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J. Appleton
*University of Liverpool*

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# X-RAY MICROANALYSIS OF GROWTH CARTILAGE AFTER RAPID FREEZING, LOW TEMPERATURE FREEZE DRYING AND EMBEDDING IN RESIN

J. Appleton

E.M. Unit, School of Dental Surgery,
University of Liverpool,
P.O. Box 147,
Liverpool L69 3BX, U.K.
Phone No.: 051 709-0141 Ext. 2942

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## Abstract

This paper reviews the work undertaken on the X-ray microanalysis of growth cartilage particularly in relation to matrix calcification. Attention is focused on the methodology available to accurately localize and retain the elements of interest. A method is described which involves rapid freezing, low temperature freeze drying in a custom built device, and embedding in Spurr resin. The results show that little tissue damage occurs and that elements of physiological interest are accurately localized at the sub-cellular level. Where damage occurs to chondrocytes as a result of freezing, however, dense intra mitochondrial granules are seen suggesting a phase transformation takes place resulting in the precipitation of calcium phosphate. Further improvements may be made in retaining the more labile elements such as K by using resins which polymerize at low temperature.

Key Words: X-ray microanalysis, cartilage, freeze drying, resin embedding

## Introduction

Growth cartilage, epiphyseal plate and mandibular condylar cartilage in particular, have been the subject of intensive investigation by X-ray microanalytical methods since the inception of the technique. Growth cartilage was given this attention because of the interest in the complex series of events which lead to the ordered deposition of an inorganic phase in the organic matrix and because the inorganic phase was present in sufficient concentration to be detected by equipment available at the time. For example the early work of Brooks et al (1962) and Tousimis (1966), showed that calcium and phosphorus could be detected in the matrix of epiphyseal growth cartilage and that its relative concentration varied according to the site undergoing analysis.

Much of the earlier work on the X-ray microanalysis of cartilage gave no regard to the artefacts of tissue preparation. Hall et al (1971, 1973), demonstrated the presence of calcium and phosphorus in the membrane bound osmiophilic extracellular matrix vesicles described by Bonucci (1967, 1970) and Anderson (1969) as the initial site of apatite nucleation in cartilage. These studies used cartilage from the costo-chondral junction of guinea pigs fixed in aqueous buffered 1% osmium tetroxide. Ali (1976) and Ali et al. (1977b) using conventionally fixed but unstained calcifying cartilage from the rabbit described the increased accumulation of calcium and phosphorus in matrix vesicles as the zone of extracellular matrix calcification approached. It is now very clear that any exposure to aqueous media, however brief, results in the loss or translocation of ions of physiological interest (Boothroyd 1964, Thorogood and Craig Gray 1975, Bishop and Warshawsky 1982).

However a repetition of the work using cryofixation and cryoultramicrotomy produced similar results with matrix vesicles which contained on X-ray microanalysis material with a high Ca/P molar ratio indicative of hydroxyapatite (Ali et al 1977a). Additionally the chondrocytes contained electron dense intra-mitochondrial granules with a Ca/P molar ratio more characteristic of amorphous calcium phosphate. Clearly the Ca/P molar ratios so

determined and presence or absence of intramitochondrial granules are related to the tissue preparation techniques.

Recent work in our laboratory (Appleton et al 1985) suggests that the presence of mitochondrial granules is indicative of chondrocyte damage which can occur during rapid freezing. The studies of Landis (1979) and Landis and Glimcher (1982) on cryosectioned cartilage confirmed that differences exist in the Ca/P molar ratios between mitochondrial granules and non-membrane bound extracellular particles which they suggest are the initial site of matrix calcification. No signal for calcium was found in the extracellular matrix vesicles and there was no significant difference between the intra-vesicular and extra-vesicular phosphorus. It was suggested therefore that the presence of intra vesicular calcium and phosphorus may be an artefact of tissue preparation. However Ozawa & Yamamoto (1983), Morris et al. (1983) using anhydrous methods for the examination of rat epiphyseal growth plate detected mineral deposits within or in close association with matrix vesicles. These mineral deposits were confirmed by using energy dispersive X-ray microanalysis. No other mineral deposits were found at other sites preceding matrix vesicle calcification. The necessity of avoiding aqueous media would tend to invalidate the study of Davis et al. (1982) who described a specific distribution of calcium and phosphorus around hypertrophic chondrocytes from chick cartilage fixed initially in aqueous solutions of glutaraldehyde.

Boyde and Shapiro (1980) using separated intracellular and extracellular components of cartilage found on X-ray microanalysis high extracellular K levels. High levels of intra and extracellular Na and K were also found in rat epiphyseal (Barckhaus et al. 1981, Krefting et al. 1981, 1984) and chicken growth plate (Hargest et al. 1985). It was also shown by Quint et al. (1982) that significant amounts of K were not extractable when treated with buffer and it was therefore suggested that these ions were bound to negatively charged macromolecules such as proteoglycans.

This study describes some of the X-ray microanalytical results obtained after rapidly freezing cartilage, freeze drying and embedding in resin. All of these measures are intended to minimise artefacts due to specimen preparation and thereby produce an accurate representation of element distribution. Improvements in fast freezing techniques and consequent minimising of ionic shifts have diverted attention from the solid intracellular and extracellular inorganic phases. Efforts are now also being focused on the distribution of ions in the chondrocytes and in the organic matrix before a solid phase appears. Therefore more effort is being directed towards understanding the role of cells in the calcification process.

## Rapid freezing and freezing drying of cartilage

A rapid rate of freezing is best achieved by using liquid nitrogen cooled liquid propane at several degrees below its equilibrium freezing point, i.e., at 93K (Elder et al 1981). The aim is to achieve a rate of cooling in which the ice crystals formed are so small, at least at the surface of the specimen, that they are beyond the limit of resolution of the electron microscope and therefore cause minimal tissue disruption or elemental redistribution. Condylar cartilages were removed from 5 day old black and white rats anaesthetised with Nembutal. The smallest pieces of tissue which could be practically manipulated were mounted on thin flat copper discs and flash frozen in liquid propane, (Lyon et al. 1985, Appleton et al. 1985). The propane was produced by allowing the gas to pass through a coiled copper tube immersed in liquid nitrogen according to the method of Elder et al. (1981) (Fig 1). The discs were removed from the propane and stored in liquid nitrogen prior to rapid transfer onto the stage of a custom built freeze drying device the chamber of which had been flushed with Argon (Lyon et al. 1985). The stage was pre-cooled to 150K prior to the transfer of the discs. There may be some slight initial rise in the temperature of the specimen but not enough to bring about freeze drying. Although specialised freeze drying devices are commercially available they were either too expensive or unable to maintain a low enough temperature to prevent ice re-crystallization during freeze drying. Some low temperature freeze drying devices have been described (Stowell 1951, Hanzon and Hermudsson 1960, Frederik and Klepper 1975, Sjostrand and Kretzer 1975, Terracio and Coulter 1975, Coulter and Terracio 1977, Edelmann 1977, Zs Nagy et al. 1977, Coutler and Terracio 1978) but the apparatus described in Fig 2 was designed to be inexpensive, ubiquitous and easy to operate. Furthermore it was designed to allow polymerization of low temperature resin on the cold stage.

The freeze drier consists of a perspex chamber containing a thermostatically controlled cold stage cooled by means of a conduction bar. The chamber was maintained under vacuum by a rotary pump. A problem with perspex is that it absorbs most incident ultraviolet radiation. A requirement was that the freeze drier could subsequently be used for the polymerization of low temperature resins in situ using ultraviolet light (Carlemalm et al. 1982). Therefore, a silica glass window, transparent to this form of radiation was incorporated into the lid. Aluminium was chosen for the conduction bar as it has a lower thermal conductivity than copper or brass, but a lower specific heat capacity, and therefore thermal mass. In practice this allowed adequately low specimen temperatures to be maintained while ensuring economical use of liquid nitrogen. Thermal integrity between the aluminium stage assembly and conduction bar was achieved by having matching tapers on these components which then interlocked. The

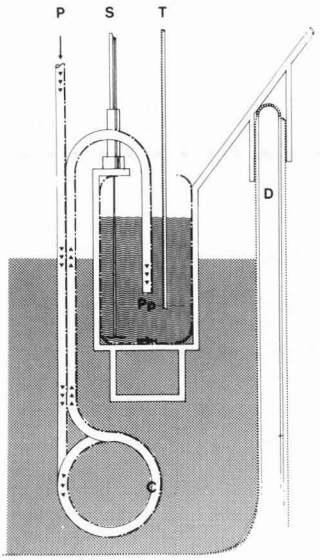


Fig 1 Diagram of apparatus used to produce liquid propane. The gas (P) enters the copper tubing which is coiled (C) and immersed in liquid nitrogen. The liquid propane (Pp) is collected in a plastic container supported by a bracket which clips onto the wall of a vacuum flask. A stirrer (S) is connected to an electric motor via a flexible drive and the temperature of the liquid propane is monitored by a thermometer (T). The whole apparatus is enclosed in a fume cupboard for safety.

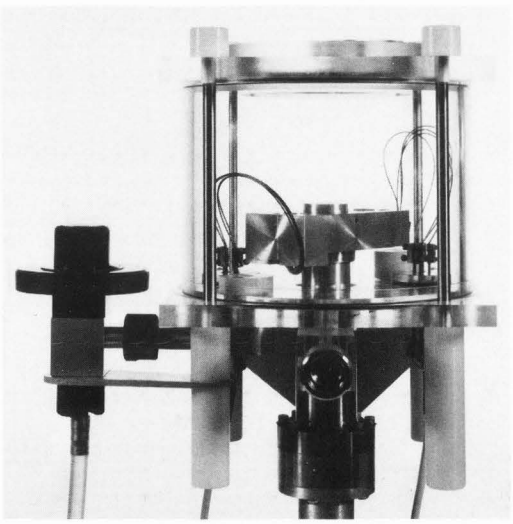

Fig 2 The cast Perspex chamber of the freeze drying device containing the specimen stage to which are connected heaters and sensors and from which a conduction bar passes through the base of the chamber into a 25l Dewar flask of liquid nitrogen which cools the stage. Vacuum ($10^{-3}$ Torr) is obtained via a rotary pump and the chamber may be flushed with Argon, through a needle valve, before opening.

conduction bar projects above the stage and being colder at 80K acts as a cold finger for water vapour from the drying specimens.

The specimen stage has depressions machined on either side of the condenser to accept specimen stubs and a vessel containing low temperature resin could thus be used. A perfectly flat ground copper plate was inserted into the specimen side and thermal contact was maintained with the stage by means of conducting paste. Beneath each depression a small resistance heater and thermocouple were fitted. These were connected to a control and monitoring device.

During freeze drying the temperature was allowed to rise to 188K and maintained at this temperature until the tissue pieces were dry. Drying was complete as determined by visual examination in 24-48h after which the specimens were removed from the vacuum and the tissue was embedded in Spurr (1969) resin. With Spurr resin the tissue was kept under vacuum for 2-3 days at room temperature and then polymerised at 333K overnight. Some tissue was also exposed to osmium vapour prior to embedding. Embedding in resin facilitates tissue orientation and routine thin sectioning while avoiding the use of chemicals and thereby minimising potential artefacts. (Hohling et al. 1970, 1972, Hohling 1972, Ingram et al. 1974, Ingram and Ingram 1975, Sjostrand and Kretzer 1975, Hohling et al. 1976, Barckhaus and Hohling 1978, Hargest et al. 1985, Appleton et al. 1985).

## X-ray microanalysis of sections

Sections of unosmicated tissue 500 nm thick were cut dry with glass knives and placed on gold or copper grids and given a thin conductive coat of carbon in an Emscope sputter coater equipped with a carbon coating attachment. Gold grids were used for analysis. The grids were placed in a graphite holder and examined at 80kV in both the transmission (TEM) and scanning transmission mode (STEM) of a JEOL 100CX electron microscope equipped with a liquid nitrogen cooled anti-contamination device, liquid nitrogen cooled baffles, and a Kevex detector together with a Link System 860 pulse processor. The holder was tilted at 30° and the peak to background ratio was improved by the use of a hard X-ray kit (JEOL UK Ltd) and molybdenum fixed apertures. Analyses were carried out in the STEM mode at 80kV with a 200 nm spot size. The chlorine present in the Spurr resin was used as a standard reference so that each analysis was carried out for a fixed integral of 1000 counts for the chlorine peak. Attention was concentrated on the early hypertrophic and hypertrophic zones of the cartilage (Appleton, 1969) mitochondria, cytoplasm and inter-cellular matrix were analysed. The relative mass fractions of Na, K, Mg, P, S and Ca were obtained. In each case a window of 160eV wide was established and the total count recorded. The background was calculated as the mean of measurements on either side of the peak. A

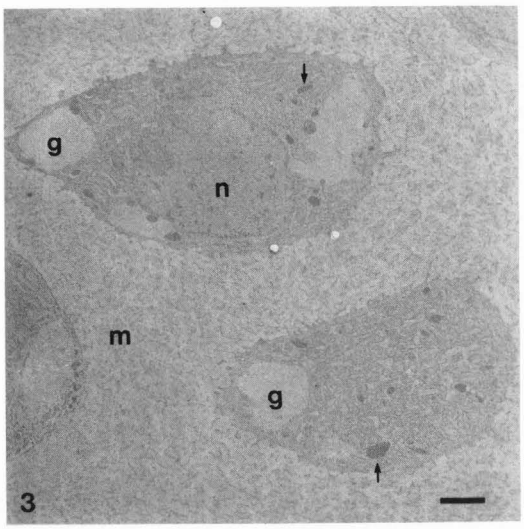


Fig 3 Chondrocytes of early hypertrophic zone after freeze drying, exposure to osmium vapour, and embedding in Spurr resin. Typically the chondrocytes contain mitochondria (→) and polarized accumulations of glycogen (g). The nuclei (n) and matrix (m) exhibit some ice crystallite damage. Bar = 2μm.

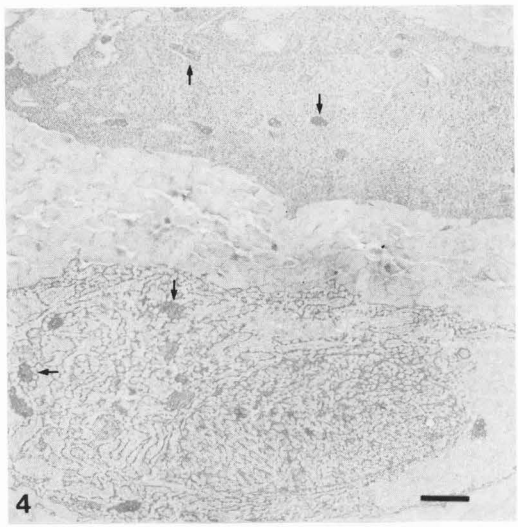


Fig 4 Adjacent chondrocytes exhibiting different amounts of ice crystallite damage. In the lower chondrocyte there is considerable damage and the mitochondria (→) contain numerous intramitochondrial granules. Osmicated cartilage. Bar = 2μm.

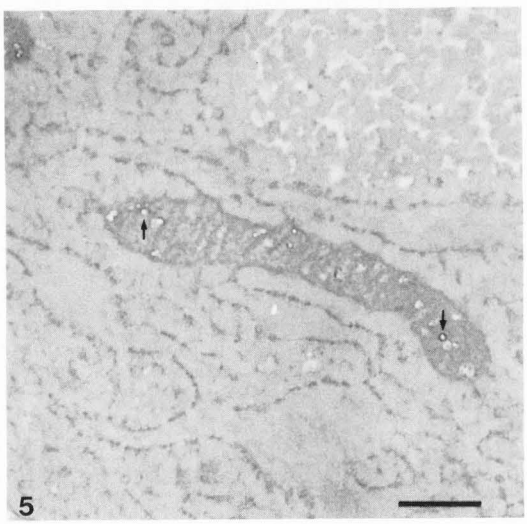


Fig 5 Detail of mitochondria from damaged cell showing spaces (→) previously occupied by mitochondrial granules removed during processing. Bar = 0.5μm.

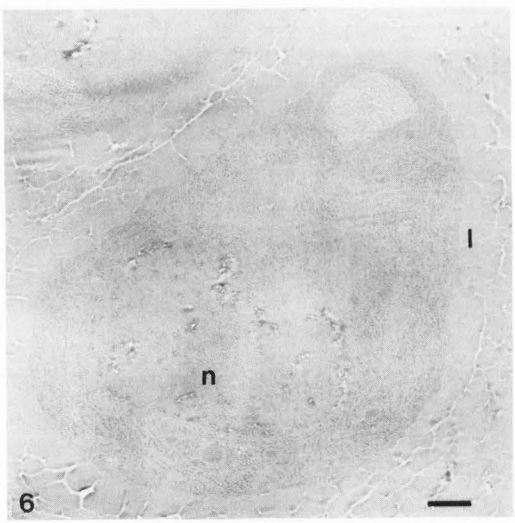


Fig 6 Hypertrophic chondrocyte with some evidence of lacuna (l) formation. Ice crystallite damage is confined to nucleus (n). Unosmicated tissue. Bar = 2μm.

continuum measurement was obtained by setting a window between 11.50 and 14.50eV (20eV/channel). If the mass of the section increased so did the continuum count. In order to establish the validity of the rapid freezing, freeze drying and embedding techniques employed in this investigation X-ray microanalysis was undertaken on selected intra-cellular and extracellular compartments to establish if there were differences in the relative amounts of elements present.

## Results

### Ultrastructure

The results of the rapid freezing produced, in most instances, a zone up to 15 μm thick at the surface of the cartilage which was free of significant ice crystal damage.

The chondrocytes of the early hypertrophic zone had a similar appearance to that described after conventional electron microscope preparation techniques (Appleton, 1969). That is

cells were flattened, often with polar accumulations of glycogen, and containing endoplasmic reticulum, Golgi apparatus, mitochondria and lysosomes (Fig 3). Resolvable ice crystal damage was confined to the nuclei and extracellular matrix. The mitochondria of chondrocytes undamaged by freezing did not usually contain intramitochondrial granules, whereas cells which were damaged had mitochondria containing granules in large numbers. This is particularly well illustrated where adjacent cells suffered different amounts of damage during the freezing process (Fig 4). Many mitochondrial granules were removed during routine thin sectioning, even though an alkaline section flotation medium was used (Fig 5).

The highly hydrated matrix suffered the most significant ice crystal damage but in some instances vesicle like structures were evident. As chondrocytes enlarged to become hypertrophic they were more rounded and were further separated by matrix. Deeper in the hypertrophic zone the beginning of lacuna formation was evident (Fig 6).

**X-ray microanalysis**

The results are given in Tables 1, 2, 3, 4 and 5.

## Discussion

The object of this investigation was to produce a routine methodology by which tissue could be prepared for the accurate localization and quantitation of ions and molecules in cartilage and other calcifying tissues using energy dispersive analysis by X-rays. There is a considerable body of evidence to support the view that rapid freezing of tissues is the best method to achieve these ends and that chemical fixation is unsuitable (Landis 1979, Landis & Glimcher 1982, Morris et al. 1983). Thereafter the principal problems are associated with the handling of the frozen tissues prior to undertaking energy dispersive analysis.

The difficulties associated with developing a reproducible freezing regime were largely overcome by using liquid propane as a cryogen according to the method of Elder et al. (1981). Liquid propane at its melting point of 86K is capable of producing the fast cooling rates necessary to avoid ice crystal damage (Schwabe & Terracio 1980, Elder et al. 1981, Plattner & Bachmann 1982). Adequate tissue preservation, however, is confined to a zone 12-15$\mu$m thick at the surface of the specimen. This zone is co-incident with the dried shell which rapidly forms around the specimen as ice sublimes during freeze drying. The frozen core will then dry as a function of the heat supplied to the specimen (Stephenson 1960). The most useful part of the specimen for morphological and analytical studies dries, therefore, at a very low temperature (167-173K) limiting ice re-crystallization to a minimum. Damage may be caused to tissue if ice re-crystallization is allowed to occur. In this laboratory tissue once frozen was maintained at or below 173K and $10^{-3}$ Torr and then allowed to rise slowly to 188K to facilitate drying. Although it has been calculated theoretically that drying under such conditions cannot take place over a reasonable time (Umrath 1983) there are numerous examples in the literature of tissues being dried well within the theoretically predicted figures (Hanzon & Hermudsson 1960, Sjostrand & Kretzer 1975, Barckhaus & Hohling 1978, Roomans & Boekestein 1978, Appleton et al. 1985, Lyon et al. 1985). In this investigation ice crystal damage was minimal and was considered to have taken place during the initial freezing.

Cryosections which are subsequently freeze dried produce energy dispersive analytical results which indicate that elements of physiological interest are retained (Ali et al. 1977a & 1978, Appleton 1978). However this technique is time consuming and technically difficult. The distinct advantage of embedding tissue in resin is that it facilitates the rapid production of reproducible sections for both morphological and analytical studies. The use of Spurr resin introduced chlorine but this was used as an internal reference standard. Resin will also dilute cellular constituents by increasing the mass of the specimen and therefore affect the lower limit of detection (Ingram & Ingram 1975). It has also been suggested that resin may leach or re-locate certain ions (Yarom et al. 1975). However the results of this study provide no evidence to support these contentions. Furthermore the recent work of Hargest et al. (1985) supports the view that resin embedding compares favourably with cryosectioning as a means of accurately retaining and localizing elements.

There were few, if any, mineral granules in those hypertrophic chondrocytes undamaged by freezing. This contrasts with the observation from numerous studies that mitochondrial granules appear in cells associated with hard tissue formation and mineralization (Ali et al. 1977a, Ali & Wisby 1975, Burger & de Bruijn 1979, Goldberg & Escaig 1984, Landis et al. 1977, Landis & Glimcher 1982, Manston & Katchburian 1984, Martin and Mathews 1969, 1970, Posner 1978, Seveus et al. 1978). However mitochondrial granules were present in large numbers in cells damaged by freezing. It is suggested, therefore, that where chondrocytes were rapidly frozen mineral granules did not form even in the presence of relatively high concentrations of calcium and phosphate ions. The presence of mineral granules may represent, therefore, the result of a phase transformation which takes place because of inadequate freezing and subsequent tissue damage (Landis & Glimcher 1982, Appleton et al. 1985).

The absence of tissue damage due to the initial freezing and the energy dispersive analysis results suggest that the methodology described in this study is reliable in retaining the localization of ions of physiological interest (Tables 1,2,3,4,5). For example there was a highly significant difference between the amount of Na present in matrix and mitochondria

TABLE 1

Relative mass fractions of elements in mitochondria of chondrocytes from mandibular condylar cartliage freeze dried and embedded in Spurr resin

| Zone | No. of analyses | Na | Mg | P | S | K | Ca |
|---|---|---|---|---|---|---|---|
| Early Hypertrophic | 6 | 33.9 | 30.3 | 66.6 | 69.6 | 21.4 | 44.0 |
| Hypertrophic | 6 | 50.3 | 29.9 | 48.5 | 107.5 | 21.5 | 69.4 |
| | P | 0.001> | NS | 0.05> | 0.01> | NS | 0.01> |

This indicates a marked difference in the relative mass fractions of all elements except K. Na and Ca are significantly higher in the hypertrophic zone while S and P are higher in the early hypertrophic zone.

TABLE 2

Relative mass fractions of elements in matrix of mandibular condylar cartilage freeze dried and embedded in Spurr resin

| Zone | No. of analyses | Na | Mg | P | S | K | Ca |
|---|---|---|---|---|---|---|---|
| Early hypertrophic | 6 | 38.1 | 29.9 | 37.9 | 74.0 | 22.5 | 36.5 |
| Hypertrophic | 6 | 62.0 | 32.3 | 38.6 | 109.1 | 22.1 | 34.0 |
| | P | 0.001> | 0.1> | NS | 0.001> | NS | NS |

Shows a marked difference in Na, Mg and S all of which are higher in the hypertrophic matrix.

TABLE 3

Relative mass fractions of elements in cytoplasm and mitochondria of early hypertrophic chondrocytes from mandibular condylar cartilage freeze dried and embedded in Spurr resin

| Early hypertrophic chondrocytes | No. of analyses | Na | Mg | P | S | K | Ca |
|---|---|---|---|---|---|---|---|
| Cytoplasm | 6 | 35.9 | 30.0 | 52.5 | 61.2 | 22.0 | 29.0 |
| Mitochondria | 6 | 34.0 | 30.3 | 66.6 | 69.6 | 21.4 | 44.0 |
| | P | NS | NS | 0.1> | 0.01> | NS | 0.001> |

Shows that there is a significantly higher mass fraction of both C and P in the mitochondria when compared with the cytoplasm of early hypertrophic chondrocytes.

TABLE 4

Relative mass fraction of elements in mitochondria and matrix of hypertrophic Cartilage freeze dried and embedded in Spurr resin

| Hypertrophic cartilage | No. of analyses | Na | Mg | P | S | K | Ca |
|---|---|---|---|---|---|---|---|
| Mitochondria | 6 | 50.3 | 29.9 | 48.5 | 107.5 | 21.5 | 69.4 |
| Matrix | 6 | 62.0 | 32.3 | 38.6 | 109.1 | 22.1 | 34.0 |
| | P | 0.01> | 0.05> | 0.05> | NS | NS | 0.001> |

Shows that mitochondria contain significantly higher mass fractions of Ca, P, Na and Mg when compared with the matrix.

TABLE 5

Relative mass fraction of elements in cytoplasm and matrix of early hypertrophic chondrocytes of cartilage freeze dried and embedded in Spurr resin

| Early hypertrophic cartilage | No. of analyses | Na | Mg | P | S | K | Ca |
|---|---|---|---|---|---|---|---|
| Cytoplasm | 6 | 35.9 | 30.0 | 52.5 | 61.2 | 22.0 | 29.0 |
| Matrix | 6 | 38.1 | 29.9 | 37.9 | 74.0 | 22.5 | 36.5 |
| | P | NS | NS | 0.01> | 0.01> | NS | 0.01> |

Indicates significantly higher mass fractions of Ca and S in the matrix than in cytoplasm but significantly lower fractions of phosphorus in matrix than cytoplasm.

of early hypertrophic cartilage when compared with hypertrophic cartilage ($P<0.001$). These results for Na are similar to those of Hargest et al. (1985) and Barckhaus et al. (1985). In both cases however they detected significant differences in the amount of K. For example, Hargest et al. (1985) using freeze drying embedding calculated 20mmol/kg wet weight of K in early hypertrophic chondrocyte cytoplasm, 208mmol/kg in early hypertrophic matrix and 47mmol/kg in early hypertrophic mitochondria. In the present study such differences were not detected and this may be due to incomplete drying at sufficiently low temperature (Edelmann 1986). Therefore the specimen temperature may be higher than that recorded for the specimen stage or cellular ice thaws during the warming of the specimen. Such difficulties will be resolved by modifying the freeze drying device so that specimen temperature can be accurately monitored.

Barckhaus et al. (1985) localized the K and Na to particles bound to the cell membrane of hypertrophic chondrocytes and furthermore these elements are extractable with water (Barckhaus et al. 1985) but not extractable with buffer (Quint et al. 1982) indicating they are bound in some way. Attempts to observe such particles in condylar cartilage were not successful. The significance of the presence of these elements is uncertain but they may reflect exocytotic processes associated with Na-K-ATPase (Barckhaus et al. 1985).

Significant differences were also noted, for example, in the relative mass fractions of Ca and P in mitochondria from early hypertrophic and hypertrophic chondrocytes. In hypertrophic chondrocyte mitochondria the relative amount of Ca was higher but P was lower. The result for Ca is in line with previous investigations (Ali et al. 1978, Althoff et al. 1982, Hargest et al. 1985, Landis & Glimcher 1982) but the result for P appears to be anomalous.

There is clearly scope for improvement in the methodology which may resolve, for example, the problem of the relatively low level of K found in this investigation when compared with other studies. The principal improvements which can be made are:

1. To the freeze drying device by ensuring complete contact between the discs, which carry the specimens, and the stage of the freeze drier. In this way the actual temperature of the specimen can be more accurately recorded. A cold finger could be placed over and close to the specimens to trap water molecules leaving the specimen during freeze drying.
2. There is evidence to suggest that the use of low temperature resin improves the retention of labile elements (Wroblewski & Wroblewski 1985). The freeze drying device was designed to accommodate low temperature resin and such work is now in progress.

## Acknowledgements

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## Discussion with Reviewers

**Reviewer 1:** Chlorine in the Spurr, which was also a constituent in the cells, was used in the present study as a standard. It is at the same time known that different cell compartments and extracellular matrix at different locations in the growth plate vary in wet weight. How would this influence your data?

**Reviewer 3:** You state that chlorine present in the Spurr resin was used as a standard reference. However it can be expected that the tissue also contains endogenous chlorine. How does this influence your results?

**Author:** In the chick growth plate (Hargest et al., 1985) and in the growth plate of pigs and rats (Krefting et al., 1981) there is little difference in the concentration of Cl between matrix and cytoplasm and this did not vary from zone to zone. In the chick, however, there was a higher concentration of chlorine in the mitochondria and there were differences in concentration particularly between prehypertrophic and hypertrophic chondrocytes. However, the differentiated state of the early hypertrophic chondrocyte and hypertrophic chondrocyte in the condylar cartilage are not comparable to the prehypertrophic and hypertrophic chick growth plate. Furthermore, counting for a fixed time, 200 secs, indicated there was no significant difference in the intensity of the chlorine peak between the different cell compartments and zones investigated in this study. Therefore, it was considered appropriate to use Cl as an internal reference standard.

**Reviewer 1:** The concentration of Na, Mg and K is

almost the same in the mitochondria, cytoplasm and the intercellular matrix of the early hypertrophic zone. Is there any possibility that redistribution of these elements occurred during specimen preparation?
**Author:** Care was taken to dissect and freeze the tissue as rapidly as possible and only the outer layer of 15 um was utilised in this study, Clearly some damage occurred due to ice crystal formation during the freezing/freeze drying process particularly in the hydrous matrix but there was no evidence of redistribution of elements in the adjacent hypertrophic zone. It is reasonable to assume, therefore, that redistribution did not occur in the early hypertrophic zone.

**Reviewer 1:** In Figs 3,4 and 6 there is evidence of severe ice crystal damage in the extracellular cartilage matrix, which suggests that some redistribution of ions can be expected. In Figure 4 different degrees of ice crystal damage is seen in two almost adjacent cells, while the size of the ice crystals in the surrounding cartilage matrix is uniform. What factors could have influenced this variation?
**Author:** The quality of preservation depends largely upon the velocity at which the freezing is achieved. The rate of cooling is a poorly controlled variable and important in this respect are the geometry of the specimen and holder, the size and nature of the tissue and the velocity at which it enters the liquid cryogen. Although some conditions may be standardised, such factors as the path the specimen takes through the cryogen cannot be determined. Also cryofixed structures are thermodynamically unstable at low temperature causing partitioning of the specimen and pure ice may form outside cells causing dehydration, distortion and shrinkage.

**Reviewer 1:** How do you explain the high concentration of S in the mitochondria especially in the hypertrophic chondrocytes? What role may S be expected to play in that compartment?
**Reviewer 3:** In several measurements of mitochondria and cytoplasm the concentration of S is higher than that of P, which is typical for matrix. Could your data have included intracellular matrix granules?
**Author:** Inorganic sulphur as part of iron sulphur proteins is an important part of the electron transport system in the inner mitochondrial membranes. The hypertrophic chondrocyte mitochondria have been implicated in the regulation of extracellular calcification by controlling the transport of calcium and phosphate ions. If this were so then it is an energy dependent process dependent on electron transport. Therefore one could reasonably expect a high concentration of sulphur. Great care was taken to ensure that mitochondria were analysed and not other intracellular organelles.

**Reviewer 2:** How is it possible to determine by visual examination complete drying of the tissue?
**Author:** Clearly this is not an objective method by which to determine complete drying. However, with the size of the pieces of cartilage used and under the conditions of temperature and vacuum described the cartilage which was judged to be dry by visual examination could be further processed and satisfactorily embedded in resin without encountering any problems indicating that the tissue was indeed dry. Visual examination, that is looking for a change in the external appearance of the cartilage to a chalky white texture after approximately 48h, therefore, was an important consideration in estimating if the cartilage was dry.

**Reviewer 2:** Did you try to determine the percentage of shrinkage of the growth cartilage during the described freeze drying and embedding procedure?
**Author:** No, no attempt was made to determine the percentage shrinkage but there is no doubt shrinkage will occur from any freeze drying procedure.

**Reviewer 3:** Your data (Tables 1,3 and 5) appear to indicate that Ca is a major element in mitochondria and cytoplasm in early hypertrophic cartilage. Its concentration is generally higher than that of K and more than half of the the phosphorus concentration. This would seem to indicate the level of at least 200/300 mmol/kg dry weight. Wouldn't one expect precipitate formation at this level?
**Author:** The hypertrophic chondrocyte is associated with the production of the cartilage matrix and its subsequent calcification. Therefore it could be reasonably argued that the high levels of calcium are not inconsistent with this progression of events. A solid phase of calcium phosphate does appear in the mitochondria but only in those early hypertrophic chondrocytes which suffer damage probably during the freezing process. At this time it is suggested that the calcium and phosphate which may have previously been bound could become available resulting in precipitation.

**Reviewer 3:** You suggest that K may be lost from the sample due to embedding at room temperature. On the other hand you discuss the fact that K is bound to matrix molecules and difficult to remove with buffer. Isn't it possible that the relatively low K levels have a biological significance rather than preparative artefact?
**Author:** The similar figures for K throughout the areas analysed would suggest that diffusion of this particular ion had taken place during the preparative procedure. However, it has been argued that cells containing high Na and low K are indicative of cell damage. Whether this cell damage is artefactual as a result of tissue processing or whether it is a result of the differentiation of the chondrocyte which eventually dies before the cartilage is replaced by bone is uncertain.

Reviewer 1- J. Wroblewski; Reviewer 2- L. Edelmann; Reviewer 3- G.M. Roomans