Cartilage Ultrastructure after High Pressure Freezing, Freeze Substitution, and Low Temperature Embedding. I. Chondrocyte Ultrastructure—Implications for the Theories of Mineralization and Vascular Invasion

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ABSTRACT Electron microscopic examination of epiphyseal cartilage tissue processed by high pressure freezing, freeze substitution, and low temperature embedding revealed a substantial improvement in the preservation quality of intracellular organelles by comparison with the results obtained under conventional chemical fixation conditions. Furthermore, all cells throughout the epiphyseal plate, including the terminal chondrocyte adjacent to the region of vascular invasion, were found to be structurally integral. A zone of degenerating cells consistently observed in cartilage tissue processed under conventional chemical fixation conditions was not apparent. Hence, it would appear that cell destruction in this region occurs during chemical processing and is not a feature of cartilage tissue in the native state. Since these cells are situated in a region where tissue calcification is taking place, the implication is that the onset and progression of cartilage calcification are, at least partially, controlled by the chondrocytes themselves. The observation that the terminal cell adjacent to the zone of vascular invasion is viable has important implications in relation to the theory of vascular invasion. This may now require reconceptualization to accommodate the possibility that active cell destruction may be a precondition for vascular invasion.

Epiphyseal cartilage, an avascular tissue consisting of chondrocytes and an intercellular matrix, is generally believed to be organized into six zones, namely: resting, proliferating, maturing, hypertrophying, mineralizing, and degenerating. This classification is based solely upon cell morphology after chemical processing and therefore a precondition for its validity is that structural integrity has been maintained during fixation. Chemical processing of cartilage tissue is, however, wrought with numerous problems including rapid uptake of water molecules by tissue with consequent swelling, loss of water-soluble components from chondrocytes and intercellular matrix, and the poor diffusion characteristics of fixative agents resulting in inhomogeneous preservation (21, 41, 47). Hence, it has been difficult to ascertain with certainty the true form and ultrastructural morphology of chondrocytes.

Although the quality of preservation during chemical fixation has been improved, particularly with respect to cell form, by the use of cationic dyes (especially ruthenium hexammine trichloride [RHT],[1] reference 41), the morphological integrity of certain intracellular organelles is still unsatisfactory. Moreover, many structural alterations are specifically induced by fixative agents during chemical fixation, and are therefore intrinsic to the technique. In the present communication we describe a novel method for the preservation of cartilage tissue combining rapid high pressure freezing with freeze substitution and low temperature embedding (HPF/FS).

The ultrastructural morphology of chondrocytes preserved in this way is compared with that of chondrocytes processed by RHT-chemical fixation (RHT-CF) and with that of cells that must be considered to represent most closely the native form and structure, i.e., chondrocytes preserved by purely

[1] *Abbreviations used in this paper.* HPF, high pressure freezing; HPF/FE, HPF and freeze-etching; HPF/FS, HPF, freeze substitution, and low temperature embedding; RHT, ruthenium hexammine trichloride; RHT-CF, RHT-chemical fixation.

physical means. These are represented by replicas of freeze-etched chondrocytes cryofixed by rapid high pressure freezing. The results indicate that the problems intrinsically associated with fixation of cartilage tissue in aqueous fixation media may be overcome or avoided when this tissue is processed by HPF/FS.

MATERIALS AND METHODS

Materials

The high pressure freezing apparatus (installed in the Department of Cell Biology at the Federal Institute of Technology, Zürich) was developed by H. Mohr with the financial support of the Swiss National Science Foundation and Balzers AG, Liechtenstein.

Hypnorm^{R} (consisting of Fluanison [10 mg ml^{-1}] and Fentanyl base [0.2 mg ml^{-1}] in physiological saline) was obtained from Philips-Duphar BV (Amsterdam, Holland). Dulbecco's modified Eagle's medium was supplied by Seromed, GmbH (München, Federal Republic of Germany), sodium cacodylate by Merck AG (Darmstadt, Federal Republic of Germany), RHT by Johnson-Matthey (Hertfordshire, England), and glutaraldehyde (highest purity, EM-grade, 70% (wt/vol)) and gelatin capsules by Elmis, International (Fort Washington, Pennsylvania). Phosphatidyl choline, Formvar^{R} (polyvinyl formal) and Epon 812 were purchased from Fluka AG (Buchs, Switzerland), Lowicryl^{R} resins (HM 20, K4M) from Chemische Werke, Lowi AG (supplied by Balzers AG, Zürich, Switzerland), and Parlodion from Mallinckrodt Inc. (St. Louis. MO).

Methods

TISSUE PREPARATION FOR HIGH PRESSURE FREEZING: 90–100 g female Wistar rats were maintained under Hypnorm^{R} anaesthesia (0.2 ml/100 g) while cartilage was removed from the proximal epiphyseal growth plates of tibia. 0.6-mm-thick tissue slices were cut and transferred immediately to Dulbecco's modified Eagle's medium (pH 7.3) at 37°C. Tissue disks of a defined diameter (0.4 mm) were cut such that they fitted exactly into the cylindrical cavities of Balzers gold specimen supporting plates (60). A second plate was then oriented such that its dome-shaped hollow was directly above the specimen. Tissue disks destined for freeze substitution were sandwiched between supporting plates both of which had been precoated with phosphatidylcholine whereas for freeze etching, only the upper plates of sandwiches were coated. Phosphatidylcholine-coating of specimen plates has been demonstrated to facilitate the dislodgement of tissue disks (60). The whole procedure, from the preparation of tissue disks to the initiation of high pressure freezing (HPF), was completed within 1–3 min.

RAPID HIGH PRESSURE FREEZING (HPF): Details of the theory of HPF and of the construction and applications of the HPF apparatus have been previously described (59, 60, 74, 75, 97).

The enclosed specimens were transferred to the HPF apparatus where the hydrostatic pressure within the tissue was built up to 2.1×10^{8} Pa (2,100 bar) at 37°C by applying a jet of liquid propanol on each side of the specimen. While maintaining this pressure (at which the freezing point of water is depressed to −22°C), we cooled the tissue to −196°C, at a rate of >7,000°Cs^{-1}, by subjecting it to a jet of liquid nitrogen (applied on both sides of the specimen). Tissue water was frozen within 40 ms of the initiation of the pressure build-up. Specimens were then transferred immediately to liquid nitrogen in which they were maintained until required for further processing.

FREEZE ETCHING: Freeze etching of tissue disks cryofixed by HPF was performed at −105°C in a modified Balzers 360 M freeze-etching device adapted with a cold trap and two electron guns (one for platinum/carbon shadowing from 45° and the other for carbon coating from 90°). Condensation of platinum and carbon vapors onto the specimen surface was monitored by a quartz oscillator. Temperature control was assured by an iron-constantan thermocouple inserted into the object support holding device. Replication was effected under a vacuum of $>4 \times 10^{-5}$ Pa ($>3 \times 10^{-7}$ torr) generated by a nitrogen-cooled oil diffusion pump.

A superficial tissue layer (80 μm thick) consisting of cells mechanically damaged during tissue excision (41) was cut from each specimen before freeze etching to a depth of 30 nm.

Freeze-etched tissue was then shadowed with platinum/carbon to a thickness of 2 nm and the replica was subsequently stabilized with a 30-nm-thick carbon coating. Tissue disks with adherent replicas were then transferred to a chloroform/methanol (1:1, vol/vol) mixture for 1–3 h to remove lipids and promote tissue hardening, which has been observed to prevent gas bubble formation within the tissue and the consequent damaging of the replica during tissue degradation. This latter process was achieved by treating tissue disks and adherent replicas initially with papain (10 U ml^{-1} of Tris-buffered saline) and subsequently with collagenase (300 U ml^{-1} Tris-buffered saline). Each incubation was carried out for a period of 2 h at ambient temperature. Replicas were then transferred to and maintained in an aqueous solution of sodium hypochlorite (1.4% wt/vol) for 14 h at ambient temperature. After being washed in bidistilled water, replicas were exposed to an aqueous solution of sulphuric acid (15% vol/vol) for 2 h at ambient temperature and finally washed in bidistilled water (four 10-min periods). Replicas were mounted on Formvar^{R}-coated copper grids in preparation for electron microscopic examination.

FREEZE SUBSTITUTION AND LOW TEMPERATURE EMBEDDING: A liquid nitrogen-cooled cryostat (based upon the models of Müller [see reference 61] and Douzou [18]), containing eight teflon vials (each with a capacity of 7 ml), was constructed for the freeze substitution processing of tissue disks cryofixed by HPF. Each specimen was maintained within the cavity of its lower supporting plate, the upper one having been removed. Continuous stirring of substitution and embedding media was assured throughout, and temperature fluctuations were kept to within ± 1°C (for details, see reference 42).

Frozen tissue water was substituted by methanol in three stages, viz., 17 h at −90°C, 13 h at −60°C, and 12 h at −35°C. One change of medium was made during the 13-h period at −60°C. Each substitution medium was identical in composition and consisted of 2% (vol/vol) glutaraldehyde and 0.5% (wt/vol) uranyl acetate in pure methanol. It is considered likely that ~95% of frozen tissue water was replaced by methanol at the end of the first stage (65, 66, 90, 100). This process was completed during the second and third stages during which mild glutaraldehyde fixation was also initiated. (Although present throughout the substitution period, glutaraldehyde probably began to take effect only when the temperature was increased to −60°C [67]).

The final substitution medium was replaced by methanol (3 h at −35°C) prior to infiltration with resin (HM 20 or K4M). The embedding process was carried out at −35°C in three stages, with a progressively increased ratio of resin to methanol, i.e., 3:1 (vol/vol) (3 h), 1:1 (vol/vol) (3 h), 1:3 (vol/vol) (14 h) and pure resin (72 h with one change after 3 h). Stirring of media during resin infiltration was conducted in the absence of oxygen, which inactivates a component of the resin responsible for UV-ray-catalyzed polymerization (5, 10).

Tissue disks were then dislodged from their supporting plates and transferred to small gelatin capsules which were filled to capacity with fresh medium, capped, and immersed (to the bases of their caps) in pure ethanol. The purpose of this latter procedure was to prevent small temperature fluctuations throughout the length of the capsule (thus preventing gas bubble formation). UV-ray-catalyzed polymerization was carried out at −35°C for a period of 24 h, after which time the process was ~95% complete (10). The capsules were then exposed to a diffuse UV-ray source at ambient temperature for 2 d.

Blocks were sectioned on a Reichert ultramicrotome OMU3. Thick sections (1 μm) were stained with toluidine blue prior to light microscopic examination. Thin sections (32 ± 2 nm) were stained with a saturated aqueous solution of lead citrate for 4–5 min before being mounted on Parlodion-coated 200-mesh copper grids for electron microscopic examination in a Philips EM 301 or 400.

RHT-CHEMICAL FIXATION (41): Primary fixation of freshly excised tissue blocks was carried out in 0.05 M sodium cacodylate buffer containing 2% (vol/vol) glutaraldehyde (after the addition of which the pH was adjusted to 7.3) and 0.7% (wt/vol) RHT for 2.5 h at ambient temperature. Tissue blocks were then postfixed in 0.1 M sodium cacodylate buffer containing 1% (wt/vol) osmium tetroxide (after the addition of which the pH was adjusted to 7.3) and 0.7% (wt/vol) RHT again for a period of 2.5 h at ambient temperature. Dehydration and embedding (in Epon 812) procedures were carried out at ambient temperature and resin polymerization at 60°C.

RESULTS

Replication of chondrocytes after processing by high pressure freezing and freeze-etching (HPF/FE) has obviated the use of chemical fixation techniques, and for this reason the replicas were considered to represent most closely the form and ultrastructural morphology of native chondrocytes. They were included in the present study as a basis for comparison with chondrocytes processed by high pressure freezing, freeze substitution, and low temperature embedding (HPF/FS). Chondrocytes preserved by RHT-chemical fixation (RHT-CF) were included as representative of one of the best-quality preservations currently attainable by use of purely chemical fixation techniques.

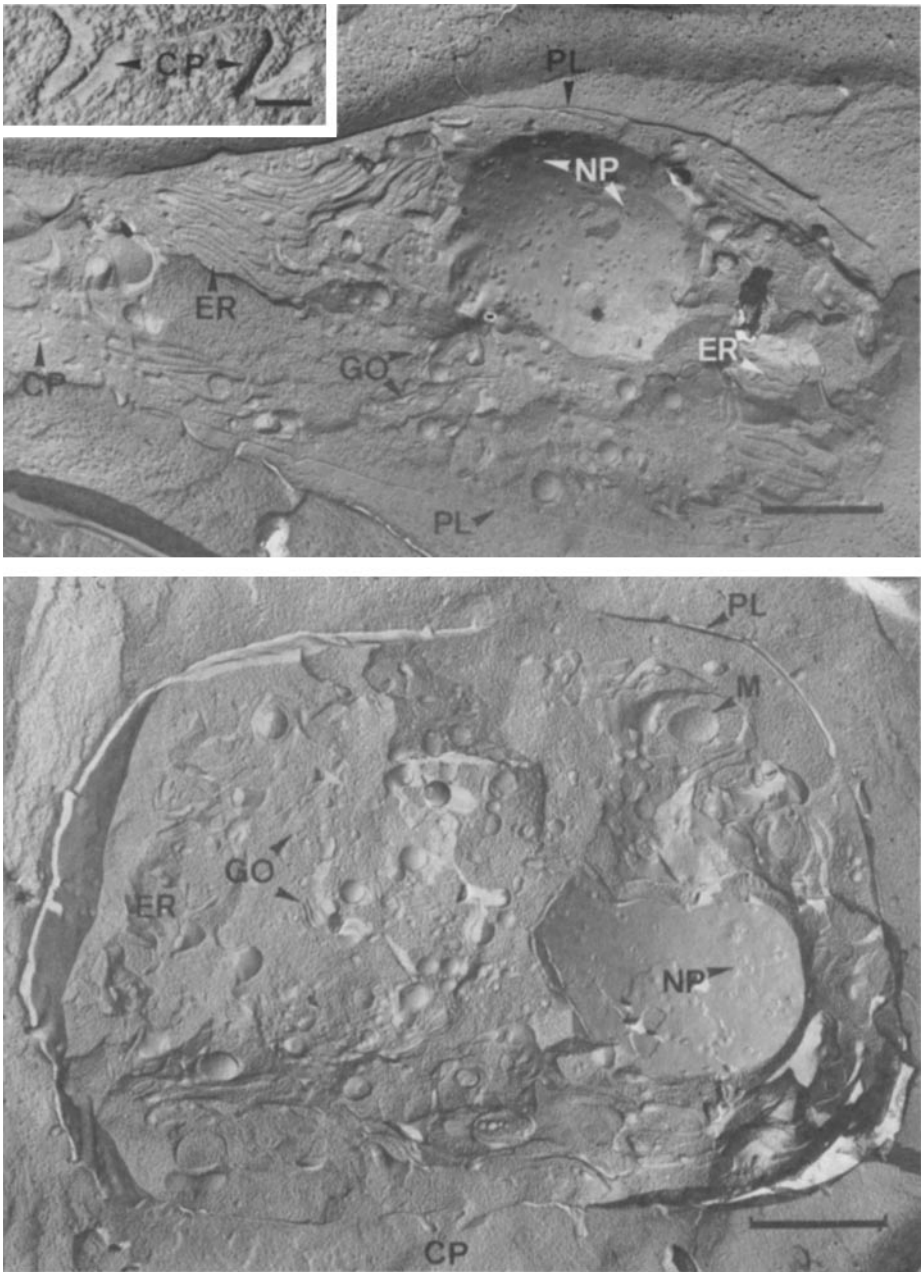
Replicas of both a proliferating and a hypertrophic chon-

drocyte processed by HPF/FE are represented in Figs. 1 and 2, respectively. The longitudinal cross-sectional form in each case, ellipsoidal for proliferating and rectangular-to-circular for hypertrophic, was found to be in accordance with light microscopic observations on native tissue (41, 46). These cell forms were preserved also within cartilage tissue processed by both HPF/FS (Figs. 3 and 4) and RHT-CF (Figs. 5 and 6) and differed considerably from those observed after routine chemical fixation (39, 43).

Chondrocytic plasmalemmata were maintained intact and in close apposition to intercellular matrix in tissue processed by HPF/FE, HPF/FS, and RHT-CF, whereas in tissue preserved under standard chemical fixation conditions they are consistently ruptured and dislocated from the intercellular matrix (39, 43). Slender cell processes, which were partially masked in chondrocytes processed by RHT-CF (Figs. 5 and 6), were clearly visible after HPF/FE (Figs. 1 and 2) and HPF/FS (Figs. 3 and 4).

Chondrocytes cryofixed by HPF/FS revealed a substantial improvement in the preservation quality of intracellular organelles compared with those in chondrocytes processed by RHT-CF. The membranes of all the intracellular organelles were preserved intact and their trilaminar structures were readily discerned (Figs. 3, 4, and 8). A feature characteristic of chondrocytes preserved by RHT-CF (and standard chemical fixation procedures [39, 41]) was the presence of many intracellular vacuole-like spaces (Figs. 5 and 6) whose formation had caused shifting of the cytoplasmic ground-substance and intracellular organelles. These vacuoles were not formed during processing by either HPF/FE or HPF/FS.

Nuclear membranes of both proliferating and hypertrophic chondrocytes processed by both HPF/FE and HPF/FS were preserved intact. Although these membranes were well preserved in proliferating chondrocytes processed by RHT-CF, nuclear membranes of cells in the hypertrophic zone of the same tissue were frequently observed to be ruptured (Fig. 6). Distinct differences between peripheral heterochromatin and central euchromatin were visible in the nuclei of chondrocytes



FIGURES 1 and 2 Fig. 1 (*top*): Electron micrograph of a proliferating chondrocyte after processing by rapid HPF/FE. The cell appears ellipsoidal in shape and is enclosed by a plasmalemma (*PL*), the smooth contour of which is interrupted by numerous slender cell processes (*CP, inset*). Within the cell, rough endoplasmic reticulum (*ER*) and Golgi organelles (*GO*) are clearly apparent. Along the nuclear surface, nuclear pores (*NP*) are irregularly distributed. Bar, 2 μm. × 7,800. *Inset*: Bar, 0.2 μm. × 37,000. Fig. 2 (*bottom*): Electron micrograph of a hypertrophic chondrocyte after processing by rapid HPF/FE. The cell appears rectangular in shape, and numerous cell processes (*CP*) are apparent projecting from the smooth plasmalemma (*PL*). Within the cell, several small Golgi organelles (*GO*) are clearly visible. Mitochondria (M); rough endoplasmic reticulum (*ER*); nuclear pores (*NP*). Bar, 2 μm. × 8,900.

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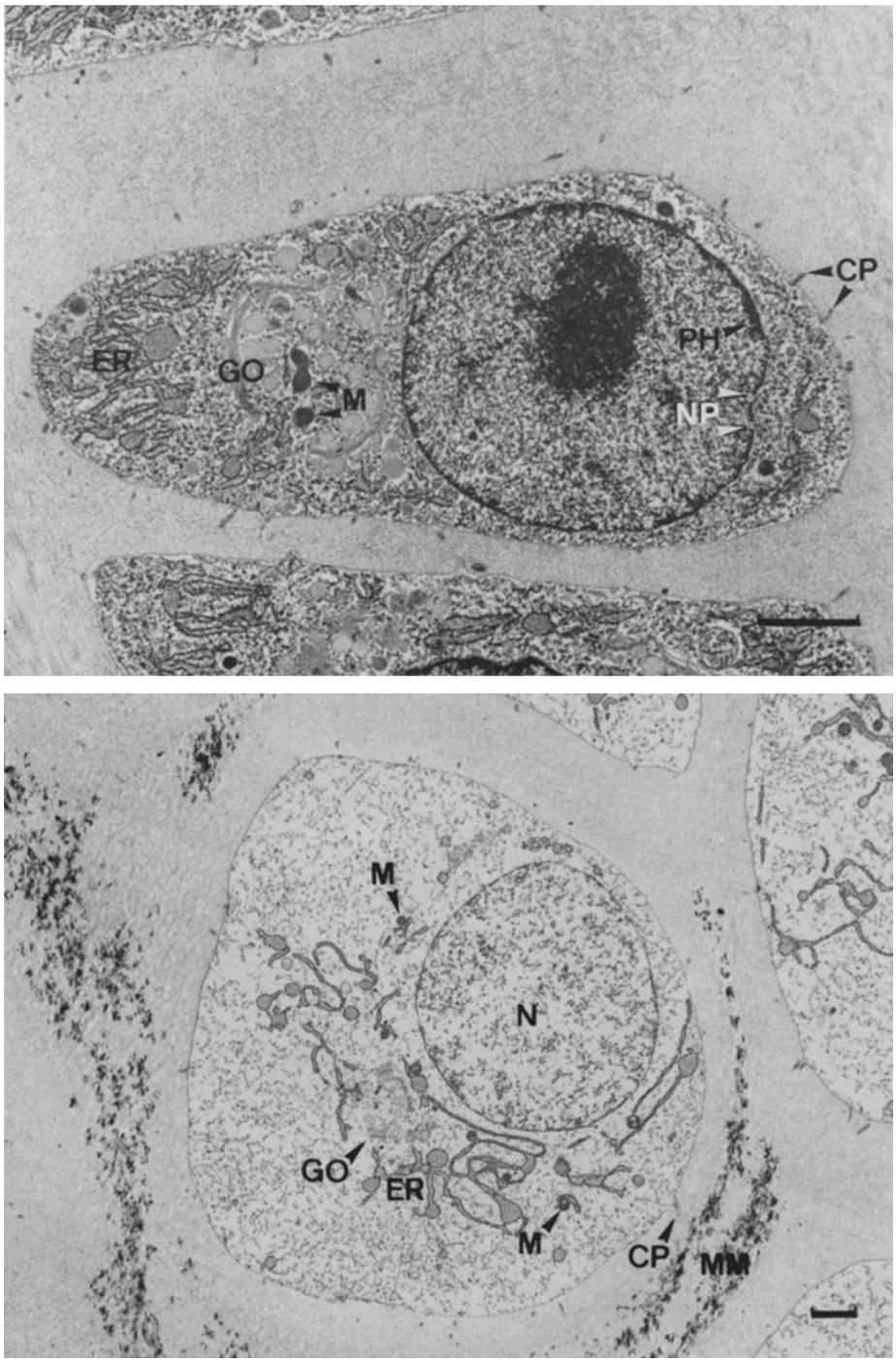


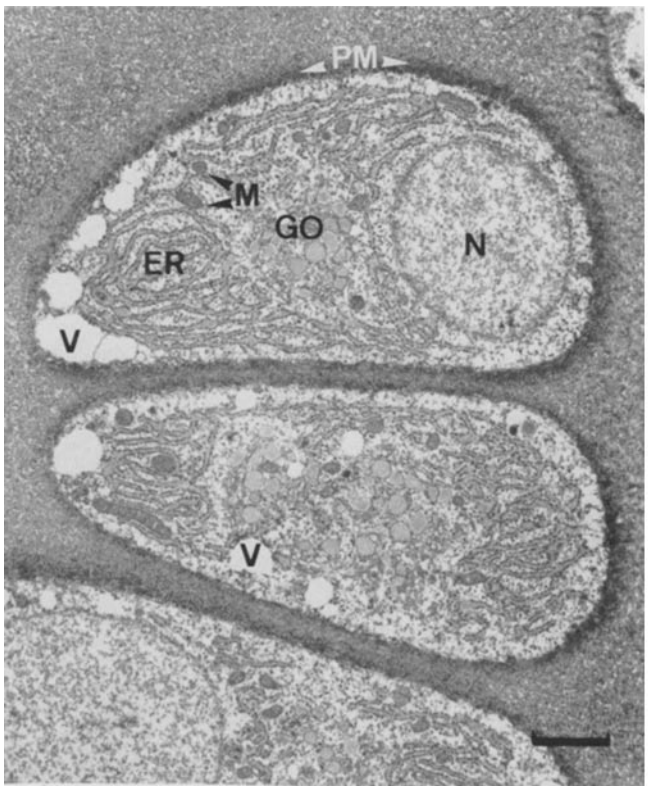
FIGURES 3 and 4 Fig. 3 (*top*): Electron micrograph of a proliferating chondrocyte following processing by HPF/FS. The ellipsoidal shape of the cell is preserved and the numerous fine cell processes (*CP*) are prominent. Nuclear pores (*NP*), peripheral heterochromatin (*PH*), Golgi organelles (*GO*), mitochondria (*M*). Bar, 2 μm. × 6,900. Fig. 4 (*bottom*): Electron micrograph of a hypertrophic chondrocyte (deep within the mineralizing zone) following processing by HPF/FS. The cell is spherical in shape and is clearly delineated by an intact plasmalemma; all cytoplasmic organelles and the nucleus (*N*) appear to be morphologically integral. Golgi areas (*GO*), rough endoplasmic reticulum (*ER*), mitochondria (*M*), mineralized interterritorial matrix (*MM*). Cell process (*CP*). Bar, 2 μm. × 2,800.

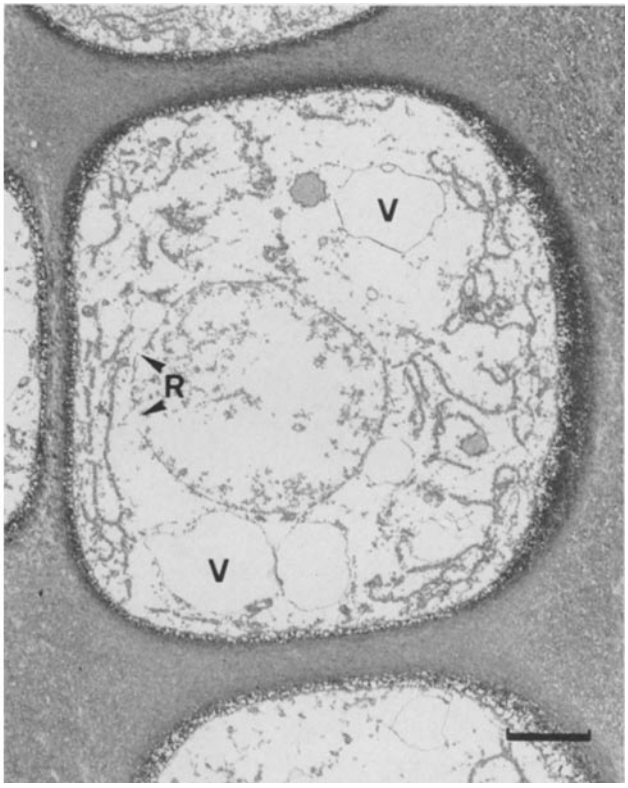
fixed by HPF/FS (Figs. 3 and 4), but such differences were not clearly discernible in the nuclei of chondrocytes processed by RHT-CF.

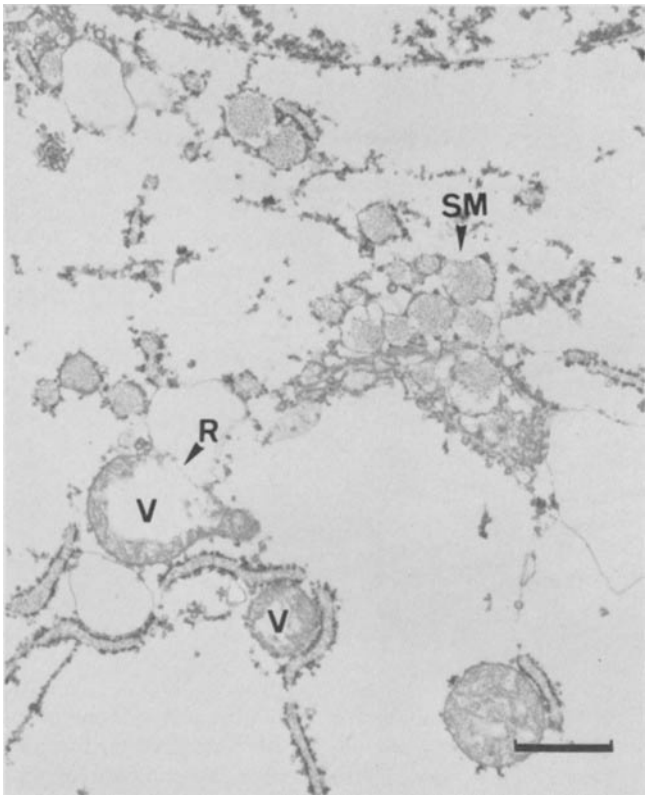
In chondrocytes preserved by HPF/FS, the fine structural details of the rough endoplasmic reticulum were immediately apparent (Fig. 8) and ribosomes were present as discrete entities lying along a clearly delineated reticular membrane. In tangential section, ribosomal polysomes could be readily distinguished. Although rough endoplasmic reticulum was discernible in chondrocytes processed by RHT-CF, fine ultrastructural details were frequently lost.

Rarely were more than one or two Golgi organelles apparent in chondrocytes of cartilage tissue processed by RHT-CF. Those within cells of the proliferating zone were well preserved but the cisternal membrane systems of Golgi complexes in hypertrophic cells were completely absent. Their former occurrence was apparent only from the presence of Golgi-associated vesicles and secretory granules (Fig. 7). It seems probable that Golgi membranes were solubilized during chemical fixation since the organelles were more numerous and composed of densely packed intact cisternal membrane stacks in hypertrophic chondrocytes of tissue processed by both HPF/FE and HPF/FS (Figs. 8, and 9) (38, 40, 78, 98).

Secretory granules were more abundant in chondrocytes processed by both HPF/FE (Fig. 9) and HPF/FS (Fig. 8) than in those preserved by RHT-CF (Fig. 7). Differences in struc-







FIGURES 5–7 Fig. 5 (*top left*): Electron micrograph of a proliferating chondrocyte processed by RHT-CF. The ellipsoidal shape of each cell is preserved, but the slender cell processes are not apparent, possibly due to obscuring by precipitated proteoglycans within the pericellular matrix (*PM*) compartment. Nucleus (*N*), Golgi-area (*GO*), rough endoplasmic reticulum (*ER*), mitochondria (*M*), artifactually formed vacuoles (*V*). Bar, 2 μm. × 5,100. Fig. 6 (*top right*): Electron micrograph of a hypertrophic chondrocyte processed by RHT-CF. The cell, rectangular in shape, is delineated by an intact plasmalemma. The nuclear membrane is, however, partially ruptured (*R*) and numerous artifactually formed vacuoles (*V*) are apparent. Bar, 5 μm. × 2,200. Fig. 7 (*bottom*): Electron micrograph of part of the cytoplasmic region of a hypertrophic chondrocyte processed by RHT-CF. The cisternae of the Golgi organelles are not apparent, probably due to solution during fixation, and the organelle-associated vesicles also exhibit partially solubilized membranes (*SM*). Mitochondrial membranes often appear ruptured (*R*) and the organellar structure is distorted due to the formation of large vacuoles (*V*). Bar, 1 μm. × 13,000.

ture and staining characteristics between granules within chondrocytes preserved by HPF/FS were so clear that it was possible to differentiate without difficulty between lysosomes, secretory granules, and vesicles (Fig. 8). Moreover, differences within the subpopulation of coated vesicles were also distinguishable.

Mitochondria preserved by RHT-CF were frequently observed to be ruptured, to contain vacuole-like spaces, and to be inhomogeneously stained (Fig. 7). In contrast, those preserved by HPF/FS were intact and densely stained (Fig. 8). In fact, the amount of stainable material retained (87) was so great that the infoldings of the inner membrane were not clearly visible. Examination of the fracture plane along the mitochondrial surface membranes in replicas of chondrocytes

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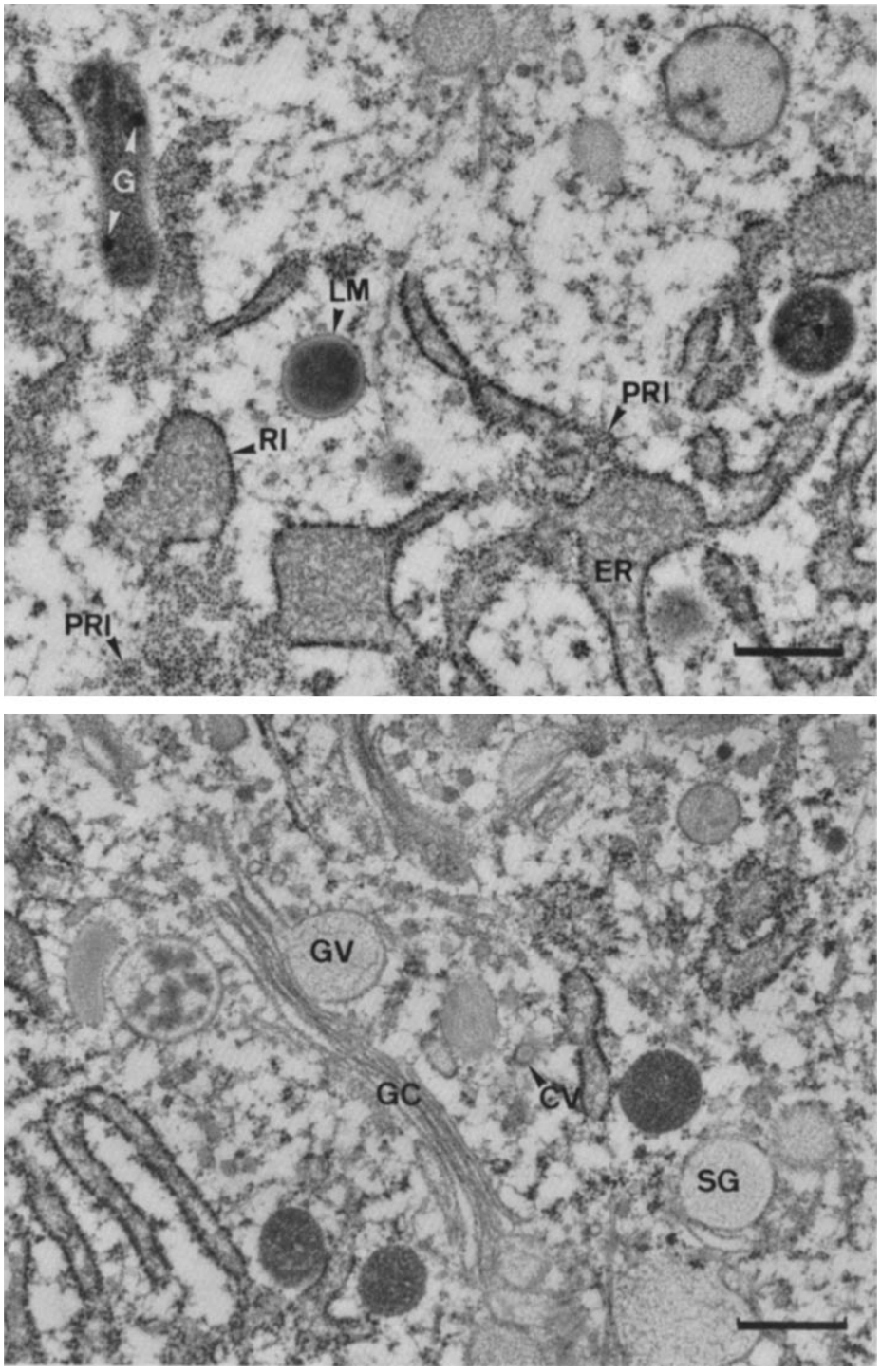

FIGURE 8 High power electron micrographs of the cytoplasmic region of a hypertrophic chondrocyte processed by HPF and freeze substitution. (*top*) Examination of rough endoplasmic reticulum (*ER*) sectioned perpendicularly reveals the ribosomes to be preserved as discrete entities (*RI*); the polysomal arrangement of these structures is clearly apparent in rough ER cut in tangential or oblique planes (*PRI*). Mitochondrial granules (*G*) may be readily distinguished within densely stained mitochondria (*M*). The well preserved trilaminar membrane of a primary lysosome is indicated (*LM*). Bar, 0.5 μm. × 30,000. (*bottom*) A Golgi complex, consisting of numerous cisternae (*GC*) and associated vesicles (*GV*), together with coated vesicles (*CV*) and a secretory granule (*SG*). Bar, 0.5 μm. × 30,000.

processed by HPF/FE revealed a patchy appearance (Fig. 9, inset) characteristic of membranes having mutual points of contact. Moreover, after processing by HPF/FS, the outer and inner mitochondrial membranes may be seen to be in continuous contact (Fig. 8). Since, on the basis of analysis after standard chemical fixation, the space existing between inner and outer mitochondrial membranes is believed to be on the order of 10–20 nm (95), it seems likely that this space is artifactually widened (40, 88). In cartilage tissue processed under standard chemical fixation conditions, mitochondrial granules were observed to be reduced in number or absent from cells in regions of mineralization and 'degeneration' (8, 9, 55, 99). Examination of cells throughout the epiphyseal plate in tissue processed by HPF/FS revealed no correlation between the numerical area density of mitochondrial granules and the extent of mineralization. All cells contained comparable numbers of calcium-rich granules, and this finding supports a recent observation made in frozen thin sections of cartilage tissue (51).

Examination of chondrocytes throughout the epiphyseal plate as a whole in cartilage tissue processed by HPF/FS revealed that all cells were morphologically integral, even those in the mineralizing and so-called 'degenerating' zones, including the terminal cell adjacent to the zone of vascular

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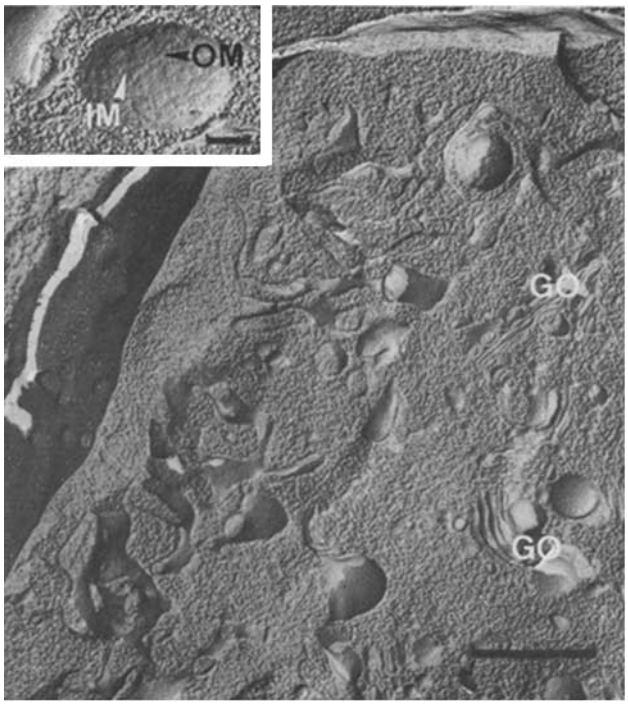

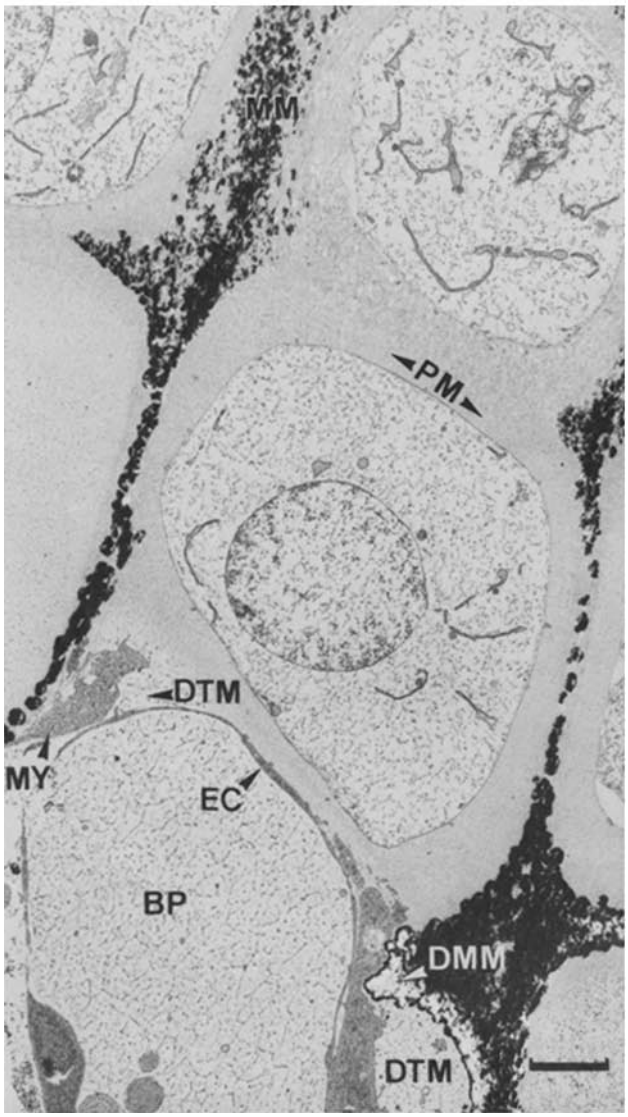

invasion (Fig. 10). On the basis of results obtained using standard chemical fixation techniques, cells in these regions were generally believed to be disintegrating. These observations are of considerable significance with respect to the theories of mineralization and vascular invasion currently debated, and their implications in this regard are discussed in the final section of this communication.

DISCUSSION

Many of the problems associated with and intrinsic to the fixation of cartilage tissue in aqueous media may be circumvented by employing purely physical methods, which in this investigation were exemplified by replicas of chondrocytes processed by HPF/FE. However, such replicas lend themselves mainly to membrane analysis studies and as such their usefulness is limited. Clearly, it is the preserved material itself that has the potential to yield most information at the ultrastructural level. However, during the preparation of such material for electron microscopic examination, the use of certain chemical processing techniques cannot be avoided. These include the substitution of tissue water with a resin-miscible substance followed by infiltration with resin itself.

In the current investigation, a method of tissue processing is described which, by the adoption of low temperature conditions, minimizes the deleterious effects upon tissue associated with these stages under standard fixation conditions. The three stages of the tissue processing were thus: rapid high pressure freezing, freeze substitution fixation, and subzero temperature embedding.

The principle advantage of HPF is that native tissue may be frozen directly without the need for quenching in liquid coolants, which necessitates the use of antifreeze agents to prevent ice crystal growth, a consequence of the slow rate of cooling (15, 20, 25, 27, 69, 96). The addition of these agents is known to induce multiple artefactual alterations including intracellular substance losses (12, 23) and dislocation of membrane proteins (4, 30, 85). Alternative methods of freezing that avoid the use of antifreeze agents include the propane jet

FIGURES 9 and 10 Fig. 9 (*top*): Electron micrograph illustrating a representative portion of the cytoplasmic region of a hypertrophic chondrocyte processed by HPF/FE in which two Golgi organelles (*GO*) are visible. Bar, 1 μm. × 16,000. *Inset*: High power magnification of a mitochondrion. Examination of the fracture profile reveals patches of both outer (*OM*) and inner (*IM*) mitochondrial membranes. Bar, 0.2 μm. × 27,000. Fig. 10 (*bottom*): Electron micrograph of a terminal chondrocyte processed by HPF/FS. The cell, bound by an intact plasmalemma, appears morphologically integral and is surrounded by a pericellular matrix compartment (*PM*) within which no sign of degradation is apparent. In the region where the invading blood capillary endothelium (*EC*) contacts the intact transverse septum, fine monocytic cell processes (*MY*) may be seen. The white patches (*DMM*) within the mineralized longitudinal septa (*MM*) are formed as a result of solubilization of mineral content in the water used for section flotation. It is apparent from the electron micrograph that the water-rich interior of the blood capillary (*BP*, blood plasma) is less well preserved than the similarly water-rich environment within the hypertrophic chondrocytes. The existence of this situation is not, however, purely fortuitous but appropriately illustrates the role of the proteoglycan-rich pericellular matrix in acting as a cryoprotective agent deep within tissue blocks (>120 μm). *DTM*: Degraded territorial matrix. Bar, 5 μm. × 2,000.

(62, 96) and cooled metal block (33, 34, 37) methods. The techniques are, however, suitable for freezing only vesicle suspensions, cells, and tissue up to a thickness of 10–20 μm (17, 20, 96).

When pure water is pressurized to 2.1×10^8 Pa (2,100 bar), its freezing point is depressed to −22°C (45, 92). Consequently, the temperature range over which ice crystal growth can occur is reduced by 22°C (i.e., over the range −22°C–−80°C instead of that from 0°C to −80°C). It is to be expected that under similar pressure conditions the freezing point of cartilage tissue was depressed at least to −22°C and most probably to a lower value (6, 45, 71) and hence the critical temperature is further reduced. Moreover, the rapid rate at which the specimen was cooled (>7000° Cs^{-1}) assured that the passage through this range was achieved very rapidly (within ~9 ms). In addition, the structures of the ice crystals formed under a pressure of 2.1×10^8 Pa (2,100 bar) are of the types II, III, and IV (19, 24, 56, 91); the nucleation and growth rates of such crystals are considerably slower than the corresponding rates for type I crystals formed at atmospheric pressure (27, 57, 71, 75). Since tissue water was frozen within 40 ms of the initiation of the pressure build-up, pressure-induced structural alterations (demonstrated to be time-dependent [58, 94]) were avoided.

Proteoglycans within the intercellular matrix exist in an underhydrated state (36), and a consequence of this is that those water molecules present are associated with and highly oriented around these macromolecules. Water molecules thus ordered offer considerable resistance to the reorientation forces that operate during ice crystal formation (6). This antifreeze property of the cartilage matrix (6, 13, 25, 27) allows that thermal energy dissipated from the enclosed chondrocytes during freezing may be more effectively conducted away (since the thermal conductivity of liquid water is considerably higher than that of frozen water) before the matrix itself is frozen (25).

The next stage of the tissue processing consisted of substituting frozen tissue water with a resin-miscible substance. Methanol was chosen because of its rapid rate of substitution (65, 66, 90, 100) and because it has the smallest denaturing effect upon tissue proteins at low temperatures (10, 18, 68). Freeze substitution was initiated at −90°C in order to prevent ice-recrystallization processes (which begin at −80°C [3, 12, 56, 64, 84, 90]), and 95% of frozen tissue water can be expected to be substituted within 10 h at this temperature (65, 66, 100). The temperature was then increased to −60°C and then to −35°C (at which temperature resin infiltration and embedding processes were carried out) in order to initiate 'mild' glutaraldehyde fixation. The small amount of tissue water remaining when the substitution temperature was increased to −60°C and then to −35°C was probably that which was tightly bound to the surfaces of PG molecules and thus resistant to ice crystal formation (6, 27). This water was nonetheless completely replaced by methanol during these final stages of substitution.

Glutaraldehyde was added during substitution in an attempt to limit disordering of protein molecules which would otherwise occur during embedding. This process together with the dehydration step are considered to have a considerable disordering and denaturing effect upon protein structure under standard fixation conditions (i.e., at ambient temperature) (12, 52). Freeze substitution with methanol serves not only to replace frozen tissue water with a resin-miscible substance but also as a dehydration step. Since protein disordering and denaturation induced during dehydration and embedding have been shown to be extremely temperature-dependent (5, 35, 83, 93, 98), it seems unlikely that this was an important factor under the temperature conditions adopted in this investigation. Resin polymerization, which under standard conditions is carried out at 60°C, is also a stage during which protein denaturation can take place. In the current investigation, UV-ray-catalyzed resin polymerization was conducted at −35°C and hence this denaturing effect was avoided.

The results indicate that the preservation quality of chondrocytes processed by HPF/FS is superior to that of chondrocytes fixed by one of the best of the currently available chemical fixation techniques (i.e., RHT-CF). All epiphyseal chondrocytes together with their associated matrix compartments, including the terminal cell in the mineralizing zone adjacent to the region of vascular invasion, were found to be morphologically intact. This finding is contrary to that found after standard chemical fixation in which cells of the mineralizing and so-called 'degenerating' zones were observed to be disintegrating (2, 7, 9, 22, 72). Although a few authors believe that some of these cells may survive (16, 32, 39), the presence of these degenerating chondrocytes is an observation that has consistently been made and is thus believed by many authors to be a naturally occurring phenomenon. It is therefore not surprising that an explanation for this cellular disintegration has been sought in terms of its possible significance to the mineralization process. Cells of the degenerating and mineralizing zones are believed by some authors to die as a result of low oxygen tension and inadequate diffusion of metabolites (7, 9, 31, 76, 89). After disintegration, released enzymes are believed to degrade matrical proteoglycans, a high concentration of which have been shown to inhibit mineralization (1, 7). Mineralization is then initiated along the interterritorial matrix vesicles which are believed to originate from budded-off processes of living chondrocytes. Some workers in the field believe that degenerating cells may contribute to this population in the 'degenerating' and mineralizing zones since the density of such vesicles increases in these regions (1, 7, 72). On the basis of our results, this possibility, together with the idea that proteoglycan-degrading enzymes are passively released from degenerating chondrocytes, may be excluded.

It is generally believed that monocytes associated with the ingrowing vessels clear away cell and matrix debris in preparation for vascular invasion (1, 79, 80, 86). Since the terminal cell and its associated matrix, adjacent to this ingrowing vessel, has been observed to be intact, the possibility is now raised that these cells may be actively destroyed. If this is indeed the case, the concept of vascular invasion needs to be reassessed, since the destruction of living tissue (14, 29, 63, 82) raises problems of quite a different nature from those associated with the removal of cell and tissue debris. In a situation such as this, monocytes would initially have to penetrate an intact transverse septum; it is conceivable that the terminal chondrocyte could secrete an inhibitor of the antiinvasive factor that is known to be present in this matrix (44, 50). Thereafter, the terminal chondrocyte would have to be destroyed, and this requires that it is recognized as foreign by the invading monocytes. It is possible that following breakdown of the pericellular matrix, specific membrane-bound antigenic sites not previously accessible to the immune system are recognized as foreign by invading monocytes and consequently destroyed in a manner analagous to that of the lysing of senescent

erythrocytes (48, 49). An alternative explanation could be that complement-mediated cell lysis is induced after destruction of the proteoglycan matrix (11, 70). It may be of significance in this context that complement-activated cell destruction has been demonstrated to occur when polyanionic substances (of which a proteoglycan molecule is one) at the cell surface interact with polycationic compounds (e.g., lysozyme, which is known to be present in cartilage matrix) (53, 73, 81). However, it should be emphasized that the suggestions made regarding penetration of the transverse septum and active destruction of the terminal chondrocyte and its associated territorial matrix are purely hypothetical; we have not undertaken experiments to disprove or substantiate these ideas. Their inclusion is intended to provoke discussion and research into the new problems now raised by our findings.

The observations made in the current investigation that all cells together with their associated matrix compartment throughout the epiphyseal plate are morphologically, and by implication, functionally intact at the ultrastructural level imply, by analogy to the bone mineralization process, that mineralization and vascular invasion are controlled not by haphazard changes in local environmental conditions but, at least in part, by the chondrocytes themselves.

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