiltration was carried out in three stages (each at -35°C) over a period of 20 h, during which the proportion of resin (HM 20 or K4M) to methanol was progressively increased. Tissue disks were then infiltrated with pure resin for 72 h (with one change after 3 h). UV-ray-catalyzed polymerization of resin

was conducted primarily at -35°C for 24 h and then at ambient temperature for an additional 2 d.

Thin sections (35 nm) were cut on a Reichert ultramicrotome (OMU 3), stained with a saturated solution of lead citrate for 4-5 min, and mounted on Parlodion-coated 200-mesh copper grids prior to electron microscopic examination in a Philips EM 301 or 400.

## RESULTS

The ultrastructural appearance of cartilage extracellular matrix in tissue processed by HPF/FS (Fig. 1c) contrasted sharply to the appearance of that in tissue preserved by either SCF (Fig. 1a) or RHT-CF (Fig. 1b). After SCF (Fig. 1a), very few PG remained within the interstices of the collagen network, most having been lost by aqueous extraction during fixation. Although the inclusion of RHT in aqueous fixation media prevented the solution of PG, it did so by precipitating these components such that they appeared as condensed 'granules' within the matrix (Plate 1b). Only when cartilage tissue was processed by HPF/FS were the PG apparent as a fine filamentous network of even density within each matrix compartment (Plate 1c). Furthermore, examination at high magnification (Figs. 2 and 4) revealed that this network was heterogeneous in composition and consisted of a reticulum of thick, darkly staining strands (T) with finer, less intensely staining branches (F).

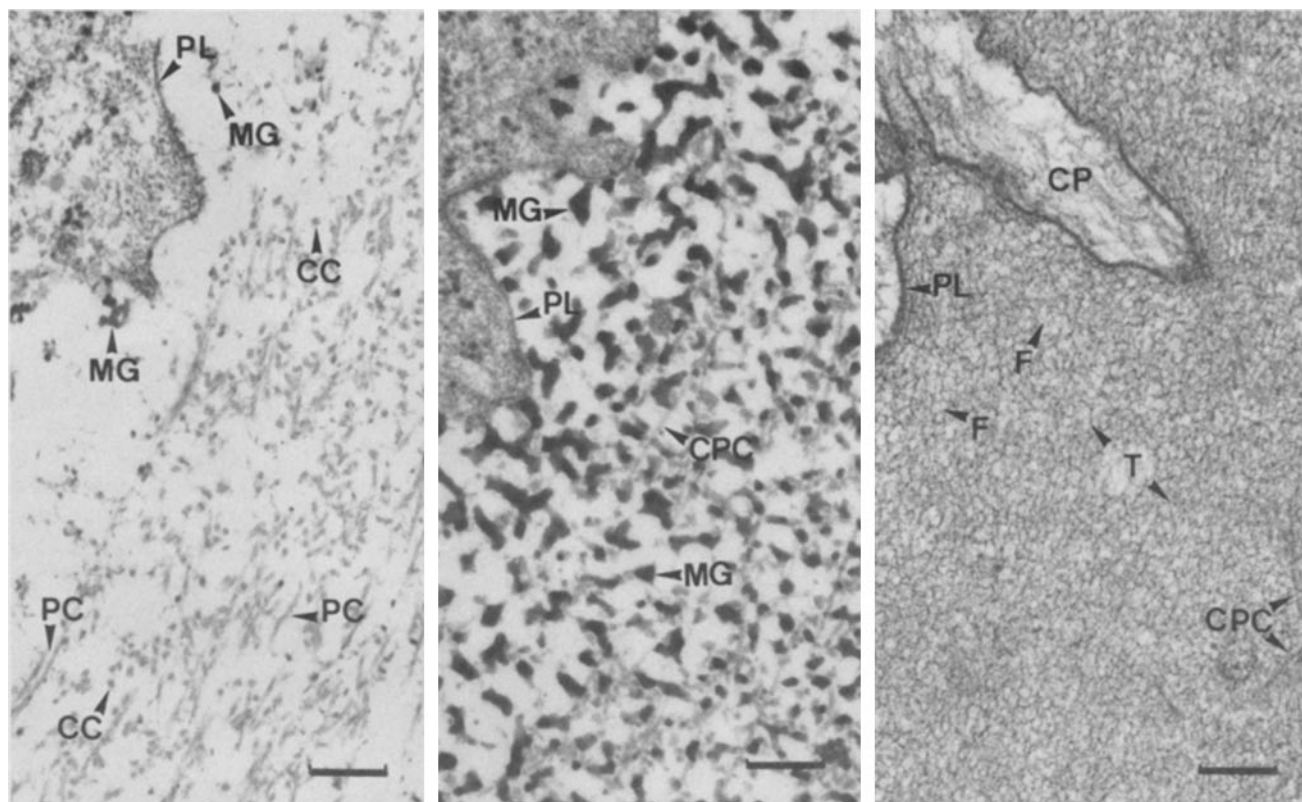


FIGURE 1 Electron micrographs of the pericellular matrix region surrounding hypertrophic chondrocytes after (a) SCF, (b) RHT-CF, and (c) HPF/FS. (a) The pericellular matrix is almost completely devoid of proteoglycan molecules. Only a few, present as condensed granules (MG), are visible along and close to the plasmalemma (PL) surface. As a consequence of proteoglycan depletion, the skeleton of collagen fibrils is clearly exposed. However, their cross-banded structure is not apparent. CC, cross-sectioned collagen fibril; PC, collagen fibril lying parallel to or obliquely within the section plane. Bar, 0.2  $\mu$ m.  $\times$  50,000. (b) Matrix proteoglycans are present as condensed granules (MG), irregularly spaced as a consequence of displacement during RHT-precipitation. The cross-banded structure (CPC) of collagen fibrils is indistinct, but apparent in most instances. PL, plasmalemma. Bar, 0.2  $\mu$ m.  $\times$  50,000. (c) The matrix is apparent as a fine filamentous network of extended proteoglycans within which collagen fibrils (CPC) with a clear, cross-banded structure may be distinguished. T, thick, darkly stained component of a proteoglycan molecule; F, fine, faintly stained component of a proteoglycan molecule; CP, cell process; PL, plasmalemma. Bar, 0.2  $\mu$ m.  $\times$  50,000.

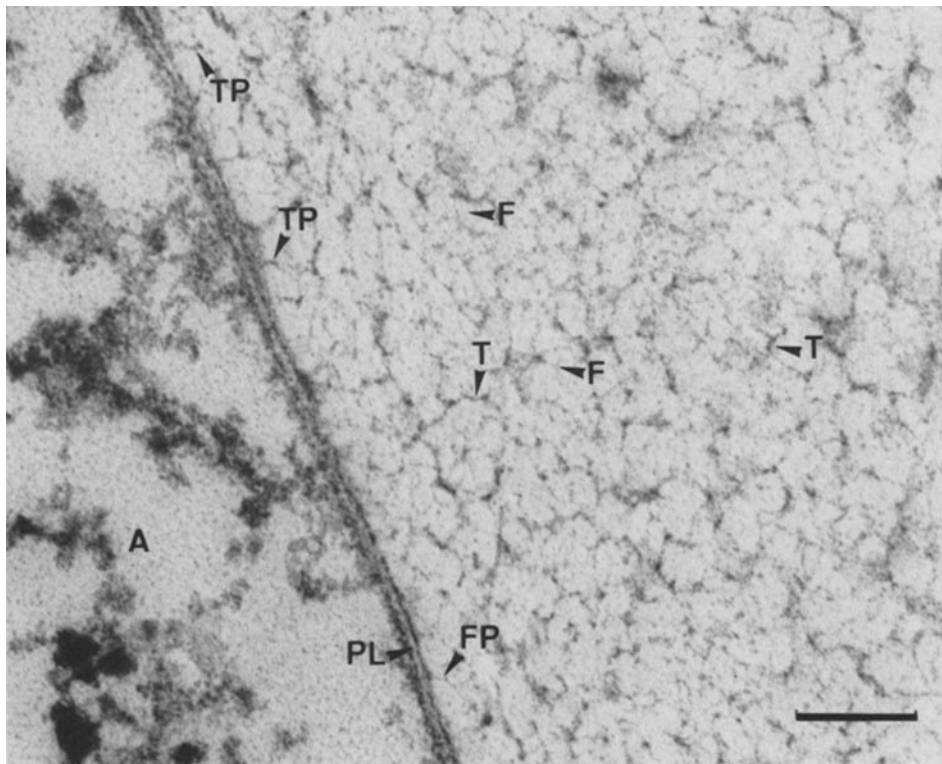


FIGURE 2 High power electron micrograph of the pericellular matrix region of a hypertrophic chondrocyte after processing by HPF/FS. The proteoglycan network is composed of both thick (darkly stained) (T) and thin (faintly stained) (F) threads. Numerous points of contact between the chondrocytic plasmalemma (PL) and both F (FP) and T (TP) threads may be clearly discerned. A, cell cytoplasm. Bar, 0.1  $\mu\text{m}$ .  $\times 160,000$ .

It was not possible to assess with any degree of accuracy the length of the T strands, due to the tremendous variability in this parameter. This observation is an indication that, in most instances, only portions of their total lengths were visible within the plane of sectioning and is typical for isotropically oriented structures whose lengths exceed appreciably the thickness of the section. The variation in length of the F strands lay within a much smaller range, 40–60 nm (Fig. 4), which suggests that the measurements are representative of structures having finite dimensions within the plane of the section. F strands were consistently found to be separated by a distance of 5–15 nm; the lateral separation between T strands lay within the range of 60–80 nm (Fig. 4). Justification for identifying the F filaments with the carbohydrate components and the T strands with the protein core components of PG is presented in the Discussion.

Collagen fibrils were apparent in tissue processed by each of the three methods. Cross-banding of these structures within the plane of the section could, however, be seen only rarely and, when apparent, was poorly resolved in tissue processed by SCF (10, 19). Resolution was improved considerably in tissue preserved by RHT-CF (Fig. 1*b*) but the striations could be discerned with clarity and consistency only in tissue processed by HPF/FS (Figs. 1*c* and 3*a*).

Examination of Fig. 3*b* reveals the presence of numerous contact sites between cross-sectioned collagen fibrils and T and F components of the PG network in tissue processed by HPF/FS. Their existence is a strong indication that such a relationship exists in the native state. Contact sites could also be seen between T and F strands and collagen fibrils lying within the plane of sectioning (Fig. 3*a*), but the appearance of these may have been a consequence of over-projection. (Since the thicknesses of T and F strands and collagen fibrils lie within the thickness range of tissue sections ( $32 \pm 2$  nm), one structure may lie above another. This relationship cannot

be resolved in a standard electron microscopic image, and the structures will thus appear to be touching.)

Many contact sites between T and F components of the PG network and the plasmalemmata of chondrocytes were discernible in tissue processed by HPF/FS, which may be indicative of an interactive relationship in the native state (Fig. 2). Owing to the manner in which PG are preserved in tissue processed by RHT-CF, any fine ultrastructural relationships existing between these components and either collagen fibrils or the plasmalemmata of chondrocytes will be lost (Fig. 1*b*). Although PG were apparent lying along the plasmalemmal surfaces of chondrocytes in tissue processed by SCF (Fig. 1*a*), it could be argued that these contacts occurred secondarily as a consequence of the gross shifting of PGs which takes place during this fixation (5, 37, 44).

## DISCUSSION

The extensive loss of PG that is incurred during conventional chemical processing of cartilage tissue (5, 44) has rendered an analysis of the structural organization of these matrix components impossible. Although the problem of PG solubilization has been overcome by the use of cationic dyes, their use has not permitted an appraisal of matrix structure at a molecular and submolecular level (29, 37, 43). The main reason for this is that these compounds precipitate PG in the form of condensed granules, the degree of condensation depending upon the precipitating capacity of the agent used. Cationic dyes combining the properties of a small mass and high charge density such as RHT induce total collapse of the PG whereas those with a larger mass and lower charge density such as alcian blue (36), cinchomeric acid (37), and cupromeronic blue (39) produce only partial collapse. A slight improvement in resolution may thus be achieved by use of these latter dyes. Nonetheless, all of these agents produce, whether to a greater

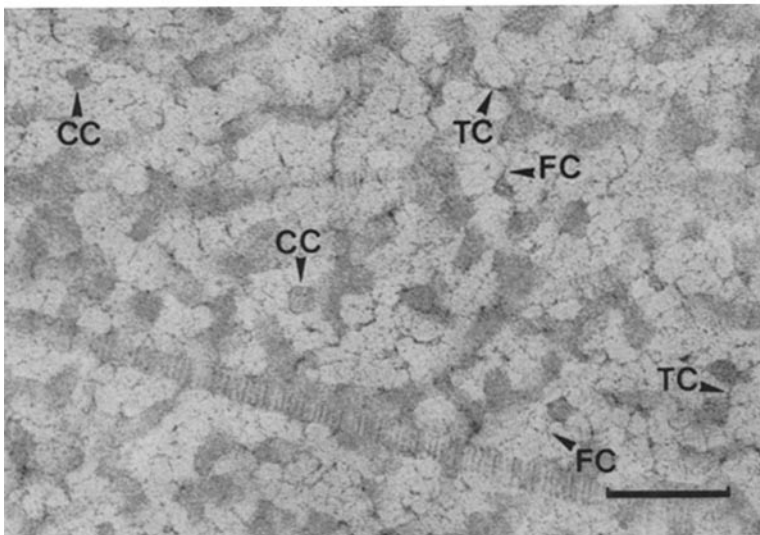
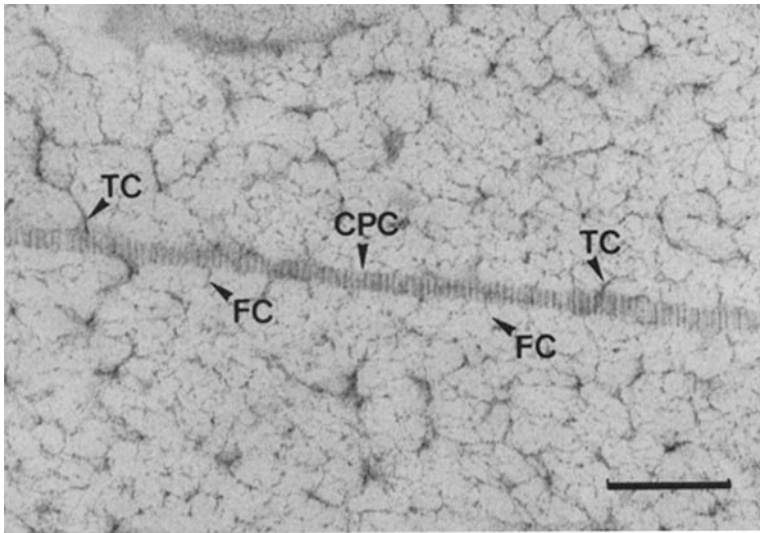


FIGURE 3 High power electron micrograph of cartilage interterritorial matrix after processing by HPF/FS. (a) A type II collagen fibril, lying parallel to the section plane, exhibits a clear cross-banded structure (CPC). Numerous points of contact between this fibril and thick (TC) and thin (FC) components of the proteoglycan network are apparent. Bar, 0.2  $\mu\text{m}$ .  $\times$  80,000. (b) Many contact sites between cross-sectioned (CC) collagen fibrils and thick (TC) and thin (FC) components of the proteoglycan network may be discerned. Bar, 0.2  $\mu\text{m}$ .  $\times$  80,000.

or lesser extent, a collapse of the PG molecules and hence do not permit an analysis of fine ultrastructural relationships (in the order of a few nanometers) within the PG network (29, 30, 37). Moreover, the unknown specificity of action of these dyes towards PG (39) and the uncontrollable local shifts produced within the matrix during precipitation are further indications of the limitations of this method of preservation with respect to analyzing PG ultrastructure.

When cartilage tissue is processed using a method that combines high pressure freezing with freeze substitution and low temperature embedding (HPF/FS), the problem of PG extraction is avoided by circumventing an aqueous immersion step during which the PG become fully hydrated and hence solubilized. The temperature conditions under which freeze substitution was carried out are such that molecular (9, 25) and even ionic (8, 9, 20) movements are drastically reduced and hence PG are virtually immobilized. Under these conditions of processing it is thus no longer necessary to prevent PG dislocation and solubilization by use of precipitating agents.

The appearance of the extracellular matrix after HPF/FS more than adequately portrayed the considerable improvement in preservation quality attainable using this technique. PG components were apparent as a reticulum of fine filamen-

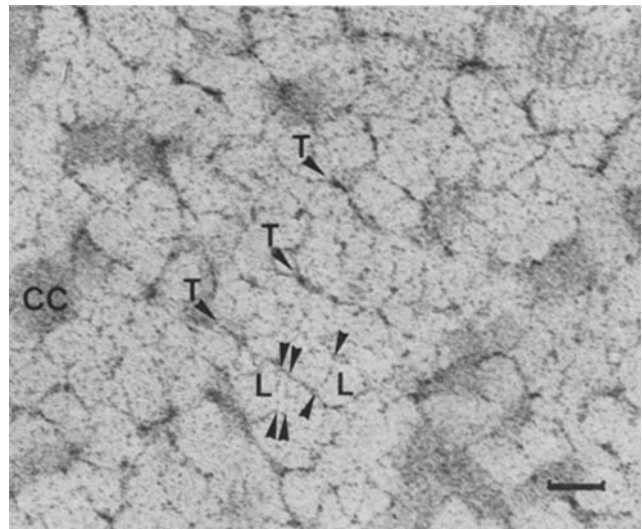


FIGURE 4 High power electron micrograph of cartilage interterritorial matrix after processing by HPF/FS. The lengths of the F strands (L) lie within the range of 40–60 nm and the distance separating these structures varies between 5 and 15 nm. T strands (T) are separated by a distance lying within the range of 60–80 nm. CC, cross-sectioned collagen fibril. Bar, 50 nm.  $\times$  150,000.

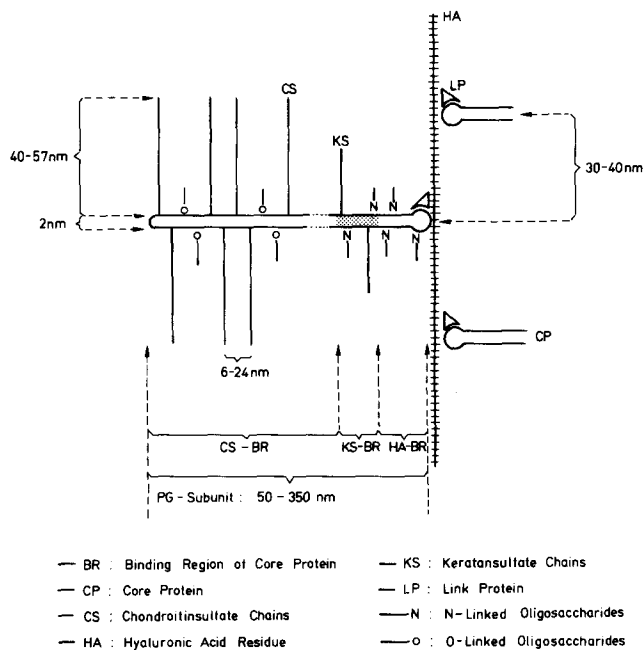


FIGURE 5 Two-dimensional representation of an extended proteoglycan subunit after Hascall (11) and Hardingham (7). The indicated dimensions are those which have been determined by Buckwalter (2), Hascall (10), Heinegard (12), Kimura (16), Rosenberg (34), and Thyberg (45).

tous threads throughout the matrix, and systematic examination of electron micrographs revealed no sign of dislocations or discontinuities within this network. Furthermore, two components were readily distinguishable and we have evidence that supports identifying the finer, faintly staining threads (F) with carbohydrate chains and the thicker more intensely staining strands (T) with the protein core components of PG molecules. This evidence is now presented.

It is well known that uranyl acetate (the staining agent applied to ultra-thin sections) has a high affinity for proteins but stains carbohydrates only weakly (6, 10, 43). The observation that T strands stained more intensely than F strands may be used as evidence in support of identifying the former as the protein core components and the latter as the carbohydrate components of the PG network. Moreover, since it is believed that each cartilage PG molecule (Fig. 5) consists of a single protein core to which are attached ~250 carbohydrate chains (100 chondroitin sulphate chains, 50 keratan sulphate chains, and 100 oligosaccharides), one would expect the carbohydrate components to be more numerous than the protein components (7, 11). Examination of Figs. 2 and 4 reveals that the density of F threads is higher than that of the T threads.

The lengths of the F strands lay within a range (40–60 nm) (Fig. 4) that is close to the value measured for chondroitin sulphate chains in isolated, fully expanded molecules (40–57 nm) (2, 10, 34, 45, Fig. 5). The distance separating F threads lay within a range (5–15 nm) (Fig. 4) that is below that measured between chondroitin sulphate chains in isolated, fully expanded molecules (6–24 nm) (2, 32, 38). Similarly, the lateral distance between T strands (60–80 nm) lay within a range below that theoretically predicted for the separation between the protein cores of two PG subunits (lying in the same plane with their longitudinal axes parallel), i.e., twice the length of an F strand (80–114 nm). Since it is believed that cartilage extracellular matrix is preserved in a state close

to that existing in native tissue after processing by HPF/FS, this discrepancy is not unexpected. Native PG exist in an underhydrated and hence highly concentrated state (11), a consequence of which is that the protein cores of separate PG subunits are closely packed and the volume occupied by a single molecule is smaller than that occupied by a similar molecule in a fully hydrated state. One would therefore expect the distance between chondroitin sulphate chains in a fully expanded PG to be greater than the corresponding distance in a PG within cartilage tissue processed by HPF/FS. However it should be borne in mind that this lateral distance range between F strands (5–15 nm) lies within the diameter range expected for microcrystals of ice that may have been formed during cryofixation. It is thus feasible that the F strands consisted of matrix components that were compressed between such crystals during their growth. Although this possibility cannot be excluded, it nonetheless represents a rather unlikely explanation since water molecules within the matrix are highly oriented around PG molecules. As such they exist in a highly ordered state and are thus able to resist the reorientation necessary for ice crystal formation and growth (1). It thus seems probable that the network of F strands represented a native structural arrangement.

Although there is a considerable amount of data that supports the existence of an interaction between PG and collagen fibrils in cartilage tissue, the evidence has been drawn principally from biochemical studies *in vitro* (3, 4, 23, 31). Regularly spaced contact sites between matrix 'granules' and collagen fibrils in cartilage tissue after fixation in the presence of cationic dyes were first documented by Smith in 1970 (43) and their existence was later confirmed independently by Myers (see reference 29; 30) and Shepard and Mitchell (40, 41). More recently (33), the existence of contact sites between collagen fibrils and 'particulate staining' products has been demonstrated using immunohistochemical techniques following preservation under standard chemical conditions. However, under conditions of processing in which aqueous fixation techniques are used, it is not possible to detect the existence of a discrete relationship (i.e., on the order of a few nanometers) between collagen fibrils and components of the PG molecules (30, 38). Nonetheless, these previous findings indicated that a relationship between PG and collagen fibrils may exist. The appearance of numerous contact sites between F and T components of the PG network and cross-sectioned collagen fibrils in tissue processed by HPF/FS now provides strong evidence that such contacts are present in native tissue (Fig. 3*b*). It should be emphasized that contact sites are defined strictly on a structural basis, and do not anticipate a specific chemical interaction between these two components.

Previous studies have provided indirect evidence that associations between PG and the surfaces of various cell types exist (17, 18, 32). Indirect evidence for the existence of membrane-bound PG in epiphyseal cartilage tissue has been extensively discussed in a recent publication (14). The abundance of contact sites that were apparent between F and T components of the PG network and the outer surfaces of chondrocytes in tissue processed by HPF/FS now provides direct morphological evidence for the existence of a relationship between matrix PG and chondrocytic plasmalemmata.

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