

# *Acta Medica Okayama*

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*Volume 47, Issue 2*

1993

*Article 5*

APRIL 1993

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## Abstract

Epiphyseal growth cartilage of the femoral head obtained from Wistar rats was investigated after fixation by a rapid-freezing and freeze-substitution. Liquid helium was used in order to achieve a fast cooling rate without ice-crystal damage during the rapid freezing. Use of the rapid-freezing and freeze-substitution procedure provided better ultrastructural preservation of the chondrocyte than conventional chemical fixation methods. This procedure allowed a more reliable approach to electron probe analysis. X-ray microanalysis of the specimens confirmed that calcium is not detected in the initial matrix vesicles as a result of the freezing process. The results suggest that calcium release from precipitates occurs in the free state without any detectable formation of hydroxyapatite at the initial stage of calcification and that calcium is not tightly bound to the matrix vesicles.

**KEYWORDS:** rapid-freezing and freeze-substitution, femoral head, epiphyseal cartilage, matrix vesicles calcification

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\*PMID: 8506756 [PubMed - indexed for MEDLINE]

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## Ultrastructure and X-Ray Microanalysis of Epiphyseal Growth Cartilage of Femoral Head Processed by Rapid-Freezing and Freeze-Substitution

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Epiphyseal growth cartilage of the femoral head obtained from Wistar rats was investigated after fixation by a rapid-freezing and freeze-substitution. Liquid helium was used in order to achieve a fast cooling rate without ice-crystal damage during the rapid freezing. Use of the rapid-freezing and freeze-substitution procedure provided better ultrastructural preservation of the chondrocyte than conventional chemical fixation methods. This procedure allowed a more reliable approach to electron probe analysis. X-ray microanalysis of the specimens confirmed that calcium is not detected in the initial matrix vesicles as a result of the freezing process. The results suggest that calcium release from precipitates occurs in the free state without any detectable formation of hydroxyapatite at the initial stage of calcification and that calcium is not tightly bound to the matrix vesicles.

**Key words :** rapid-freezing and freeze-substitution, femoral head, epiphyseal cartilage, matrix vesicles, calcification

Many attempts have been made to preserve the natural state of cartilaginous tissue and its ultrastructure without artifacts. However, it has been difficult to maintain the structure of the chondrocytes during the conventional chemical fixation with an aqueous media (1). Epiphyseal growth cartilage is an avascular tissue containing numerous proteoglycans (2). The chemical fixatives diffuse poorly from the extracellular matrix to the chondrocytes even by the perfusion method, and various structural alterations are induced by the chemical fixatives.

It has been revealed that the rapid-freezing and freeze-substitution procedure can minimize such alterations in the cartilage. The quality of ultrastructural preservation of the cryofixed specimens depends upon the speed of cooling (3). Previous reports have focused on the use of liquid nitrogen ( $-196^{\circ}\text{C}$ ) for the coolants (4-6). In the present study, we used liquid helium ( $-269^{\circ}\text{C}$ ) instead of liquid nitrogen in order to achieve a faster cooling rate. The rapid-freeze and freeze-substitution method can fix the

cartilage for a moment and retain the natural form including water-soluble components.

The mechanism of the initial calcification have been the subject of electron microscopic observations. The matrix vesicles with membrane-bound structure were detected in the calcifying zone of epiphyseal growth plates (7-8). Mineralization in the mitochondria or matrix vesicles was found in the chondrocytes of the growth plate by cryosection (9) and by anhydrous method (10-11). A histochemical stain with potassium pyroantimonate was employed with low specificity for the demonstration of calcium by the formation of an electron-dense precipitate visible at the ultrastructural level (12).

The rapid-freezing and freeze-substitution make it possible to retain and stabilize diffusible and extractable materials (13-14). This procedure was adopted for a more reliable approach to electron probe analysis of initial calcification in the cartilage for this study.

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## Materials and Methods

The epiphyseal growth cartilage in the present study was obtained from female Wistar rats, 3-4 weeks old of age, 65-75 g in weight. Initial calcification appears in the epiphysis at this stage of development. Femoral heads were removed quickly from the hips under ether. The specimens were divided parallel to their long axis into two blocks. They were transferred and mounted onto the holder of the Quick Freezing Device (QF-5000, Slammer, Polar-on Co. Ltd.). They were brought into contact with the polished copper surface which had been precooled with liquid helium (LHe:  $-269^{\circ}\text{C}$ ). After rapid freezing, they were transferred immediately into the liquid nitrogen (LN:  $-196^{\circ}\text{C}$ ).

The frozen specimens were substituted with 1% tannic acid in pure acetone at  $-80^{\circ}\text{C}$  for 24 h, and fixed with 2% osmium tetroxide ( $\text{OsO}_4$ ) in acetone at  $-80^{\circ}\text{C}$  for 48 h. In order to avoid recrystallization of the residual water in the samples, the fixed specimens were completely substituted with pure acetone refrigerated at  $-80^{\circ}\text{C}$  in acetone on dry ice.

The specimens thus treated were warmed gradually to room temperature in three steps (at  $-30^{\circ}\text{C}$  for 12 h,  $4^{\circ}\text{C}$  for 6 h and room temperature for 6 h), and rinsed several times in pure acetone. They were substituted with propylene oxide, infiltrated with epoxy resin (Epon 812) and embedded in this resin. Ultrathin sections were cut within 10-20  $\mu\text{m}$  below the tissue surface which were frozen onto a copper block. The sections were doubly stained with uranyl acetate and lead citrate. For control study, the epiphyseal growth plates were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) (15) and postfixed in 2%  $\text{OsO}_4$  in 0.1M cacodylate buffer (pH 7.4). They were sectioned in the same way.

Sectioned samples were examined with a transmission electron microscope (H-700, Hitachi, Co. Ltd.), and were analyzed in the electron probe X-ray microanalyzer (JEM-100C system) equipped with multichannel analyzer (Kevex Corp.). The electron beam, focused to 50-70 nm in diameter, was used for analysis of mitochondria, cytoplasm, and matrix vesicles with budding, or those disintegrated in the epiphyseal cartilage.

## Results

Initial calcification of the femoral head was early observed in female Wistar rats, 3-4 weeks old, 65-75 g (Fig. 1), extending from the epiphyseal to the diaphyseal ends of the growth plate. Proliferative (Fig. 2A) and hypertrophic (Fig. 2B) zones at the site of initial calcium deposition was rapidly frozen and freeze-substituted. Use of the rapid-freezing and freeze-substitution procedure yielded a marked improvement in the survey of the chondrocyte characteristics in comparison to those fixed with the conventional chemical fixation methods. Intracel-

lular organelles fixed with the rapid-freeze method were well preserved compared with conventional chemical fixation. Within approximately 10-15 micron below the tissue surface, the area of the sample in contact with the copper block precooled by liquid helium, appeared to be well preserved. Intercellular ice crystal damage spread at depths greater than 20 micron, those areas not in contact with the copper block.

The Golgi complex consisted of flattened disc-shaped cisternae, secretary granules and transport vesicles (Fig. 3A). Secretary granules containing electron dense materials were closely observed. Mitochondria matrices after the freeze-substitution appeared to be more dense than those in the conventional chemical fixation samples (Fig. 3B). Numerous mitochondrial dense granules were detected in the leaflets of the mitochondria. The rough endoplasmic reticulum (RER) appeared to be composed of parallel and flattened cisternae which contained more dense materials than by conventional chemical fixation. Terminal end of the expanded cisternae of the RER without rupture were observed. Plasma and nuclear membranes were observed to be intact and a part of opening of the membranes were detected (Fig. 3C). Dense bodies appeared to contain higher electron density than any other cellular components (Fig. 3D).

Instant budding of the matrix vesicles from the plasma membrane was clearly found in the hypertrophic zone (Fig. 4A). Extruded vesicles from the plasma membrane seemed to contain its intracytoplasmic dense content. Electron density of the matrix vesicles in the extracellular matrix varied. Rupture of the matrix vesicle and extracellular release of the contents was observed in the hypertrophic zone (Fig. 4B).

There were observations of artificial vacuoles, cell shrinkage, rupture of the intracellular membrane, loss of cytoplasmic matrix, and expansion of cellular organelles in the conventional chemical fixed specimens of the control study (Fig. 3E, F) but not in those processed by the rapid-freezing and freeze-substitution procedure.

The sectioned samples were examined with an electron probe X-ray microanalyzer system in order to detect the mineralization of the initial calcification of the cartilage. Electron microprobe analysis of the cartilage mineralization showed that calcium is either not detected in the initial matrix vesicles with budding, or is disintegrated in the epiphyseal cartilage as a result of the freezing process (Fig. 4). Spectrograms revealed no distinct peaks of a combined spectrum of calcium. Discharge and release of

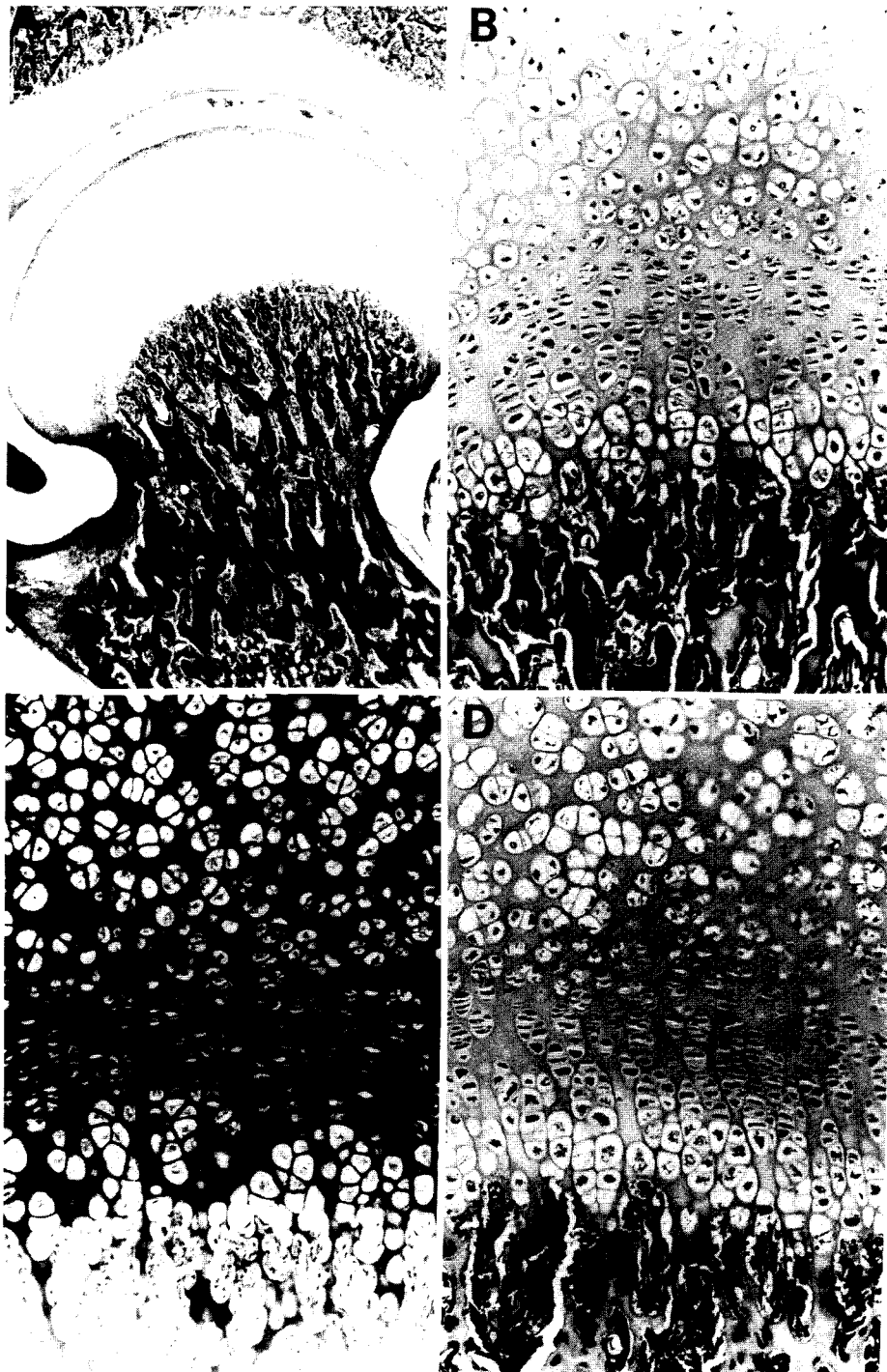


Fig. 1 The calcification developed in the epiphyseal cartilage of femoral head of Wistar rats, 3-4 weeks old, 65-75 g, female. A, B: H.E. stain, C: Safranin-O, D: Masson trichrome; Magnification: A  $\times$  110, B  $\times$  270, C  $\times$  270, D  $\times$  270.

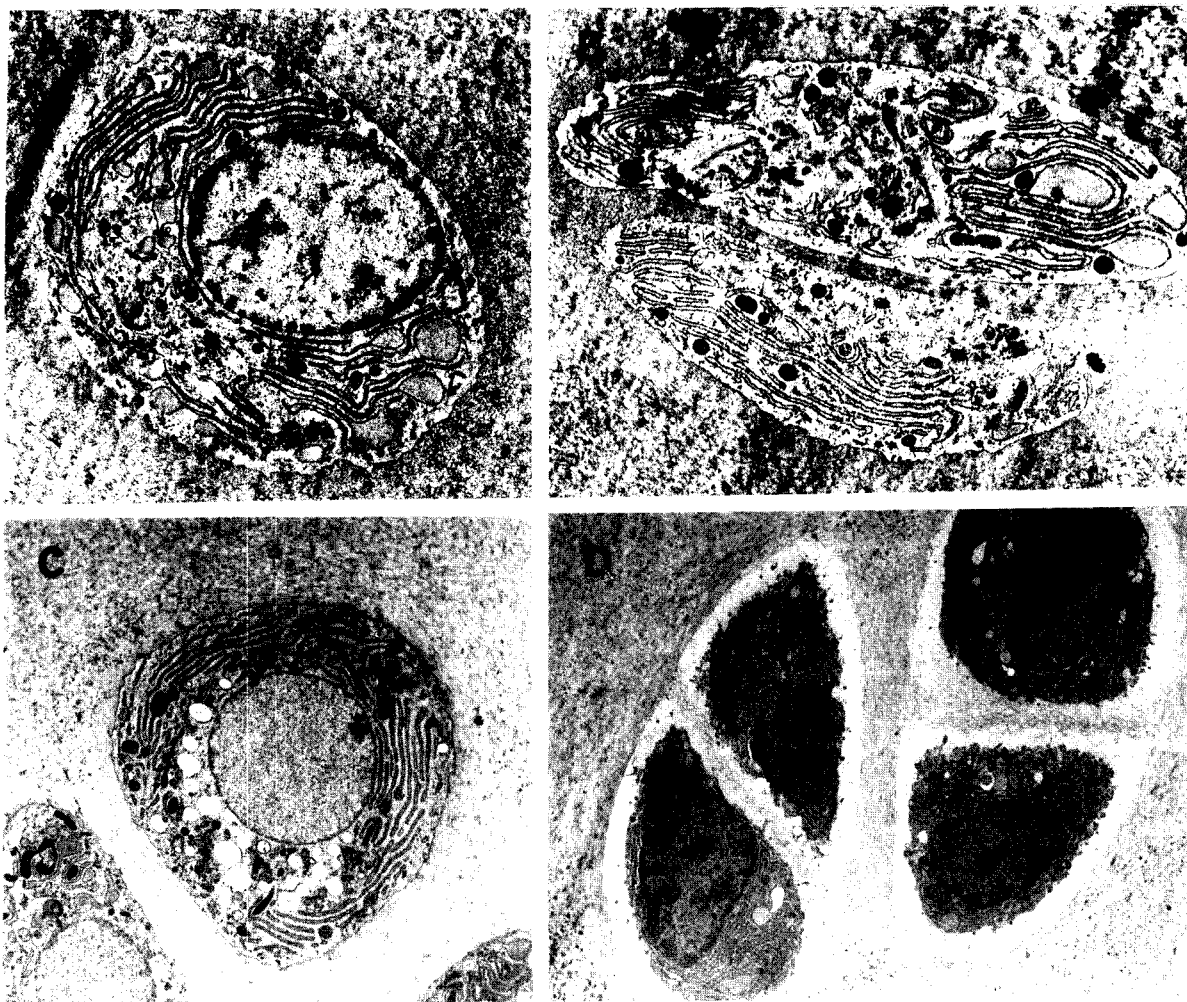


Fig. 2 A, B: The ultrastructure of the chondrocytes in the proliferative zone processed by rapid-freezing and freeze-substitution. A, B  $\times 6,000$ . C, D: The ultrastructure of the chondrocytes in the hypertrophic zone processed by rapid-freezing and freeze-substitution. C, D  $\times 6,000$ .

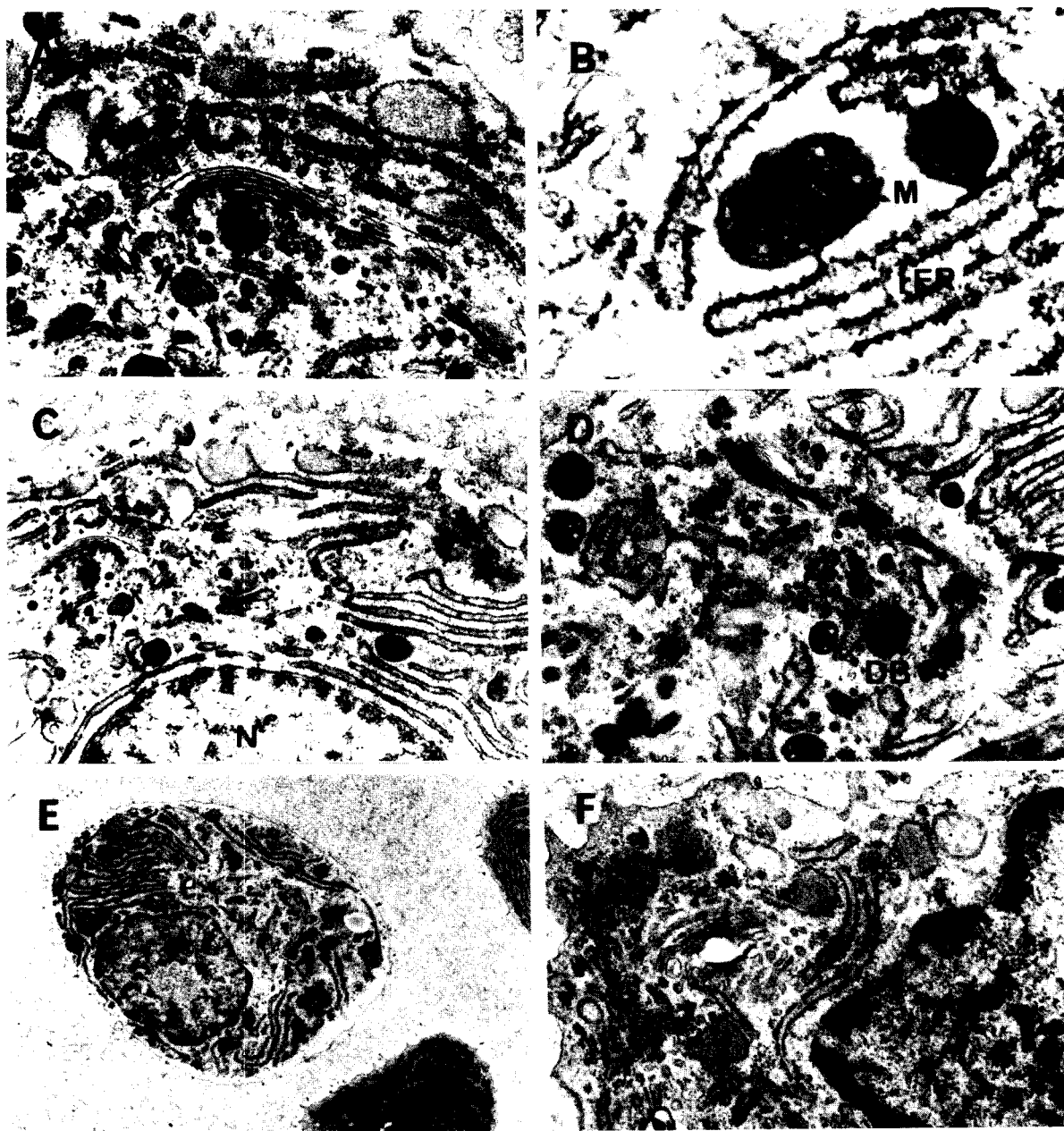
calcium was not found even at the different site or surroundings of the matrix vesicles.

### Discussion

Aqueous fixation has been the conventional technique used to observe ultrastructure of the cartilage (16-17). However, artifacts cannot be avoided during the processing of specimens for electron microscopy with chemical fixations. Infiltration of aqueous fixatives into the chon-

drocyte is a lengthy process due to a deficiency of vessels in the femoral head which contains abundant proteoglycan and collagen in the cartilage matrix. Osmotic imbalance of the conventional chemical procedures during the fixation renders the membrane structure fragile, distorted and vacuolated. Macromolecules and electrolytes are extracted and lost from the cell components during the aqueous fixation.

The rapid-freezing and freeze-substitution method is advantageous in the observation of the natural state of specimens. The quality of preservation of the ultra-



**Fig. 3** A: The Golgi complex (Go) consists of flattened disc-shaped cisterna, secretory granules and transports vesicles.  $\times 22,000$ .  
 B: Mitochondria (M) and rough endoplasmic reticulum (rER) following the rapid-freezing and freeze-substitution.  $\times 50,000$ .  
 C: The plasma membrane and nuclear membrane are preserved intact by the use of the cryofixation.  $\times 13,000$ .  
 D: Dense body (DB) seems to contain higher electron density than any other components.  $\times 17,000$ .  
 E, F: Alterations of the chondrocyte are often observed in the conventional chemical fixation. E  $\times 3,900$ , F  $\times 22,000$ .

structures in cryofixed specimens is a function of the size of crystal formation during the freezing procedure (3) which depends upon the speed of cooling. The cartilage

matrix contained a large quantity of proteoglycan but the cellular components are deficient in proteoglycan and abundant in water. It is suggested that proteoglycans

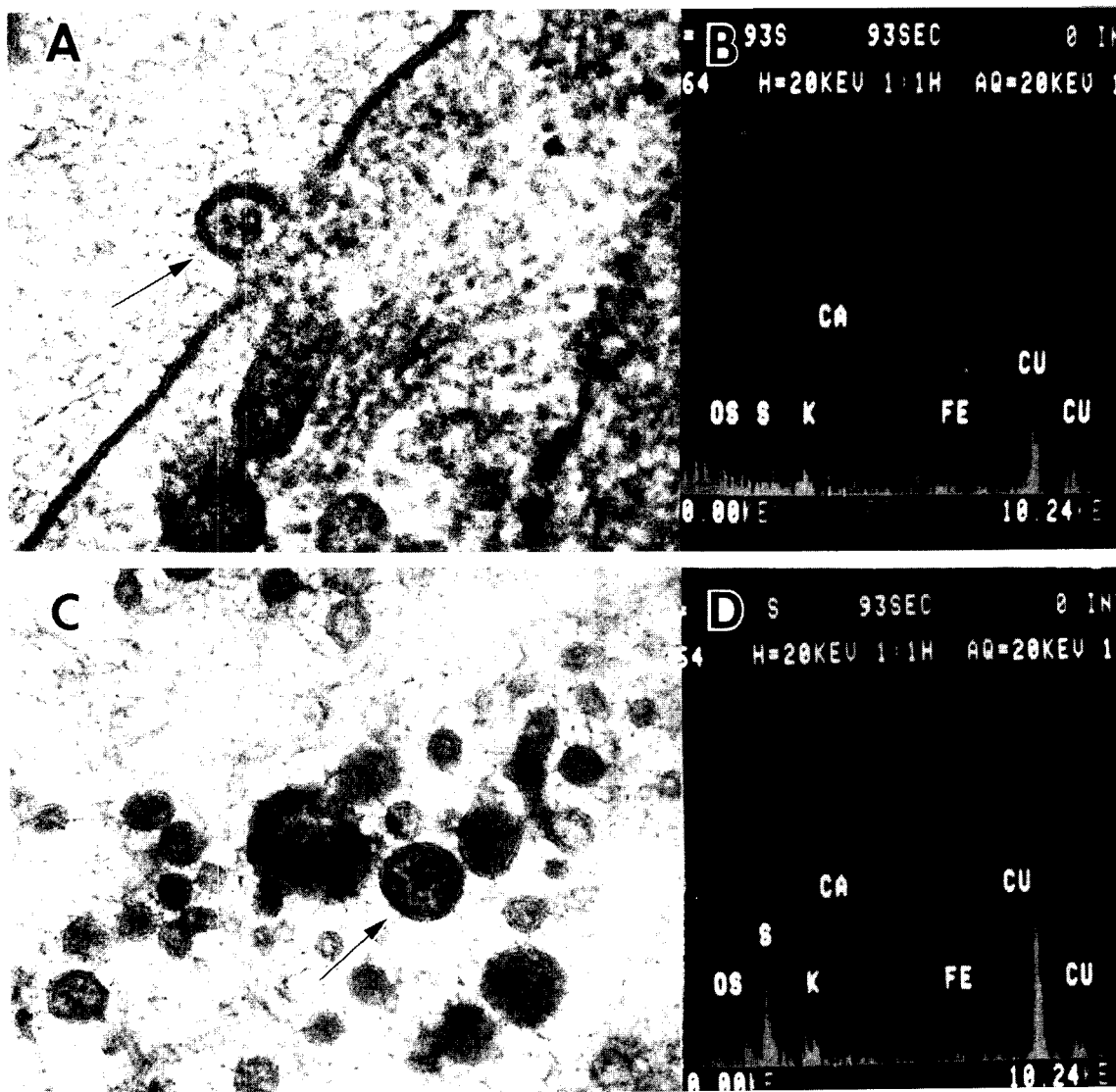


Fig. 4 A: The budding of the matrix vesicle from the membrane is detected in the hypertrophic zone.  $\times 126,000$ .  
 C: Various matrix vesicles are observed in the hypertrophic zone.  $\times 90,000$ .  
 B, D: X-ray microanalysis to the initial matrix vesicles where the arrows point is performed. Mark identified Ca peak.

seem to act as natural cryoprotectants. It is also suggested that the mineral component has a relatively low thermal conductivity (18). Thermal conductivity of the cartilage tissue differs between the cartilage matrix and cellular components. Therefore, in order to prevent damage during ice crystal formation, an extremely rapid cooling rate at the moment of the cryofixation is required. Previous studies used liquid nitrogen ( $-196^{\circ}\text{C}$ ) for the

coolants during the cryofixation (4-6). In the present study, liquid helium ( $-269^{\circ}\text{C}$ ) was used instead of liquid nitrogen in order to achieve a faster cooling rate. Moreover, the rapid cooling rate of the specimens was enhanced by the use of metal contact freezing with a copper block precooled in liquid helium. The cartilage matrix can absorb the shock of the metal contact and revert to a stable configuration. It is preferable to maximize cooling



rates by using cold blocks closer to the temperature of liquid helium instead of liquid nitrogen.

Phase change and the fluidity of membranes are particularly dependent upon the lipid composition (19). In a lipid bilayer, the hydrophobic chains of the lipids can be aligned to provide a rigid structure. As the temperature is decreased, the hydrophobic chains of fatty acid in the membrane will transform to an ordered state and conform the membrane to a stiff structure. As the fluidity of the membrane decreases, the permeability of the membrane to water and water-soluble molecules will be reduced. Hydrophobic molecules and water-soluble molecules can remain well preserved in the cytoplasm. Interaction of protein to protein on the plane of the membrane such as gap junction, tight junction, patch or cap is reduced. Use of the rapid-freeze and freeze-substitution can avoid certain undesirable alterations of the protein-protein interactions that occur within the membrane during the use of the chemical fixation (20-21). The rapid-freezing and freeze-substitution procedure is able to detect the existence of the integral membrane protein transporting the membrane. Since the ultrastructure of the membrane is well preserved by cryofixation, this technique may provide additional evidence of the transport across the membrane, and intercellular contact and communications such as signal transmission, or endocytosis in the future.

The matrix vesicles are the most probable sites for the initial calcification of the cartilage matrix (22). Ali and others, in the observation of the epiphyseal growth plate by the use of cryosection methods and electron probe microanalysis of the matrix vesicles, revealed that Ca/P mass ratio in the matrix vesicles increases with approach to the calcified zone. They also report that the matrix vesicle plays the most important role of the initial calcification of the cartilage (9). However, Landis and Glimcher, using the anhydrous method, reported an absence of Ca and P at the solid phase in the matrix vesicles. Heterodispersed electron dense granules (HEDG) were detected by them without the matrix vesicles and HEDG become hydroxyapatite in the calcified zone (11).

Rapid-freezing and freeze-substitution was adopted for a more reliable approach to electron probe analysis of the initial calcification. X-ray microanalysis of the present specimens confirmed that calcium is not detected in the mitochondria or matrix vesicles as a result of the freezing process. The results suggest that calcium release from precipitates occurs in the free state without any detectable

formation of hydroxyapatite at the initial stage of calcification and that calcium is not tightly bound to the initial matrix vesicles. The mechanism of this calcium release in the matrix vesicles may be related to the weak concentration and bond of the calcium binding protein. Uptake and retention of calcium in the mitochondria at the initial mineralization would require little energy. The mitochondria exhibits a low affinity for Ca and slow initial rate of uptake. Calcium release from the mitochondria may be related to the low oxygen retention due to the avascular area in the cartilage at the initial calcification. The matrix vesicles, which originate by budding from chondrocytes and then disintegrate, contain no insoluble amorphous calcium phosphate (ACP) or hydroxyapatite (23). Our results suggest that  $\text{Ca}^{2+}$ , phosphate and phospholipid are organized in the inner leaflets of the matrix membranes, and the formation of ACP or hydroxyapatite structures which subsequently lead to calcification occurs on the outer surface of the disintegrated matrix vesicles.

**Acknowledgments.** We are grateful to Prof. Takuzo Oda and Prof. Toshitaka Akisaka for their kind guidance and encouragement. The skillful technical assistance of Miss Fumiko Kanoh is kindly acknowledged. A part of this study was given as an oral presentation at the 5th Meeting of SIROT, Montreal, September 7-10, 1990.

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Received July 24, 1992; accepted September 16, 1992.