MASTER

Elemental distributions in the growth plate analyzed with a proton microprobe

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Elemental distributions
in the growth plate
analyzed with a proton microprobe

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Abstract

Bone formation starts in the embryonal phase from a cartilaginous template. This formation of calcified bone on a cartilage scaffold, called endochondral ossification, also plays a major role during growth. Postnatal longitudinal growth of long bones is limited to a relatively thin region, called the growth plate. In this region, typically a few millimeters thick, cartilage is rapidly converted into bone. Understanding the mechanisms involved in chondrocyte apoptosis is vital for understanding the process of endochondral ossification. A hypothesis of Mansfield is that: Phosphate ions (Pi) induce apoptosis of terminally differentiated hypertrophic chondrocytes and $\text{Ca}^{2+}$ enhances Pi entry into chondrocytes. Furthermore, it is expected that Pi and $\text{Ca}^{2+}$ concentrations are high in the terminal zone of the growth plate, due to turnover of mineralized cartilage into bone and that there is a calcium and phosphorus concentration gradient towards the bone [1].

To assess the latter hypothesis, this study aims at measuring concentration profiles across the growth plate of the femur of fetal pigs. To obtain this information in high detail, nuclear analysis techniques are used. Forward and backward scattering spectrometry is used for the determination of the amount of main matrix elements (H, C, N, and O) of the cartilage material, while particle induced X-ray emission (PIXE) is used for determination of the amount of the other elements that play a major role in the development of the bone (P, S, Cl, K and Ca). The results are compared with results found in literature, from scanning transmission electron microscope measurements on rat rib growth plate material [1] and from histochemical measurements on bovine tibial growth plate material [2].

The existence of a phosphate gradient, as found in literature [1, 2], is not proven in this project. But the determined concentration of phosphorus in the resting zone of about 2 mg/g dry weight, is consistent with the results of the electron microscope measurements [1]. The concentration of calcium determined in this study, is about 2 mg/g dry weight in the resting zone and increases along the proliferative and hypertrophic zone towards 4 mg/g. Again, these data are consistent with the results of electron microscope measurements [1]. These findings support the theory about the presence of a calcium gradient [1] which might enhance the phosphate induced apoptosis of terminally differentiated epiphyseal chondrocytes [3].

This study also found that sulphur and potassium concentrations increase step-wise between the proliferative zone and the resting zone. This could not be concluded from the spot measurements at different locations in the growth plate with the electron microscope described in literature [1].

Finally, it is concluded that the concentration distributions depend on the state of maturation of the growth plate. The further the growth plate is developed, the clearer the resting zone and proliferative zone become visible as two different zones. Also, an asymmetry between the primary and secondary growth zone shows up. Although the same process takes place in both zones, the secondary growth zone is thinner than the primary.
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1 Introduction

The mammalian skeletal system is built from individual bones and connective tissue joining them. Bone formation starts in the embryonic phase from a cartilaginous template. This formation of calcified bone on a cartilage scaffold, called endochondral ossification, also plays a major role in postnatal growth. The longitudinal growth of long bones is limited to a relatively thin region, called the growth plate. In this region, typically a few millimeters thick, cartilage is rapidly converted into bone. The chondrocytes (cartilage cells) start to proliferate, become hypertrophic (increase in size) and finally undergo apoptosis (programmed cell death) leaving behind a calcified cartilage template which is finally remodeled into bone. The properties of the tissue change drastically from soft sulphur-rich cartilage into stiff calciumphosphate-rich bone. Understanding the mechanisms involved in chondrocyte apoptosis is vital for understanding the process of endochondral ossification. Many research is carried out to understand the way growth plate development is controlled. With regard to the last part of the process, chondrocyte apoptosis, Mansfield [3] hypothesized that: Phosphate ions (Pi) induce apoptosis of terminally differentiated hypertrophic chondrocytes and Ca\(^{2+}\) enhances Pi entry into chondrocytes. Furthermore it is expected that Pi and Ca\(^{2+}\) concentrations are high in the terminal zone of the growth plate, due to turnover of mineralized cartilage into bone [1].

Validation of this hypothesis requires mapping of calcium and phosphorus concentrations across the growth plate. Gradients in the concentrations of elements or chemical compounds of interest have previously been measured by extracting them from the tissue by solvents followed by chemical analysis [2]. Results of such studies support the hypothesis, but the spatial resolution of these measurements is insufficient for accurate validation of the hypothesis.

The goal of this project is to determine whether a Ca or P gradient is present, with a spatial resolution in the order of the size of the individual cells. An approach based on particle induced X-ray emission (PIXE) and scattering spectrometry is used for assessing the concentrations of calcium, phosphorus and other elements throughout the growth plate with a high resolution. The Eindhoven microprobe setup has the ability of scanning the ion beam microprobe over the sample, producing a two dimensional map of the elemental distributions, using only a single histological slice as sample. Not only the resolution is superior to the mentioned histochemical methods, it is also possible to project the measured distribution afterwards on a microscope image of the same histological slice, which is not possible using these histochemical methods.

The analysis will be carried out on bones in different stages of development, because it is known that the morphology of the growth plate changes during development.
Elemental distributions in the growth plate analyzed with a proton microprobe
2 Theory

This chapter starts with a description of the properties of bone and cartilage tissue, followed by the process of long bone formation in the mammalian skeleton [4, 5]. Subsequently, it is explained why the concentrations of different elements in the growth plate are of interest and why nuclear analysis techniques are powerful analysis methods to assess these elements quantitatively. Finally, the physics behind the used techniques is explained in detail [6, 7].

2.1 Properties of bone and cartilage tissue

The skeletal system is built from individual bones and connective tissues joining them. The bones provide a hard and rigid skeleton for the body to maintain shape and conduct movements due to contraction of the connected muscles. It also protects the soft tissues of organs in the cranial, thoracic and pelvic cavities and functions as a framework for the bone marrow located inside the bones.

In contrast to its rigid appearance, bone is a self-repairing tissue, constantly adapting its apparent density, shape and properties in order to deal with changes in mechanical requirements.

As a simplification, bone can be described as a matrix of collagen type I fibers covered with minerals. The system has both biomechanical and metabolic importance. The minerals give the bone the strength and the fibers toughen the bone. Calcification starts with the precipitation of amorphous calcium-phosphate, which is transformed into hydroxy-apatite \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \) [8]. The \( \text{Ca/P} \) ratio found in the material can be used as a measure for the maturation of the mineralization process [9].

The mineral content of the bones also serves as a reservoir for calcium and phosphorus ions. Calcium plays a major role in the process of muscle contraction and the conductive behavior of the nerves. It is also an essential component for cells to survive. Phosphorus, as an element in adenosine triphosphate (ATP), has its role in the life of all cells. Phosphorus also plays a major role in the activation of proteins and enzymes.

The cartilage matrix surrounding the chondrocytes (cartilage cells) consists of macromolecules like proteoglycan aggregates and collagen fibers, which are built up from organic material. The matrix material mainly consists of the elements H, C, N and O. The proteoglycan aggregates look like a central chain with bottle brush chains, called proteoglycans attached to it (fig. 1). The side chains of the proteoglycans, called glycosaminoglycans (GAG), have also side chains (not shown in fig. 1) containing large amounts of sulphur in the form of sulphate \( \text{SO}_4^- \) [8].

Potassium in the form of \( K^+ \), which is a typical intracellular cat-ion, is an important electrolyte. However, due to the high concentration of negative charge on the proteoglycans, cartilage has a high extracellular concentration of potassium [8]. The an-ion \( \text{Cl}^- \) has a very high mobility due to the fact that it is the main solute of cartilage extracellular fluid.
Figure 1: Part of a proteoglycan aggregate consisting of a central chain with bottle brush side chains (proteoglycans). The side chains of the proteoglycans called glycosaminoglycans on their turn have side chains (not visible) containing large amounts of sulphur in the form of sulphate $SO_3^-$. 

2.1.1 Bone formation

The formation of long bone starts with primary mesenchymal condensation in the embryonal phase, from which a cartilaginous template, called anlage, is formed. Bone formation on this cartilaginous template is called endochondral ossification (fig. 2). It plays a major role during skeletogenesis, postnatal growth and fracture repair.

Chondrocyte differentiation in the center of the anlage leads to chondrocyte hypertrophy (increase in size) and finally apoptosis (programmed cell death). The matrix in between the apoptotic cells becomes calcified, and the invading vessels allow migration of osteoblasts, cells capable of depositing bone on the calcified matrix scaffold. Both proliferation and hypertrophy result in longitudinal growth of the bone.

In late fetal life, formation of secondary ossification centers starts in the epiphyseal cartilage. These secondary ossification centers finally result in the formation of bone in the regions called epiphysis. The diaphysis and epiphysis are now separated by a thin region of cartilage called growth plate. In this region different zones can be distinguished (fig. 3). The resting zone, containing the original cartilage material of the anlage, the proliferative zone, the early and late hypertrophic zones and the mineralization zone. Chondrocytes pass through all these zones, until they become apoptotic in the late hypertrophic zone, where the matrix mineralizes.

In the resting zone, chondrocytes are arranged randomly in a matrix containing large amounts of type II collagen and proteoglycans. On the bottom of the resting zone, chondrocytes start to proliferate (reproduction of living cells) resulting in columns of chondrocytes forming the proliferative zone. Elongation of the growth plate in this section is mainly caused by the division of chondrocytes and matrix production. In the prehypertrophic zone, chondrocytes start to increase in size. Proliferation ceases in this zone, so the growth is fully dominated by the increase in cell size.
Elemental distributions in the growth plate analyzed with a proton microprobe

Figure 2: Embryonic endochondral formation of long bones. Mesenchymal condensation forms a cartilaginous template in which chondrocyte differentiation leads to chondrocyte hypertrophy and finally to apoptotic death. The resulting cartilage matrix is covered with bone minerals by osteoblasts. Proliferating chondrocytes continue the process of bone formation, while secondary ossification centers develop in the epiphyseal cartilage. The remaining cartilage is called growth plate, in which longitudinal growth of the bone takes place by continuous conversion of cartilage into bone.

In the hypertrophic zone, the cells finally enlarge to the point that their height, in the direction of the bone, has increased nearly five times.

In the proximal tibia of a rat for example, the elongation of bone due to cell proliferation is about 9%, due to matrix production in the proliferative and hypertrophic zones about 18% and 14%, while the hypertrophy of the cells causes about 59% of the total bone elongation [10].

Once the glycogen stores are depleted [4], the cells undergo apoptosis. The cartilage matrix separating the lacunae that are left behind by the cells, becomes calcified. Further vascularization finishes the process of endochondral ossification by making it possible for osteoclast cells to resorb the bone and osteoblast cells to remodel it in trabecular bone. This process of continuous bone growth finally ceases with the closure of the growth plate, in which the growth plate slowly but fully becomes calcified.
Time

Figure 3: Schematic representation of the growth plate. The resting chondrocytes below the epiphyseal bone start to proliferate resulting in formation of columns of chondrocytes inside a cartilage matrix. Towards the bottom of the growth plate, these chondrocytes become hypertrophic resulting in apoptosis. The cartilage separating the lacunae inside the matrix becomes calcified by osteoblasts building up the bone mineral. The invading blood vessels supply the nutrients.

2.1.2 Elemental distributions in articular cartilage

The transformation of cartilage into bone is a complex process and is not yet completely understood. This section gives an overview of four studies related to the processes involved in bone growth. Mansfield et al. [3] investigated the role of calcium and phosphorus ions on the apoptosis of terminally differentiated chondrocytes. They hypothesized that extracellular phosphate ions, which are a main product of bone resorption, cause apoptosis of the terminally differentiated chondrocytes in the growth plate [3]. Mwale et al. [2] found a gradient of free calcium and phosphate in the growth plate towards the bone. These findings support the theory of Mansfield. Finally, studies of Reinert et al. [11] using nuclear analysis techniques on articular cartilage and Wroblewski et al. [1] using electron microscopy on growth plate material are presented.

Mansfield found that cell cultures of embryonic tibial chondrocytes treated with inorganic phosphate (Pi) show a significantly reduced viability in a dose- and time-dependent manner. The observed death process of the cartilage cells shares many common biological features of the apoptotic process, suggesting a Pi induced apoptosis. Cells in an earlier stage of differentiation remain viable despite a large increase in the extracellular Pi concentrations, suggesting that Pi-induced apoptosis is a stage-specific process which might be linked to the chondrocyte maturation and mineralization of the extracellular matrix. Blockage of Pi uptake inhibits apoptosis almost completely [12], suggesting that the uptake and not just the extracellular concentration is important for cell viability. Besides Pi, also Ca$^{2+}$ is released in the bone resorption process, therefore it is suggested that the Pi-induced cell death might be modulated by Ca$^{2+}$ [13]. High concentrations of extracellular calcium alone have little effect on the viability of chondrocytes, but the Ca$^{2+}$ ions greatly increase Pi uptake and enhance Pi-dependent cell
death. Therefore it is suggested that the increased Pi and Ca\(^{2+}\) concentrations at the cartilage mineralization front trigger apoptosis of the terminally differentiated chondrocytes.

**Figure 4:** Changes in the total growth plate tissue contents of (A) calcium and (B) phosphate. The hypertrophic zone (HZ) is indicated.\[^2\]

Because the remodeling of the bone takes place only at the end of the growth plate, a gradient of Pi and Ca\(^{2+}\) towards the bone is expected in the growth plate [2]. To study this hypothesis, growth plate material from bovine fetuses was obtained by Mwale et al. and cut in transverse sections each 20 \(\mu\)m thick. Five consecutive sections were pooled to form samples containing 100 \(\mu\)m material from all positions within the growth plate (A,B,C... in fig. 4). From these samples the calcium and Pi concentrations were measured using extracts created from the samples. The results show an increase in calcium and Pi content with decreasing distance from the bone front (fig. 4). This supports the theory about the Ca\(^ {2+}\) enhanced Pi induced maturation of the chondrocytes. The spatial resolution that can be achieved with this histochemical analysis of tissue is limited to about 100 \(\mu\)m and is only one-dimensional, because of the minimum amount of tissue needed for one measurement.

Modern nuclear/electron microprobe techniques have a superior resolution. Element distributions can be detected even down to sub-micrometer resolution. Although these techniques are employed frequently in materials/tissue research, there is only a small amount of literature on bone or cartilage research.
The rest of this section presents a summary of a study [11, 8, 14] in which nuclear microprobe techniques are employed to analyze the elemental distributions in joint cartilage samples from a porcine knee. The interpretation of the measured elemental distributions is emphasized rather than the exact concentrations, since the joint cartilage might differ from the growth plate cartilage. The nuclear microprobe techniques themselves will be discussed in section 2.2.

A light microscopy image of the joint cartilage is shown in fig. 5 in which the same histological zones can be seen as discussed in section 2.1.1. Simultaneously particle in-

![Figure 5: Light microscopy image (1 mm x 1 mm) of a cross-section of a porcine tibial cartilage (top: articular surface, bottom: calcified cartilage, dark spots: chondrocytes). The different histological zones are indicated [8].](image)

duced X-ray emission (PIXE), Rutherford backscattering spectrometry (RBS) and elastic recoil detection analysis (ERDA) are performed in order to obtain spatially resolved distributions of the elements (H: ERDA), (C, N, O: RBS) and (P, S, Cl, K, Ca: PIXE). Scanning transmission ion microscopy (STIM) analyzes the density distributions in the sample (fig. 6).

**STIM**

The STIM analysis (density distributions in fig. 6) of the cartilage samples reveals zones of different mass density. These zones with increased mass density match the hypointense zones in the NMR-images (fig. 6), which are related to aligned collagen fibres. Histochemical staining indicates a higher collagen content. Thus, the density distribution shows mainly the distribution of collagen in the cartilage.
Figure 6: Representative qualitative elemental distribution [8] (PIXE-intensity maps, 1.2 mm x 1.2 mm) of P, S, Cl, K and Ca of femoral (top) and tibial (bottom) cartilage and density distributions (STIM). The NMR-microscopy overview images indicate the position of the investigated regions (white box). The region between the dashed lines corresponds to the histological zone III. For explanation of the zones see fig 5.

Phosphorus
In the non-calcified cartilage, phosphorus is mainly concentrated in the organelles of the chondrocytes. Therefore the phosphorus-distribution is dominated by the cell distribution. In the hypertrophic zone (IV), the chondrocytes form clusters visible in the P-distribution (fig. 6). The accumulation of phosphorus at the superficial zone (I) beneath the articular surface of the cartilage shows a strong correlation with the calcium distribution, indicating calcification of the surface.

Sulphur
Due to the large amounts of sulphur in the side chains of the proteoglycans the S-distribution is dominated by the distribution of the proteoglycans. In the superficial zone (I), the concentration of sulphur is low since the collagen fibers form a narrow matrix in which the proteoglycans cannot enter due to their size. With increasing depth towards zone IV the proteoglycan concentration increases toward its maximum in the hypertrophic zone. Finally the concentration drops off rapidly in the calcified zone. The decrease of sulphur and therefore decrease of proteoglycan concentration is again probably caused by a denser collagen network. Histochemical staining also shows a decrease in proteoglycan content and an increase in collagen content.
Chlorine
The Cl-distribution shows the opposite behavior of the S-distribution (proteoglycan). This can be explained by the fact that the negative charge of the $SO_3^-$-groups repels the negative charge of the highly mobile $Cl^-$-ions.

Potassium
The qualitative distribution of the potassium equals the S-distribution. This can be explained by the attraction of mobile positive ions by the negative charge of the fixed sulphate groups. The concentration is the highest in the hypertrophic zone stimulating the calcification, and the concentration rapidly drops off in the calcified zone.

Calcium
The calcium concentration increases towards the calcified zone. In the hypertrophic zone, high Ca concentrations are found in the mitochondria of the chondrocytes and in the matrix vesicles (extracellular organelles), suggesting that matrix vesicles play a role in the initiation of the calcification process. Calcium accumulations (spots) are detected in the high density regions and because there are no phosphorus accumulations at the position of the spots, calcium-phosphate accumulations can be excluded. As described before, the accumulation of calcium in the superficial zone is likely to be caused by calcium-phosphate depositions.

Hydrogen, carbon, nitrogen and oxygen
These elements (matrix elements) are the main components of the matrix molecules and show a rather inhomogeneous distribution pattern. The highest concentration for each of the four elements is measured in zone III, indicating that the higher mass density is indeed due to a higher concentration of matrix elements.

In this study [8, 11, 14], a calcium gradient towards the bone is measured, which supports the $Ca^{2+}$ enhanced Pi-induced maturation hypothesis for chondrocytes. However, it must be noted that chondrocytes in articular cartilage do not mature as they are doing in growth plate cartilage. The latter is intended to grow while the former is intended to serve as a load-distributing tissue that lubricates the joint. On the other hand, the secondary ossification center is surrounded by a growth zone, so a growth zone can be found below the articular cartilage if the samples were taken from a not yet fullgrown animal.

Another microscopic technique used for elemental analysis is scanning transmission electron microscopy [1]. This technique uses an electron beam instead of a proton beam. As in PIXE it is possible to detect the elements P, S, Cl, K and Ca. However it is not possible to detect hydrogen, carbon, nitrogen and oxygen like with ERDA/RBS. Measurements on rat rib growth plates [1] show an increase in calcium and phosphorus concentrations towards the bone. This result is more comparable with the aforementioned biochemical analysis [2], since both studies used growth plate material. The scanning transmission electron microscopy has a sub-micrometer resolution, which makes it possible to discriminate between
intra and extracellular concentrations. The ion beam analysis techniques have a superior sensitivity compared to the electron microscopy techniques but this only plays a role in the detection of trace elements. Therefore it is of no importance for the detection of P, S, Cl, K and Ca in growth plate material.

2.2 Ion Beam Analysis Techniques

Ion beam analysis techniques are based on the interaction processes of highly energetic (order of MeV) projectiles and photons with matter. The interactions take place in both the sample and the detector. In ion beam analysis techniques, MeV ions hit the sample under investigation. X-rays, resulting from the excitation of target atoms in the sample and scattered projectiles are detected. The energy of the X-rays and scattered particles allows to determine the composition of the sample under investigation. In this section, the interactions are explained first, followed by the explanation of the used techniques.

2.2.1 Energy loss of projectiles

Energetic projectiles which travel through matter loose energy due to collisions with electrons and nuclei. The energy loss \( \Delta E \) of a projectile per distance target \( \Delta x \) depends on the properties of the projectile, the properties of the target and the kinetic energy \( E \) of the projectile itself:

\[
\lim_{\Delta x \to 0} \frac{\Delta E}{\Delta x} = \frac{dE}{dx}(E) = S(E). \tag{1}
\]

Assuming that the stopping \( S(E) \) is known for any energy, the energy of the projectile at a depth \( x \) below the surface can be written as:

\[
E(x) = E_0 - \int_0^x \frac{dE}{dx} \, dx. \tag{2}
\]

Note that the stopping \( S(E) \) is available as a function of the projectile energy and not as a function of depth. In order to evaluate the integral in eqn. 2, the depth of the ion in the sample can be considered as a function of the ion energy:

\[
dx = \frac{dx}{dE}(E) \, dE. \tag{3}
\]

This is shown in fig. 7 and leads to the reformulation of eqn. 2 to

\[
x = \int_{E_0}^E \frac{1}{S(E)} \, dE. \tag{4}
\]

Once an \( E_0 \) is chosen, \( x \) can be plotted (fig. 7) as function of \( E < E_0 \) and the energy \( E(x) \) can be read from this graph.
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Figure 7: Left: the stopping as function of the projectile energy. Right: the energy as function of the position given a certain energy $E_0$.

A more convenient way to calculate the energy of the projectile as function of depth ($x$) is to divide the target in a number of slabs ($n$) with thickness $dx = \frac{\Delta}{n}$ (fig. 8). Each slab gives rise to a certain energy loss. If the thickness of the slab is small, the energy loss per slab is also small and the stopping can be approximated constant over the individual slab.

Figure 8: The energy loss of a projectile passing through matter can be iterated by dividing the sample in individual slabs with constant stopping.

The energy after the transmission through $n$ slabs can be iterated with eqn. 6 and eqn. 5 as boundary condition.

\[
E_{n=0} = E_0
\]
\[
E_{n+1} = E_n - \Delta E_{n-n+1} \approx E_n - S(E_n)dx
\]

The stopping of a compound material can be calculated from the stopping of the individual elements contained in the sample, using Bragg’s rule:

\[
S_{\text{comp}}(E) = \frac{\sum_i n_i S_i(E)}{\sum_i n_i}
\]

with $n_i$ the stoichiometry of the individual element in the compound. The program SRIM [15] (the Stopping and Range of Ions in Matter) uses this rule to calculate the stopping of user-defined samples.
2.2.2 X-ray attenuation

When an X-ray beam passes through matter the intensity (I) of this beam is attenuated by scattering and absorption of the photons. The linear attenuation coefficient $\mu$ is the probability of removing a photon from the beam passing a slab of matter. The loss of intensity $dI$ per thickness $dx$ is given by:

$$dI = -\mu I dx$$

Integrating eqn. 8 over the slab thickness ($x$) gives the intensity (I) of a mono-energetic beam after traveling through homogeneous material for a given initial intensity ($I_0$) (Beer's Law).

$$I(x) = I_0 e^{(-\mu x)}$$

The attenuation can also be described in relation to areal mass density. The mass attenuation coefficient is defined as $\left( \frac{\mu}{\rho} \right)$ with $\rho$ the mass density of the material. This mass attenuation coefficient depends on the target element and on the X-ray energy ($E_x$). The elementary X-ray mass attenuation coefficients are tabulated [16] for the elements $Z = 1$ to 92, and for X-ray energies from 1 keV to 20 MeV. The mass attenuation coefficient of a compound can be calculated using Bragg’s rule,

$$\left( \frac{\mu}{\rho} \right)_{\text{compound}} = \sum_i w_i \left( \frac{\mu}{\rho} \right)_i$$

based on the linear combination of the mass attenuation coefficients of the constituents and their weight fractions ($w_i$).

In general, the mass attenuation coefficient decreases monotonic with increasing X-ray energy (fig. 9) but shows discrete steps at the X-ray energy matching the binding energy of the electrons in the shells of the atom. The implications of this behavior are discussed in the next section.

![Figure 9: Typical mass attenuation coefficient as function of the X-ray energy (phosphorus)](image-url)
2.2.3 Particle induced X-ray emission

Particle induced X-ray emission (PIXE) is an analysis technique based on the detection of X-ray photons emitted by a sample under investigation, while irradiated by high-energy ions. The target atoms are ionized by the incident particles through removal of an inner shell electron (fig. 10). After the creation of a vacancy in the inner shell of the atom, the process of de-excitation can emit an Auger electron or an X-ray photon. The energy of the emitted photons depends on the element and shell and is typically in the range of 1-30 keV. The photons are detected and the target element is identified by the measured photon energy.

![Figure 10: Principle of PIXE. The incoming ion knocks an electron out of the shells of the target atoms forming the sample. The created vacancy is filled by an electron falling back from a higher energy level while emitting an X-ray photon.](image)

The production of X-ray photons can be calculated using the production cross section:

\[
\sigma_{Z,K}^{\text{prod}} = \sigma_{Z,K}^{\text{ion}} \omega_{Z,K} b_{Z,K}
\]  

which contains the probability for a projectile to ionize the target atom called ionization cross section \( \sigma_{Z,K}^{\text{ion}} \), the branching ratio \( b_{Z,K} \) and the fluorescence yield \( \omega_{Z,K} \). The subscript \( Z \) indicates the element for which the coefficient applies and \( K \) indicates the electron shell to which decay occurs.

The Binary Encounter Approximation (BEA) gives a semi-empirical formula for the ionization cross section

\[
\ln(\sigma_{Z,K}^{\text{ion}} U_i^2) = \sum_{n=0}^{5} b_{i,n} \ln \left( \frac{E_p}{\lambda U_i} \right)^n
\]

with \( U_i = \frac{1}{2} m_e V_i^2 \) the binding energy of shell \( i \), \( E_p \) the energy of the projectile and \( \lambda = 1836 \) for protons. The coefficients \( b_{i,n} \) result from the fitting of experimental data.

The probability of X-ray emission called the fluorescence yield \( \omega_{Z,K} \) is related to the probability of Auger electron emission,

\[
\frac{P_{\text{x-ray}}}{P_{\text{auger}}} = \frac{\omega_{Z,K}}{1 - \omega_{Z,K}}
\]
The semi-empirical formula of Bambynek

\[
\frac{\omega_{Z,K}}{1 - \omega_{Z,K}} = \sum_{n=0}^{5} B_{i,n} Z^n
\]  

(14)
is used to calculate the fluorescence yield. The coefficients \( B_{i,n} \) are determined empirically by fitting experimental data. The fraction of X-rays appearing in the \( K_\alpha \) line compared to the \( K_\beta \) line is given by the branching ratio

\[
b_{Z,K_\alpha} = \frac{I(K_\alpha)}{(I(K_\alpha) + I(K_\beta))}.
\]  

(15)

Experimentally determined branching ratios can be found in literature, together with the ionization cross-sections and fluorescence yields for different target elements, projectiles and projectile energies.

For thin films, the energy of the ion can be considered constant in depth. Consequently the production cross sections are taken constant in depth. In the thin film approach also the attenuation of X-ray can be neglected. This leads to a simple expression for the X-ray yield of an element \( Z \):

\[
Y_Z = N_p N_z \sigma_{K,Z}^{\text{prod}}(E_p)
\]  

(16)

with:

- \( N_p \), the number of protons impinging on the sample
- \( N_z \), the number of target atoms of element \( Z \) per square centimeter [cm\(^{-2}\)]
- \( \sigma_{K,Z}^{\text{prod}}(E_p) \), the production cross section [cm\(^2\)] for a given projectile energy \( E_p \)

The assumption that the scattering and X-ray production cross sections are independent of the depth is valid for samples with an areal density smaller than 2 mg/cm\(^2\). For thick films two effects have to be considered. The first effect is the attenuation of emitted X-rays by the sample. The second effect concerns the decrease of the projectile energy in the sample, which leads to a depth dependent X-ray production cross section.

Photons emitted by an atom of a certain element have a high probability to be absorbed by other atoms of the same element. This self-absorption is the dominant factor in the thick target effects and gives a deviation from the thin target approximation of about 10\% for biological samples with an areal density of 1 mg/cm\(^2\).

The thick target PIXE formula can be found in literature and for the complete derivation, the reader is referred to [6].
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- $c_z$, the concentration of the element $Z$ in the sample [$g/g$]
- $A_Z$, the atomic mass of element $Z$ [$g/mol$]
- $E_0$, the energy of the incident particle [MeV]
- $E_f$, the energy of the particle after transmission through the sample [MeV]
- $\left( \frac{\mu}{\rho} \right)$, the mass attenuation coefficient of the sample [cm$^2$/g] for a given X-ray energy
- $\theta$, the angle between the incident beam and the line connecting the sample and the detector
- $\epsilon^*(E_p)$, the mass stopping power [MeVcm$^2$/g]

In the experiments, the sample behaves like an isotropic point source of X-rays and the detector has a limited solid angle $\Omega$, so only a fraction $\frac{\Omega}{4\pi}$ of the total emitted X-ray photons is detected. The detector has an entrance window, which gives rise to attenuation of the X-rays. The thickness of the entrance window of the detector sets a lower limit to the detectable photon energy, typical 1 keV. Therefore the photons of elements with $Z \leq 11$ (sodium) can not be detected due to the absorption of the entrance window. This is represented by the transmission $T(E_x)$ of the window. The efficiency $\epsilon(E_x)$ of the detector detecting an X-ray photon depends on its energy $E_x$. The detected number of counts $I_{det}$ can be calculated from the PIXE yield with:

$$I_{det} = Y_Z \frac{\Omega}{4\pi} \epsilon(E_x)T(E_x) \tag{18}$$

A total PIXE spectrum consists of Gaussian shaped peaks (will be discussed in section 2.2.6), superimposed on a continuous background. This background is due to bremsstrahlung of projectiles and free electrons slowing down in the sample. Also, experimental contributions like noise and pile up add to the background. Pile up appears at high countrates, when two or more events are recorded within the dead time of the measurement setup. The energies of these X-rays or projectiles can not be separated and appear in the spectrum at the sum energy. Pile up can be reduced by reducing the countrate by using a low beam current or by a beam-on-demand system [6].
2.2.4 Scattering spectrometry

Scattering spectrometry relies on the detection of projectiles after scattering on target atoms. The energy of the scattered projectiles can be derived from non-relativistic collision kinematics. In the laboratory frame a projectile scatters on a stationary atom (fig. 11).

\[ \frac{1}{2} m_1 v_0^2 = \frac{1}{2} m_1 v_1^2 + \frac{1}{2} m_2 v_2^2 \]  \hspace{1cm} (19)

and conservation of momentum:

\[ m_1 v_0 = m_1 v_1 \cos \theta + m_2 v_2 \cos \phi \] \hspace{1cm} (20)

\[ 0 = m_1 v_1 \sin \theta - m_2 v_2 \sin \phi \] \hspace{1cm} (21)

and substitution of \( x = \frac{m_1}{m_2} \) it follows:

\[ \frac{v_1}{v_0} = \frac{x \cos \theta \pm \sqrt{1 - x^2 \sin^2 \theta}}{1 + x} \] \hspace{1cm} (22)

The kinematic factor for light projectiles is then defined as

\[ K_1 = \frac{E_1}{E_0} = \left( \frac{x \cos \theta + \sqrt{1 - x^2 \sin^2 \theta}}{1 + x} \right)^2 < 1 \] \hspace{1cm} (23)

A single element in a sample gives in general a single peak in the PIXE spectrum with a peak-width that is determined by the energy resolution of the detector. However, in a scattering spectrum the width of a peak corresponding to a single element depends also on the properties of the sample.
This can be seen as follows from figure 12. A projectile with energy $E_0$ that is scattered at the surface of the sample towards the detector ($\theta = 147^\circ$) and has an energy $E_1(147^\circ, \text{surface}) = K_{147^\circ} E_0$. A projectile that scatters at the backside of the sample first loses energy due to the stopping on the way in and again loses energy on the way out: $E_1(147^\circ, \text{depth}) = K_{147^\circ}(E_0 - \Delta E_{1-3}) - \Delta E_{3-4}$. $E_1(147^\circ, \text{depth})$ is always smaller than $E_1(147^\circ, \text{surface})$ due to the stopping. Projectiles scattering between the two surfaces have an energy which depends on the depth where the scattering takes place (fig. 13). Scattering in forward direction reverses the depth-energy relation, which implies that particles scattered at the surface appear at a lower energy than particles scattered at depth. This is a consequence of the increase of $dE/dx$ with a decrease of the particle energy. Consequently particles scattered at the surface loose more energy than particles scattered at depth, so $E_1(45^\circ, \text{surface})$ is smaller than $E_1(45^\circ, \text{depth})$. As a consequence, energy loss provides the possibility for depth profiling in scattering spectrometry.
Although depth profiling is not used in this thesis, the energy loss has as consequence that a peak can not be fitted with a Gaussian shaped distribution.

The peak area from an element $Z$ in the thin target approximation is given by

$$Y_Z = N_p N_Z \frac{d\sigma}{d\Omega} Z \Omega$$

(24)

with:

- $N_p$, the number of protons impinging on the sample
- $N_Z$, the number of target atoms of element $Z$ per square centimeter $[cm^{-2}]$
- $(\frac{d\sigma}{d\Omega}) Z$, the differential scattering cross section $[cm^2/srad]$ for a given target element and projectile energy
- $\Omega$, the solid angle of the detector $[srad]$

In eqn 24 the differential scattering cross section is taken constant, for thick targets the energy dependence of $(\frac{d\sigma}{d\Omega}) Z$ has to be taken into account. In our experiments only peak areas will be evaluated. Only for extremely thick targets, the scattered projectile can be stopped completely and remain undetected. Backscattering spectra can be simulated with the computer code XRump [17]. For a given sample composition and layer structure, this program is able to calculate the spectrum of the scattered projectiles (fig. 13a) taking stopping and changing scattering cross sections into account.

2.2.5 Data analysis

The formalism of PIXE and scattering spectrometry can be used to calculate the yield when all the parameters of the sample are known. In practice however, experiments are carried out to determine the unknown composition of a sample.

The composition of a thick sample can be obtained starting from the thin target approximation to get a first estimation of the sample composition and the amount of material present. The X-ray attenuation and stopping of a sample with this composition can be calculated using Bragg’s law for X-ray attenuation and stopping. These values are then used to correct for the thick target effects. After correction, a new sample composition is calculated which is a better approximation of the real sample. By iterating this process, the calculated sample composition converges to the real composition.

Correction factors for the X-ray attenuation and the stopping are evaluated in a computer program written for the interpretation of the measurements in this thesis. In the following paragraphs the correction for X-ray attenuation and stopping are explained separately and the total flowchart of the program is presented on page 22.
X-ray attenuation correction
The self absorption of X-ray photons is the dominant factor in the thick target effects. For a sample with thickness \(d\) the PIXE yield of a layer with thickness \(dx\) becomes

\[
\begin{align*}
  dY_Z &= N_p \sigma_{K,Z}^{\text{prod}} \frac{\Omega}{4\pi} \epsilon(E_x) T(E_x) N_z \frac{1}{d} \exp \left[-\left(\frac{\mu}{\rho}\right)(E_x) x\right] dx.
\end{align*}
\]

Note that the production cross section is taken constant.

The total yield of the sample becomes

\[
\begin{align*}
  Y_Z &= N_p \sigma_{K,Z}^{\text{prod}} \frac{\Omega}{4\pi} \epsilon(E_x) T(E_x) N_z \frac{1}{d} \int_0^d \exp \left[-\left(\frac{\mu}{\rho}\right)(E_x) x\right] dx \\
  &= N_p \sigma_{K,Z}^{\text{prod}} \frac{\Omega}{4\pi} \epsilon(E_x) T(E_x) N_z \frac{1}{d} \left(1 - \exp \left[-\left(\frac{\mu}{\rho}\right) d\right]\right) \\
  &= \left( N_p \sigma_{K,Z}^{\text{prod}} \frac{\Omega}{4\pi} \epsilon(E_x) T(E_x) N_z \right) (C_{\text{corr}})
\end{align*}
\]

The correction factor is a function of the composition of the sample and the X-ray energy via the mass attenuation coefficient and areal density.

Stopping correction
The stopping correction is calculated with the assumption that the stopping power remains constant as a function of depth, which implies that the energy of the ion decreases linear in depth:

\[
\begin{align*}
  S(E_p) &= S(E_0) \\
  E_p &= E_0 - Sx
\end{align*}
\]

For the layer thicknesses considered in this thesis, a linear function can be used for the production cross sections as a function of the projectile energy between 2800 and 3000 keV.

\[
\sigma = aE_p + b
\]

The values and dimension of \(a\) and \(b\) are given in appendix C.

Substitution of eqn. 30 in eqn. 31 gives

\[
\sigma = aE_0 - aSx + b.
\]

For a sample with thickness \(d\) the PIXE yield of a layer with thickness \(dx\) becomes

\[
\begin{align*}
  dY_Z &= N_p \frac{\Omega}{4\pi} \epsilon(E_x) T(E_x) \frac{N_z}{d} (aE_0 - aSx + b) dx.
\end{align*}
\]
Elemental distributions in the growth plate analyzed with a proton microprobe

The thick target correction for the scattering yield can also be derived using a linear function for the energy dependence of the differential scattering cross section \( \frac{d\sigma}{d\Omega} \) instead of the production cross section \( \sigma \) giving

\[
Y^{\text{scat}} = N_p \Omega N_z \int_0^d (aE_0 - aSx + b) \, dx
\]

(36)

(37)

With the detector characteristics for the PIXE detector, forward scattering (FS) detector and backscattering (BS) detector, the cross sections (appendix C5), mass attenuation coefficients (appendix C6) and the mass stopping powers (appendix C7) known for all involved elements and for all involved X-ray and projectile energies, the thin target calculations and corrections can be carried out (page 22) with the use of Bragg's rule for X-ray attenuation and stopping. The iterative process is stopped after a certain number of iterations (typically 10), which is set by the user.
2.2.6 Statistics

The pulse height distribution $\frac{dN}{dH}$ from a detector measuring a mono-energetic source of X-rays or particles is a special case of the binomial distribution

$$P(x) = \frac{n!}{(n-x)!x!} p^x (1-p)^{n-x}$$  \hspace{1cm} (38)

in which the probability for $x$ successes $P(x)$ depends on the number of trials $n$ and the success probability $p$. In nuclear detection techniques the success probability is very small ($p<<1$) and the number of trials $n$ is large, so the binomial distribution can be approximated with the Poisson distribution

$$P(x) = \frac{(pn)^x e^{(-pn)}}{x!}$$  \hspace{1cm} (39)

which in turn can be approximated with the Gaussian distribution

$$P(x) = \frac{1}{\sqrt{2\pi \sigma^2}} e^{\left(-\frac{(x-x_0)^2}{2\sigma^2}\right)}.$$  \hspace{1cm} (40)

The variance $\sigma$ of the Poisson distribution is given by $\sigma^2 = pn = \bar{x}$ so the pulse height distribution can be written as

$$P(x) = \frac{A}{\sigma \sqrt{2\pi}} e^{\left(-\frac{(x-x_0)^2}{2\sigma^2}\right)}.$$  \hspace{1cm} (41)

\textbf{Figure 14:} Gaussian pulse height distribution for a mono-energetic source.

Gaussian shaped distributions will be used in the routines for fitting the PIXE-spectra. The variance of the distribution is a main factor in the uncertainty in the final result of the data-analysis. The square-root behavior of this variance will be used in the accuracy calculations.
Elemental distributions in the growth plate analyzed with a proton microprobe
3 Experimental setup

This chapter gives a description of the used setup for ion beam analysis and the sample preparation procedure. First the ion beam setup is briefly explained and the target chamber in which the measurements are conducted is discussed in more detail. Finally the preparation of the samples is explained.

3.1 Ion beam setup

Accelerator

A 3 MeV Singletron\textsuperscript{(TM)} accelerator of High Voltage Engineering Europe produces a high energy proton beam with a very low energy spread (\(\Delta E/E < 10^{-4}\)), which is guided into the beam line (fig. 15).

![Ion beam setup diagram]

Figure 15: Top view of the ion beam setup.

Electrostatic steerers deflect the beam in such a way that it is sent through a energy selecting setup, containing two slit systems and a 90° magnet, decreasing the energy spread of the beam even further (\(\Delta E/E < 10^{-5}\)). The switching magnet guides the proton beam into the beam line towards the target chamber.
Beam line
A set of correcting magnets, object and aperture diaphragms and four quadrupole magnets complete the ion beam optics. These optics enable to shape and focus the beam down to a sub-micrometer spot. A scanning magnet placed just before the target chamber scans the beam over the sample giving this setup its experimental advantage. All the methods discussed in 2.2 can now be used to retrieve 2D spatial information of the sample.

Target chamber
The target chamber at the end of the beam line contains the X-ray and particle detectors. The forward and backward scattered projectiles are detected with the two passivated implanted planar silicon (PIPS) detectors (Canberra), while the ultra-LEGe detector (Canberra) detects the X-rays. The angle of the detector for forward scattered ions with respect to the direction of the beam is adjustable from -90 degrees to +90, as indicated in fig. 17, and is set at +45 degrees for the scattering spectrometry. With the detector at an angle of 0 degrees bright field STIM measurements can be conducted. The detector for backscattered ions is placed at a fixed angle of -147 degrees and the Ultra LEGe X-ray detector is placed at an angle of -45 degrees.

The samples are attached to the targetwheel which can hold up to 8 targets. Four of these positions are filled with samples needed for beam focussing/alignment and calibration. The remaining four are available for use as desired. A Faraday cup in line with the beam measures the beam current or deposited charge, giving a measure for the number
of projectiles hitting the target. The mirror allows the use of an optical microscope for a rough selection of the region of interest. The sample is positioned in such a way that the region of interest is within the scan range of the scanning magnet. After a quick large scale scan used as an overview, final adjustments of the sample position are possible. The vacuum chamber can be sealed of the beam line with a vacuum valve allowing ventilation of the chamber without ventilation of the accelerator.

![Figure 17: Top view of the target chamber.](image)

### 3.2 Sample preparation

The sample preparation starts with the dissection of a leg of a fetal pig (fig. 18). The fetal pigs arrive from the abattoir (Ballering Export C.V. The Netherlands) within a few hours after the death of the female animal. After determining the size of the animal, the femurs are taken out of the leg as fast as possible. The distal part of the femur is used in the ion beam analysis.

![Figure 18: The skeleton of a (fetal) pig comparative position to the body. Some of the bones and joints in the limbs are denoted.](image)
The differences in tissue properties of the cartilage and bone will make it difficult to cut thin histological slices without ripping the growth plate from the calcified bone. The perichondrium/periosteum covering the cartilage/bone as a thin sheet holds the growth plate attached to the calcified part of the bone. Once the perichondrium/periosteum is removed, the growth plate easily detaches from the bone [19]. To reduce the risk of detachment, the bone is cut through, parallel to and just below the growth plate, leaving a small amount of diaphysial bone to the growth plate.

First the bone is cut through in the middle, perpendicular to the long axis of the bone by handsaw or scalpel. The diaphyseal side of the bone is then clamped in the sample holder of a processor controlled circular saw (Accutom, Streurs). The diamond covered saw blade can be fed through the tissue with a predefined cutting speed and force.

The sample containing the growth plate is frozen (at -20°C), placed on a sample holder (also at -20°C) and embedded in TissueTec. The TissueTec is liquid at room temperature and becomes solid within seconds in contact with the cold sample and sample holder gluing the sample to the sample holder.

10 μm thick histological slices are cut from the sample using a cryotome (Reichert-Jung Frigocut Microtome Frigocut 2800 N). The slices are transferred to backing foils for ion beam analysis or to glass object slides for light microscopy analysis. The slices for ion beam analysis are kept in the cryotome at -20°C for about 2 hours, for freeze-drying. The samples are then also stored in a vacuum desiccator for at least 4 days, until the ion beam analysis. The freeze-drying minimizes the diffusion of mobile elements during the thawing of the samples while placing them into the vacuum desiccator. The vacuum desiccation prevents re-absorption of moisture from the air and reduces the amount of free water after freeze-drying even further. The slices for light transmission microscopy are air dried for about one day before staining them with Saffranin O (Appendix. A) for contrast enhancement.
4 Pilot study

This chapter contains the results of both a theoretical and experimental pilot study. Simulations will show that the correction for thick target effects is necessary and that the iterative process corrects for thick target effects. The results of the sample preparation and the experienced difficulties during preparation are discussed. Finally, the results of a ion beam analysis are presented.

4.1 Simulations and calculations

A program is written based on the flowchart on page 22. A hypothetical sample is used to find out whether the iterative process described in section 2.2.5 really converges to the actual composition of the sample. The hypothetical yield from this sample, with the composition given in Tab. 1, is calculated using the thick target formulas. These calculated yields are then used as input for the iterative process. The difference between the calculated sample composition and the real sample composition can be calculated because the real sample composition is exactly known.

| Table 1: Properties of human cartilage (1.10 g/cm³) from SRIM 2003 [15] compound library. |
|---------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Stoichiometry                  | H         | C         | N         | O         | Na        | P         | S         | Cl        |
| Stoichiometry                  | 6.23e1    | 5.39      | 1.03      | 3.04e1    | 1.42e-1   | 4.65e-1   | 1.84e-1   | 5.54e-2   |

In order to gain insight in the relative importance of the correction for the X-ray attenuation and the stopping, the correction for X-ray attenuation has been evaluated separately. The correction for stopping can be switched off in the iterative process by setting the variable a in the linear functions (giving the cross sections as function of the energy) zero. For correction of X-ray attenuation only, (fig. 19a) the difference between the given and calculated elemental concentrations analyzed with PIXE (Na, P, S and Cl) decreases as expected with increasing number of iterations (iteration 0 is the thin target calculation). For the elements analyzed with scattering (H, C, N and O) the difference remains constant. This is due to the fact that for calculating the H, C, N and O concentrations constant cross sections are applied. The absence of a correction for the stopping also causes the difference in the concentrations of the PIXE elements not to converge to zero but to a constant value. However, with correction for both the X-ray attenuation and stopping (fig. 19b) the sample configuration converges to the real composition.

The difference after correction for X-ray attenuation only is about 2% for all the elements, whereas the difference for the PIXE elements is as high as 12% in thin target approximation. This shows that the X-ray attenuation is the dominant factor of the two thick target effects.
Figure 19: The error in sample composition is plotted against the number of iterations for a simulated cartilage sample of 10 μm thick. With correction for X-ray attenuation only (a) and with corrections for both X-ray attenuation and stopping (b). For a 5 μm thick sample (c) and (d).

The simulation of a target with half the thickness (fig 19c and d) shows the same behavior regarding the corrections. It also shows that the deviation from the thin target approximation, decreases with decreasing sample thickness.

Note that the composition of the cartilage from the SRIM compound dictionary does not contain any amount of calcium or potassium. From literature, on the other hand it is known that cartilage does contain calcium and potassium, with concentrations depending e.g. on the type of cartilage (growth plate or articular), the position in the cartilage and the state of maturation of the cartilage. Therefore, the iterative process is also checked with different compositions and even concentrations far outside the range of biological relevance. The results are not presented because the trend is similar, regarding the convergent behavior and the influence of target thickness. It must be noted that, for compositions with extreme differences in concentrations the number of iterations needed to reduce the
error to a certain level increases. In this case the self-absorption of the element present in large amount far exceeds the self-absorption of the other elements. The effect of a high concentration of a single element on the X-ray attenuation is comparable to the effect that appears with increasing target thickness. But in this case only the self-absorption for this single element is increased, instead of for all compound elements.

4.2 Sample preparation

Sample support
For the ion beam analysis experiments described in this thesis, the sample support consists of a 0.25 mm thick plastic sheet with a 13 mm hole in the middle (fig. 20), covered with a thin backing foil. This backing foil gives enough support to the histological slice without adding too much material to the sample so forward scattering analysis is still possible. The supports for the samples are home made.

![Sample support diagram](image)

**Figure 20:** The sample support consisting of a plastic sheet with a hole covered with a thin backing foil.

The backing foils covering the hole in the plastic sheet are made as described in Appendix B using the "volatile solvent" method because this gives the best results as described. Formvar is used as resin because of the availability, and no large differences are to be expected compared to using the rare Piooloform.

The thickness of the foil depends on the speed at which the glass is pulled out of the Formvar solution, the concentration of the solution and the rate of evaporation of the solvent. Measurements of the foil thickness with a profilometer show that by varying the speed the thickness can be varied between about 80 nm and 120 nm. Foils with a thickness of 100 nm have a gold color so the color can be used as an indication of the thickness.

Dissection
Fig. 21 (bottom picture) shows the individual bones (from left to right: shoulder-plate, humerus, ulna and radius still connected to the wrist joint) of a fully dissected front leg. The bone of the diaphysis is red due to the blood while the avascular cartilage, covering the secondary ossification centers in the epiphysis, has a white color.
Figure 21: Example of the left front limb before and after full dissection. The limb has a total length of about 15 cm. From left to right in the bottom picture: shoulder-plate, Humerus, Ulna/Radius and finally the foot. The surface of the shoulder and elbow joint are visible while the wrist joint is still connected.

The first trials preparing the bone were done on legs already removed from the body of the animal and stored for several weeks at -20°C. To isolate the bone, incisions on both the front and the back side of the leg are made. The incision must be as deep as possible almost touching the bone. A pair of tweezers is used for fixating the bone while the large pieces of muscle tissue are removed. This approach minimizes the risk of cutting into the soft fragile epiphyseal cartilage. Once the bone is almost free of soft tissue, the knee and hip joint are detached and the bone is taken out. Then the last pieces of tissue are removed and the bone is cut just below the growth plate removing as much calcified bone material as possible as described in section 3.2. Finally, the sample is frozen at -20°C for cryo-sectioning.

Histological slices and staining
The histological slices are cut on a cryotome, in the frontal plane of the bone parallel to the direction of growth zone (fig. 22a). If the slices are cut perpendicular to the direction of the growth zone, two directions are possible. Cutting from the cartilage towards the bone (fig. 22b) compresses the cartilage even though it is frozen and cutting from the bone towards the cartilage (fig. 22c) increases the risk of detaching the cartilage from the bone. The parallel direction also reduces the smearing of bone particles from the brittle bone towards the growth plate.

Figure 22: Three directions of cutting a histological slice in the frontal plane of the bone. The knife is indicated in grey.
The slices are transported either to a backing foil or to a microscope slide. The microscope slides are at room temperature and have a high specific heat, so the frozen slice melts once it comes in contact with the slide. After drying in air, the slices are attached very strongly to the slide and do not even de-attach during the staining procedure. Unfortunately, the slices do hardly attach to the backing foils even though they are also at room temperature. The low specific heat prevents the slice from melting. An advantage is that this reduces the diffusion of mobile elements in the sample.

![Image](image-url)

**Figure 23:** The ankle side of a pig tibia (fig. 3,18), showing from left to right: bone material of the long bone (dark purple), the growth plate (pink), secondary ossification center (dark purple) and finally the joint surface. The coupe is stained with Saffranin O (App. A). The oval shaped holes in the tissue are blood vessels that are cut through. The two dark lines are caused by the folding of the tissue.

The Saffranin O stains nuclei black, the cytoplasm gray/green and the cartilage red to pink (fig 23). This staining enhances the contrast of the picture depending on the type of tissue. The resting zone is stained red, while the proliferative zone is stained pink and the calcified bone material is dark purple. The closeup of the growth plate (fig. 24a) clearly shows that the growth plate is symmetrical in growth process towards the primary and secondary ossification centers but that the dimensions of the proliferative zones are not symmetrical. The secondary ossification center has a much smaller proliferative zone than the primary ossification center. This remains also the case in a more developed secondary ossification center.

The staining method for alkaline phosphatase, which is secreted in the hypertrophic zone of the growth plate, shows that the hypertrophic zone around the primary ossification center is about twice as thick as that around the secondary ossification center (fig. 25). Alkaline phosphatase cuts phosphate ions from phosphorylated proteins and the extra cellular matrix components. These ions are necessary for the production of bone material consisting of calcium-phosphate.
Figure 24: Part of fig. 23 magnified 4 times, showing the growth plate in more detail. From left to right: bone material, hypertrophic zone, proliferating zone and resting zone. At the most right side of the picture the hypertrophic zone around the secondary ossification center can be seen (a). A magnification of the wall of the vessel in the box in fig. 23, showing the layer of epithelial cells covering the wall of the blood vessel (b).

Figure 25: Staining shows the presence of alkaline phosphatase in the hypertrophic zones.
The microscope images show that the extracellular matrix is swollen, while the cells are reduced in size [19]. This shrinkage is caused by the freezing of the cartilage [20], which is necessary for making of the cryosections. The effect does not seem to correlate to storage temperature, but rather strongly to storage time [20]. Fast freezing might reduce extracellular matrix swelling [19], so instead of freezing the sample in a deep-freeze unit, freezing in liquid nitrogen is also tried, but unfortunately this caused cracking of the sample. The cracks might result form the fast freezing of the tissue compared with the large differences in tissue density (bone/cartilage), but also from the fact that the liquid nitrogen starts to boil in contact with the sample causing large vibrations of the sample. A possible way to prevent these vibrations is to cool liquid iso-pentane in the liquid nitrogen and then cool the sample in the liquid iso-pentane. The iso-pentane is almost as cold as the liquid nitrogen but has the advantage of a much higher boiling point. This preparation method is not used in this thesis.

4.3 Ion beam analysis

This section describes the calibrations and fitting procedures used for data analysis. Also radiation damage of the sample and the influence of the backing foil on the measurements is explained.

Detector solid angles, energy calibrations and beam diameter

Before the actual ion beam analysis of the samples can take place, the solid angle of the detectors have to be established by calibration. The calibration foil consists of a free standing polymer film with a thin layer of nickel on top of it. The areal density of the nickel is known to be $7.014 \, \mu g/cm^2$ and the total areal density of the sample is small enough to be assumed as a thin target. The cross sections of Ni for PIXE, backward and forward scattering are: $1.03E2 \, \text{barn}, 1.34E-1 \, \text{barn/sr}$ and $5.26 \, \text{barn/sr}$. The measurement gives a certain yield and beam charge, so the only variables not know in the thin target approximations are the solid angles of the detectors. The solid angles are determined to be: $\Omega_{\text{PIXE}} = 4.86E-02 \, \text{sr}$, $\Omega_{BS} = 1.07E-02 \, \text{sr}$ and $\Omega_{FS} = 5.89E-03 \, \text{sr}$.

The spectra exported from the software contain the number of counts versus the channel number of the multichannel analyzer (MCA). The channel numbers can be converted into an energy scale if necessary. The channel/energy conversion is - in good approximation - linear so the energy of only two peaks in the spectrum have to be known for an energy calibration of the full spectrum. The calibration can be carried out using a calibration sample or two identified peaks in the spectrum of the actual sample.

For the forward and backward scattering spectra no accurate energy calibration is needed, but for the fitting of the PIXE spectra it is. WinAxil 4.5 is a program used for the analysis of the X-ray spectra. The program allows to obtain an accurate energy calibration of the spectra and is used for the quantification of the PIXE yield by a fitting procedure. It is developed at the university of Antwerp and distributed by Canberra Packard Benelux.
WinAxil has a build-in calibration procedure in which the user has to identify at least two X-ray lines in the spectrum. Once all the X-ray of the elements of interest are set, WinAxil uses the first rough calibration as a initialization for the fitting routine in which the energy calibration is optimized using all the X-ray lines of the elements of interest.

Before the actual measurement is carried out the diameter of the focussed beam is measured by scanning the beam across a tungsten cross-wire. Because of the wire diameter of 2.5 μm beam sizes below 2.5 μm can not be determined. The beam diameter is focussed automatically based on scans of the cross-wire. For the measurements an indicated beam diameter of about 3 μm is used. It is found out that the beam diameter of the focussed beam is smaller than measured with the cross-wire so the indicated beam diameter is an upper-limit.

Due to the absence of background signal in the scattering spectra and the fact that the width of the peaks depends on the sample thickness, the forward and backward scattering spectra are obtained by a a straightforward summation of the counts of all the MCA channels in a single peak.

**Fitting of the PIXE spectra with WinAxil**

The PIXE spectrum consists of Gaussian shaped peaks, superimposed on a continuous background as described in section 2.2.6. Although the shape of the background in PIXE spectra is fully determined by physical processes, WinAxil has three fitting methods which are based on mathematical tricks for the determination of the background.

![Figure 26: An X-ray spectrum with the background fitted with the smooth filter. The arrows indicate increasing number of iterations.](image)
The methods are named linear, exponential and smooth filter and are discussed only briefly in the helpfile of WinAxil (Appendix D). All three fitting methods have a single user-defined parameter. For the linear and exponential filter the order has to be set while for the smooth filter the number of iterations has to be set. For a spectrum with enough statistics (smooth Gaussian shaped peaks), the parameters of all three methods can be set in such a way that the results of the background fit become consistent.

![Graph](image1)

**Figure 27:** PIXE spectra fitted in WinAxil without background (a) and with background (b).

![Graph](image2)

**Figure 28:** PIXE spectra fitted in WinAxil with the exponential background fit (a) and with the linear background fit (b).

The shape of the spectrum from the cartilage material differs from the shape of the spectrum from the bone material, due to the differences in mainly phosphorus and calcium concentrations. It is possible to fit these different spectra with the smooth filter method but, it needs adjustments of the user-defined number of iterations, resulting in subjective results. It must be noted that the background fitted with the smooth filter does not converge with an increasing number of iterations.
The exponential and linear filter give comparable results for different spectra and there appears to be no reason for choosing one above the other. With increasing order parameter (Appendix D) for the linear filter, the background is fitted better (fig. 27a and b) which can be seen from the reduction of the fit errors of the Gaussian shaped peaks. This effect becomes increasingly important with higher background levels. The exponential method shows the same behavior but the fitting of spectra with less smooth Gaussian shaped peak (fig. 28a) shows that the fitting method fails. This is probably caused by the way the start values are established (Appendix D). The fourth order linear fit method (fig. 28b) is used for the fitting of all PIXE spectra because this method is able to fit different shaped spectra without failing and with the same settings.

**Radiation damage**

The samples are free standing films in vacuum, so the energy deposited by the ion beam has to be dissipated by conduction in the sample towards the surrounding. The thermal conductivity of biological tissue is limited, so radiation damage caused by the dissipation of energy of the beam in the sample must be be taken into account. The beam current used for PIXE experiments (typical 100 pA for reduction of pile up while scanning in regions with dense calcification) measured with the Faraday cup, decreases (about 1 pA) when the sample is placed in the beam path. But the vast majority of projectiles is transmitted through the sample, due to the thin targets that are used.

The power dissipated in the sample can be calculated by multiplying the stopping of the sample with the beam current. For example: the energy of a 100 pA 3 MeV beam dissipated in 10 μm cartilage with a stopping of 19 keV/μm is 1.9E-5 watts. For a spot size of 3 by 3 μm this gives a power density of 2,1E6 W/m².

A measurement is carried out with a beam current of 300 pA during 14 hours to show the effects of radiation damage (fig. 29 and 30). Note that for regular measurements the beam current is limited to about 100 pA and the measurement only takes about 3 hours. After such a measurement no damage is visible with normal light transmission microscopy.

**Figure 29:** Severe radiation damage visible on a light transmission microscopy image even with beam scanning (dark pattern) caused by a high beam current of 300 pA during 14 hours.
Two types of radiation damage can be distinguished. The first is the damage due to the high energy projectiles and X-rays causing chemical bonds in the material to break and light elements like hydrogen to be removed from the sample due to collisions of projectiles and light target atoms. These processes are correlated to radiation dose and cannot be minimized because a certain dose is needed to gain enough signal. The second effect is damage due to the raise in temperature during the experiment. This can be minimized by reducing the dose rate, by either reducing the beam current or scanning the beam over the sample. Reduction of the beam current has the disadvantage of increasing the experimental time because still the same dose is needed. Continuous scanning of the beam over the sample keeps the local dose rate low without decreasing the total dose rate. By increasing the irradiated surface using scanning, for homogeneous samples the radiation damage during the same experimental time is reduced significantly.

Scanning also gives the possibility of retrieving 2D information of non-homogeneous samples. It must be noted that the measurement time increases, compared to the homogeneous sample, because the dose per point must be high enough to gain enough counts for statistically valid results.

![Graph showing radiation damage](image)

**Figure 30:** Radiation damage visible as decreasing yield for the light elements during the experiment.

The radiation damage is also visible if the yield is plotted against the measurement time (fig. 30). The light elements (H, C, N, O) show a decrease in signal during the experiment. The loss of signal for H and O is clearly visible, and is presumably caused by the loss of water from the sample. It is known that cartilage contains large amounts of water, up to 70% of its mass. The sample used for this experiment was cut from fresh tissue and only vacuum desiccated for a few hours between the sample preparation and the beam experiment. The effect visible in fig. 30 is partially caused by drying of the sample in the vacuum chamber during the experiment. The samples for the actual measurements are first freeze-dried a few hours and vacuum desiccated for at least 4 days as described in section 4.2 to reduce/prevent the drying effects. Because the scan pattern remains the same during one measurement it is also possible to correct for the radiation damage by
following the yield as a function of dose and finally extrapolating the measured yield to a zero dose.

**Influence of the Formvar foil on the measurements**

With the Formvar foils, a small amount of material with unknown composition is added to the samples. Pure Formvar is a polymer consisting of carbon, hydrogen and oxygen but the resin might be contaminated with other elements. Furthermore due to the use of chloroform in the preparation of the Formvar foils, traces of chlorine can be expected in the foil. To get an idea about the size of this contamination a Formvar foil without sample is used as a target for beam analysis (fig. 31a). The PIXE spectrum of the Formvar foil indeed shows a chlorine contamination as expected, but also traces of silicon, sulfur and calcium. The silicon signal results from glass fragments on the foil due to the scoring with

![PIXE spectra](image_url)

**Figure 31:** A PIXE spectrum of a Formvar foil (a) shows apart from a chlorine contamination also a silicon, sulphur and calcium contamination. A Formvar foil partially covered with a sample is measured to get an idea of the amount of signal added by foil (b,c and d). On the left the spectra from the sample and on the right the spectra of the foil.
a scalpel. The calcium trace might result from the fact that normal tap water is used in the soap solution. The source of the small sulphur contamination is unknown but it results from either the Formvar resin, chloroform or the soap solution. The foils used for the ion beam analysis of the bone samples are prepared using distilled water and are cut instead of scored to reduce the contamination of the foils.

To get an idea of the influence of the presence of the foil on the analysis of bone samples, a measurement is conducted in which a foil is partially covered with a bone sample (fig. 31b, c and d). Thus, a covered and an uncovered part of the foil can be measured simultaneously assuring the same measurement conditions. The spectra on the left side of fig. 31(b,c and d) show the yields of the part covered with the sample and the spectra on the right side show the yields of the uncovered part. The overall conclusion is that the contribution of the foil to the yield of the sample is less than 1%. The ratio of the calcium/chlorine signal (fig. 31 right) is high compared to that of fig. 31a. This is caused by the fact that in the former case, a clean foil is used and in the latter case small bone fragments from the sample are scattered over the uncovered part of the foil. This scattering occurs at the brittle bone structures of the primary ossification center. For the measurements of the growth plate this is less of a problem because by cutting the histological slices in a direction parallel with the growth plate, smearing of bone fragments onto the region of interest is minimized.

4.4 Elemental distributions in the growth plate

During all the measurements the beam is scanned in a predefined pattern (120 x 32 steps with a stepsize of 10 μm and a beam diameter of 3 μm). Each time an event (X-ray or scattered projectile) is measured, the position of the beam and the energy of the photon/particle is written to file. During the measurement this file is continuously monitored with a home made data-analysis program, SOFI [21]. This gives the opportunity to monitor the experiment during the actual measurement. SOFI not only gives the spectra per pixel, but is also able to construct pictures of the elemental distribution.

4.4.1 Pilot measurement

This section describes the total analysis of a pilot measurement to give an overview of the consecutive steps in the data analysis. Although the elemental distributions (fig. 32) only display the raw data in an arbitrary color scale, they are very useful for selecting an undamaged part of the sample for the measurements.

Because the spectra per pixel do not contain enough counts for the fitting procedures, the spectra are summed in the direction perpendicular to the direction of the growth plate, i.e. over the smallest axis of the image in fig. 32. It must be noted that the growth plate
Figure 32: The yield distribution of the elements of interest from SOFI. The sum yield of carbon, nitrogen and oxygen are presented in one picture. On the bottom a light transmission microscopy picture is presented of the same region.

is assumed homogeneous in the direction of the summation, which is valid (fig. 32) for the cartilage part if there are no vessels present in the region of interest.

The fitting routines fit the PIXE, RBS and FS spectra, resulting in a yield per element per position (fig. 33a).

Although the total yield distributions are not recalculated to actual concentrations and corrected for thick target effects, some conclusions can be drawn. The yields of hydrogen, carbon, nitrogen and oxygen, which are the main parts of tissue, hardly vary within the cartilage and only increase sharply in the calcified material due to an increase in density. The yields of calcium and phosphorus are so high in the calcified material that, based on these profiles, the presence of a calcium gradient in the soft tissue is not proven. The sulphur and potassium distributions show a remarkable increase in yield in the proliferative zone which can be correlated to the change in tissue appearance under normal light transmission microscopy (fig. 32 bottom image). The chlorine yield shows an increase in the hypertrophic zone.

These patterns are not likely to be caused by density effects because the yields of the main components (H, C, N and O) do not vary that much. From section 2.2.5, it becomes clear that due to the X-ray attenuation caused by self-absorption, patterns visible in the yield profiles of the PIXE elements (P, S, Cl, K and Ca) will become even more pronounced in the concentration profiles after correcting for thick target effects.
Elemental distributions in the growth plate analyzed with a proton microprobe

This can be seen as follows: The larger the concentration of an element, the larger the mass attenuation coefficient for the characteristic X-rays of this element. So if there is an increase in yield measured, the absorption is also increased, so the actual increase in concentration is even larger than is expected from the increase in yield.

In order to come up with more objective conclusions, the data has to be analyzed as described in section 2.2.5. The yield distributions (fig. 33a) are calculated to concentrations in mg/g (fig. 33b) and corrected for thick target effects. It can be seen that density effects due to the blood vessels disappear and the concentrations of the organic components (H, C, N and O) are almost constant apart from the decreasing hydrogen concentration in the calcified tissue. The patterns in the sulphur, chlorine and potassium are emphasized by the thick target correction as expected from the theory.

Figure 33: (a) The yield profiles of the elements of interest from the ROI in fig. 32. The error bars visualize the square root statistical variance of the yield. (b) The concentrations [mg/g] dry weight after corrections for thick target effects.
Besides a factor 100 increase in calcium concentration between the cartilage and bone, there is also a small gradient visible towards the mineralization front, from the beginning of the proliferative zone (at 60 μm in fig. 33b) up to the mineralization front. From somewhere between 100 and 110 μm the probe reaches the beginning of the mineralization front and due to the irregular shape of the trabecular bone/cartilage structure a gradient in the calcium content is caused until the probe reaches more homogeneous bone trabeculas at 128 μm. A phosphorus gradient is not visible.

4.4.2 Error calculations

In the measurements described in this thesis the uncertainty in the yield of the backscattered projectiles is the most important contribution to the uncertainty in the final concentrations. A detailed study of the accuracy of thick-target PIXE can be found in [22]. The estimation of the accuracy in this thesis is confined to the error caused by the uncertainties in yield. The variance in the PIXE yield is equal to the square root of the yield, just like the variance in the scattering yield, but the fitting of the background introduces another uncertainty. The pilot measurement is representative for all the measurements in this thesis regarding the measurement time, beam current and sample composition. The yields of this pilot measurement are therefore of the same order as the yields in the other experiments. So, the variance in the concentrations of the different elements are comparable between the different measurements.

<table>
<thead>
<tr>
<th>Table 2: Variance in yield in the cartilage part of the sample.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variance of yield as % of yield</td>
</tr>
<tr>
<td>Variance of background as % of yield</td>
</tr>
<tr>
<td>Variance of concentration in %</td>
</tr>
</tbody>
</table>

The total variance of the yield is also visible in fig. 33a. The variance of the concentrations depends not only on the variance in the yield of the particular element, but especially on the variance of the main components. The variance of the PIXE elements is estimated to be about 11%.
5 Results and discussion

This chapter presents the results from concentration profiles measured on bones of different ages to follow the development of the growth plate in time. These results will be discussed in detail in the last paragraph of this chapter.

5.1 Concentration profiles at different stages of development

Samples were taken from four animals of different age (Tab. 3). From now on these samples will be referred to as sample 1, 2, 3 and 4 as indicated in fig. 34.

| Table 3: Size [cm] of the fetal pigs (tail not included), their hind limb and the femur as a measure for the age of the animal. |
|---------------------------------|---|---|---|---|
|                                | Sample 1 | Sample 2 | Sample 3 | Sample 4 |
| Crown-tail                     | 15.5      | 16        | 30        | —         |
| Hind limb                      | 4.5       | 4.5       | 16        | 20        |
| Femur                          | 2         | 3         | 6         | 8         |

Figure 34: A sketch of the morphology of the four samples used in the experiments. Black indicates bone, white indicates cartilage and the gray squares indicate the scanned areas. Sample 1 and 2 contain only a primary growth zone, while sample 3 and 4 contain both a primary and secondary growth zone.

Light transmission microscopy (fig. 35) reveals that the layered structure of the different zones (resting zone, proliferative zone and hypertrophic zone) becomes more pronounced with increasing age. In the first sample only the hypertrophic zone can be discriminated from the rest. In the pictures of the second and third sample the proliferative zone becomes more and more distinguishable as a separate zone, with a different appearance than the resting zone. In the third and fourth sample the growth plate has a symmetrical construction regarding the different zones but the primary growth zone is significantly thicker than the secondary growth zone.
Figure 35: Light microscopy images of the samples from top to bottom increasing in age. The magnification of all four images is the same and the primary ossification center is on the right side of the images.

The concentration profiles (fig. 36) show that the order of magnitude of the concentrations of the different elements is the same for all four samples. The concentration profiles of the matrix elements are not presented because they do not show any spatial dependence and are comparable to the profiles in fig. 33. The shape of the sulphur and potassium profiles do depend on the age of the bones, the layered structure becomes more and more distinguishable with age. The results from the maturest sample (number 4) also show the asymmetry in the dimensions of the different zones. The secondary growth zone (rightside of fig. 36f) is smaller than the primary growth zone.

The close ups (fig. 37) show the calcium and phosphorus concentration profiles in more detail. The results from the very young bone (sample 1) show no sign of a calcium gradient. The calcium concentrations in sample 2 and 3 show a gradient towards the calcified part of the bone. The concentration increases in the proliferative and hypertrophic zone from 2 mg/g towards 4 mg/g. The concentration profile of sample 4, with the fully developed growth plate shows a slow increase in the primary growth zone and a sharp increase in the secondary growth zone. On both sides the increase is about 2 mg/g. It must be noted also that the minimum concentration is almost doubled compared to the concentrations measured in the three younger samples.

In none of the four samples a phosphorus concentration gradient is found (fig. 37b).
Figure 36: Results from measurements on: a very young bone (a,c), a bone where the layered structure starts to appear (b,d), a bone with a small secondary ossification center (e,g) and a bone with a fully developed growth plate (b,d). The right sides of all figures corresponds to the primary growth zone. Concentrations are in [mg/g] dry weight.
**Figure 37:** The calcium (a) and phosphorus (b) distributions in the growth plate. Concentrations are in [mg/g] dry weight. The primary growth zone is on the right side of the graphs.
5.2 Discussion

Forward and backward scattering spectrometry was used for the determination of the amount of main matrix elements (H, C, N, and O) of the cartilage material, while particle induced X-ray emission (PIXE) was used for determination of the amount of the other elements that play a major role in the development of the bone (P, S, Cl, K and Ca). The ability of measuring the amount of matrix elements is an advantage compared to scanning transmission electron microscopy, which only measures the amounts of P, S, Cl, K and Ca. Using the measured H, C, N and O amounts, dry weight concentrations of any constituent can be calculated, whereas without (e.g. in STEM) the amount of matrix needs to be estimated. The measured concentrations at different locations of the all elements are quantitatively in good correspondence with the scanning transmission electron microscopy measurements on rat rib growth plates [1] (fig. 36, 37 and tab. 4).

Table 4: Elemental composition of cartilage matrix in rat rib growth plate [1]. The concentrations are in mg/g ± SEM.

<table>
<thead>
<tr>
<th>Zone</th>
<th>P</th>
<th>S</th>
<th>Cl</th>
<th>K</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>2.20±0.40</td>
<td>14.53±2.75</td>
<td>5.88±0.88</td>
<td>13.38±1.83</td>
<td>2.00±0.52</td>
</tr>
<tr>
<td>Proliferative</td>
<td>3.04±0.34</td>
<td>37.66±3.17</td>
<td>9.84±0.84</td>
<td>21.49±1.95</td>
<td>3.08±0.32</td>
</tr>
<tr>
<td>Hypertrophic</td>
<td>3.97±0.56</td>
<td>26.40±7.04</td>
<td>9.66±1.05</td>
<td>30.19±3.20</td>
<td>3.92±0.72</td>
</tr>
</tbody>
</table>

The used field of view (typically 1.2 mm) is larger than that used in the electron microscope measurements [1], giving the ability to conduct a large scan across the total growth plate and therefore to determine the actual distributions.

The close ups in figure 37a show the calcium concentration profiles in more detail. The results from the very young bone (sample 1) show no sign of a calcium gradient towards the bone. The calcium concentrations in sample 2 and 3 show a gradient towards the calcified part of the bone. The measured calcium concentration in the resting zone is 2 mg/g dry weight, and increases in the proliferative and hypertrophic zone towards 4 mg/g which compares well to the results of [1] (Table. 4). The concentration profile of sample 4, with the fully developed growth plate, shows a slow increase in the primary growth zone and a sharp increase in the secondary growth zone. On both sides the increase is about 2 mg/g, but the minimum concentration is almost doubled. For a growth plate that has reached such a state of developed, no values for the calcium concentrations are found in literature. The rise in the minimum concentration might be caused by the fact that the two mineralization zones are close together and that the two gradients towards each side overlap.

The results from the histochemical analysis by MWale et al. [2] show a gradient in the free calcium ion concentration towards the bone similar to our results. Unfortunately, it is difficult to compare the exact concentrations found by MWale et al. with the findings from the electron microscope measurements of Wroblewski et al. [1] and our measurements, since
the free ion concentrations are determined as wet weight concentrations \([2]\) instead of dry weight concentrations. Due to the large amount of extracellular cartilage water, the difference between dry and wet weight is large (60-80\%) and also differs for the different zones.

A phosphorus gradient towards the bone was not found (fig. 37b), in contradiction with the gradient measured with the biochemical analysis \([2]\). Again it must be noted that the biochemical analysis is based on extracts and therefore gives a measure for the free phosphate whereas the ion beam analysis techniques can not distinguish between free or bound ions. Differences in chemical binding states cannot be distinguished using neither ion beam analysis techniques nor electron microscope techniques.

From the staining for alkaline phosphatase (fig. 25), it is known that this enzyme, responsible for cutting phosphate groups from proteins, is only present at the end of the growth plate. So the phosphorus might already be present in the region but in a bound form, undetectable with the biochemical measurements based on extracts. Once the alkaline phosphatase cuts the phosphate free, it can play its role in the Pi-induced apoptosis and becomes also detectable with the biochemical analysis.

The measurements with the nuclear analysis techniques and the electron microscope technique give the same concentration of phosphorus of about 2 mg/g in the resting zone (tab. 4 and fig. 37). The gradient in phosphorus concentration measured with the electron microscope is not confirmed in this thesis, although the measurements on sample 4 show signs of an increase in phosphorus concentration in the proliferative zone. A possible explanation for the absence of a gradient in phosphorus concentration, is that the existence might depend on the state of maturation of the growth plate. Which is also the case regarding calcium, the gradient only shows up after a certain state of maturation (the results from sample 1 versus sample 2, 3 and 4).

Figure 36 shows that the shape of the concentration profiles depends on the age of the bones. The layered structure becomes more and more distinguishable with age in the increase in the sulphur and potassium concentration in the proliferative zone. These findings are supported by the different appearance of the zones in light microscopy in fig. 35 and can be related to the different processes taking place in the zones.

The increase in sulphur is likely to be caused by the increase in proteoglycan synthesis in the proliferative zone \([1]\). The amount of extracellular matrix material per cell is known to increase in both the proliferative and hypertrophic zone \([10]\). Sulphur is mainly present in the form of sulphate in the proteoglycans which has, due to its negative charge, the ability to attract free potassium ions. Because the potassium is mainly present as free potassium ions, the distribution of potassium is likely to be dominated by the distribution of sulphur. A comparison between the two measured profiles supports this vision.
The profiles from the oldest sample show the asymmetry in the dimensions of the different zones. The secondary growth zone (right side of fig. 36f) is more compact than the primary growth zone. This asymmetry was also found from the alkaline phosphatase staining (fig. 25).

The increase in chlorine in the hypertrophic zone might be caused by the uptake of water containing chlorine by the hypertrophic cells. The increase in chlorine signal might also be caused by the fact that chlorine is mainly an intracellular element and the cell/matrix volume ratio increases strongly in the late hypertrophic zone [10]. It must be noted that chlorine in biological tissue is highly mobile which speaks for the measured concentration difference. Concentration differences would level out due to the high mobility, so the real increase in concentration is at least as high as the measured increase.
Elemental distributions in the growth plate analyzed with a proton microprobe
6 Conclusions and recommendations

6.1 Conclusions

This study aimed at evaluating the calcium and phosphorus concentrations across the growth plate, starting from the hypothesis of Mansfield et al. that: "Phosphate ions (Pi) induce apoptosis of terminally differentiated hypertrophic chondrocytes and Ca^{2+} enhances Pi entry into chondrocytes."

Concentration profiles of Ca and P as well as S, K and Cl have been measured across the growth plate of the distal femur of fetal pigs using nuclear analysis techniques. Using H, C, N and O measurements, these concentrations were quantified. These results compare well with results from scanning transmission electron microscope measurements on rat rib growth plate material [1] and with results from histochemical measurements of bovine tibial growth plate material [2].

The existence of a calcium gradient towards the bone is proven in this thesis, in the electron microscope measurements by Wroblewski et al. [1] and in the histochemical measurements by MWale et al. [2]. So, in contrast to the phosphorus, not only the concentration of free calcium, but the total concentration of calcium increases towards the bone. The results of the electron microscope measurements and the results in this thesis, are comparable and consistent. The concentration of calcium is about 2 mg/g dry weight in the resting zone and increases along the proliferative and hypertrophic zone towards 4 mg/g dry weight. These findings support the theory about the calcium enhancement of phosphate induced apoptosis of terminally differentiated epiphyseal chondrocytes [3].

We detected a calcium gradient similar to [1], but we did not detect a phosphorus gradient as proven in the electron microscope measurements by Wroblewski et al. [1]. However it has previously been shown by MWale et al. [2] that the free phosphate concentration increases towards the bone [2].

Comparing these results leads to the formulation of a new hypothesis on the role of calcium and phosphorus in relation with apoptosis of chondrocytes. We propose that sufficient phosphorus is present in the growth plate in a bound form, undetectable with the biochemical analysis based on extracts [2]. Alkaline-phosphatase, present at the end of the growth zone, cuts phosphate-groups from proteins. Therewith, the free Pi concentration increases in the hypertrophic zone and the amount of phosphorylated proteins decreases. The free phosphate can play its role in the Pi-induces apoptosis. The de-phosphorylated proteins may stimulate apoptosis and may as well serve as sites for the precipitation of calcium-phosphate starting the early phase of mineralization [1].
Finally it is concluded that sulphur and potassium have increased concentrations in the proliferative zone compared to the concentrations in the resting zone. These increased concentrations of sulphur and potassium in the proliferative zone were already known [1], but the profile of the distribution was unknown. This project shows that the increase between the resting- and proliferative zone is step-like, rather than a gradual gradient. It is also found that this effect develops slowly with the maturation process of the growth plate. The further the growth plate is developed, the clearer the resting zone and proliferative zone become visible as two different zones. These are new insights in the development of the growth plate. Also an asymmetry between the primary and secondary growth zone appeared in the elemental distribution, in agreement with the histological appearance of the tissue. Although the same process takes place in both zones, the secondary growth zone is more compact than the primary.

6.2 Recommendations

In the presented work, the techniques ability of two dimensional mapping is not fully exploited because in the data analysis, the results are reduced to one dimension to increase the statistics. The increase of statistics can also be done by increasing the measurement time or by increasing the beam current. Increasing the measurement time is not practical because a single measurement will then last a few days. Increasing the beam current increases the dose-rate dependent radiation damage which must be corrected for. More problematic becomes the high countrate of the PIXE detector, which causes large amount of pile up. The countrate of the PIXE detector while using a high beam current, can be brought in balance with the count-rate of the scattering detectors by placing an attenuation filter in front of the detector. The characterization of this filter is a tedious procedure because the attenuation depends on the X-ray energy [23]. One way of characterizing such a filter is using a reference sample with known composition that contains all the (PIXE) elements of interest [21] which is not easy to assemble.

Now the large scale concentration profiles are known, localized high-resolution measurements become interesting. With a sub-micrometer beam high resolution measurements of the extracellular matrix and chondrocytes can be made in the different zones. For example it is known that there are differences in the concentrations between chondrocytes and extracellular matrix [1] and between territorial and interterritorial matrix [24]. It is also known that there are regions of relatively high density in the late hypertrophic zone, containing large concentration of calcium and phosphorus [1]. These are thought to be sites of primary mineralization and are worthwhile to the investigate. In a PhD project on atherosclerosis [21], high resolution research on primary mineralization is carried out, showing the ability of the setup to determine the exact stoichiometry of calcifications in the material.
Dankwoord

Bij deze wil ik iedereen die mij op welke manier dan ook heeft geholpen in het afgelopen jaar, bedanken voor zijn of haar hulp en enthousiasme. Zonder iemand te kort te doen wil ik enkelen met naam noemen:

Arthur de Jong, René van Donkelaar en Leo van IJzendoorn allereerst voor het bieden van de mogelijkheid om af te studeren, voor alle hulp en het kritisch bekijken van dit verslag.  
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Verder alle technici, Frits, Eric, Eddy, Harry, Ad en Wim zonder wie alle problemen met de techniek nooit zo snel verholpen zouden zijn.  
Elemental distributions in the growth plate analyzed with a proton microprobe
References


[19] Private conversation with C.C. van Donkelaar. Assistant professor in the bone and orthopaedic mechanics section of the biomechanics and tissue engineering department of the faculty of biomedical engineering.


Elemental distributions in the growth plate analyzed with a proton microprobe
A Staining protocols

A.1 Saffranin O Staining for Cartilage

Description: This method is used for the detection of cartilage, mucin, and mast cell granules on frozen sections as well. The cartilage and mucin will be stained orange to red, and the nuclei will be stained black. The background is stained green.

Fixation: Formalin fixed, paraffin embedded sections.

Solutions and Reagents:

Weigert’s Iron Hematoxylin Solution:

Stock Solution A:
Hematoxylin: 1 g
95% Alcohol: 100 ml

Stock Solution B:
29% Ferric chloride in water: 4 ml
Distilled water: 95 ml
Hydrochloric acid, concentrated: 1 ml

Weigert’s Iron Hematoxylin Working Solution:
Mix equal parts of stock solution A and B. This working solution is stable for about 4 weeks.

0.001% Fast Green (FCF) Solution:
Fast green, FCF, C.I. 42053: 0.1 g
Distilled water: 1000 ml

1% Acetic Acid Solution:
Acetic acid, glacial: 1 ml
Distilled water: 99 ml

0.1% Safranin O Solution:
Safranin O, C.I. 50240: 0.1 g
Distilled water: 100 ml

Procedure:

1. Stain with Weigert's iron hematoxylin working solution for 10 minutes.

2. Wash in running tap water for 10 minutes.
3. Stain with fast green (FCF) solution for 5 minutes.
4. Rinse quickly with 1% acetic acid solution for no more than 10–15 seconds.
5. Stain in 0.1% safranin O solution for 5 minutes.
6. Wash in running tap water for 10 minutes.

Results:
- Nuclei: Black
- Cytoplasma: Gray green
- Cartilage: Orange to Red

A.2 ALPase localisation

Materials: Deionised water

Alkaline phosphatase staining kit (Sigma) containing:
Naphtol AS-BI alkaline solution: Naphtol AS-BI phosphate, 4 mg/mL in 2-amino-2-methyl-1,3-propanediol (AMPD) buffer, 2 mol/L, pH 9.5.
FBB-alkaline solution: Fast blue BB base, 5 mg/mL in hydrochloric acid, 0.4 mol/L.
Sodium-nitrite solution: Sodium nitrite, 0.1 mol/L
Citrate solution: Citric acid, 18 mmol/L, sodium citrate, 9 mmol/L, sodium chloride, 12 mmol/L with surfactant buffered at pH 3.6.
Neutral red solution, buffered: Neutral red, certified, 0.5% w/v, in acetate buffer, pH 5.2.

Acetone

Formaldehyde 37%

Procedure:
1. Prepare the diazonium salt solution:
   Add 0.1 mL sodium nitrite solution to 0.1 mL of FBB-alkaline solution.
   Mix gently by inversion (ondraaiing) and allow standing for 2 minutes.
2. Measure 4.5 mL deionised water and adjust temperature to 18-26 C and add to the solution of 1.)
3. Add 0.1 mL naphtol AS-BI alkaline solution to the diluted diazonium salt solution (solution obtained after step
4. Mix thoroughly. This mixture is called the alkaline-dye mixture.
5. Prepare a citrate-acetone-formaldehyde fixative solution by adding 6.5 mL acetone and 0.8 mL of 37% of formaldehyde to 2.5 mL of citrate solution.


7. Add slides to alkaline-dye mixture (step 4) and incubate at 18-26 °C for 15 minutes. Protect immersed slides from direct light. Discard alkaline-dye mixture after use.

8. After incubation, remove slides from Coplin jar and rinse for 2 minutes in deionised water. Do not allow slides to dry.

Results:

ALPase staining results in ALPase to colour purple.
B Backing foils

Backing foils can be made in two different ways depending on the type of solvent. The most commonly used resins are Formvar and Pioloform and the solvents are cyclohexanon and chloroform, both resins dissolve in both solvents. Cyclohexanon which is water soluble but less volatile than chloroform, does only contain the oxygen, hydrogen and carbon which are also present in biological tissue. Chloroform also contains chlorine which is also present in small amounts in tissue but which is an element of interest. Chlorine in the foil can not be distinguished from chlorine in the tissue and can therefore compromise the measurement.

- Volatile solvent [25] [26]

A 2% solution is made by adding 2 grams of resins to 100 ml chloroform. Then a microscope slide is submerged in the solution (Fig. 39 and pulled out with a constant speed. The chloroform evaporates when the slide is pulled out of the solution leaving behind a film of Formvar or Pioloform on the slide. The edges of the slide are scored with a scalpel to disconnect one side of the film from the other sides. Now the slide is lowered into distilled water leaving the film to float on the surface. This film is transferred onto the plastic sample slide (with a hole in the middle) by lowering the slide into the water. The sample on which the experiments are carried out will be situated on this film which is covering the hole

- Water soluble solvent

A 2% solution is made by adding 2 grams of resins to 100 ml cyclohexanon. Because the cyclohexanon is not as volatile as chloroform so the solvent flows of the
object slide without leaving a film behind. But because cyclohexanon is soluble in water it is possible to drop a small amount of solution on a water surface. The cyclohexanon quickly dissolves in the water leaving behind a thin film on the surface of the water. The procedure of transferring this film to the sample slide is the same as described before.

The color of the reflected light is a measure for the thickness of the film. The aim is to produce silver/gold films with a thickness of about 1000 Å or 100 nm.

Both techniques and used resins have their own advantages and disadvantages. Formvar and Pioloform do not show large differences but some people claim that the coupes better hold on the Pioloform films compared to the Formvar films. The big difference shows up comparing the two techniques. The second technique is much faster than the first, but results in a poor films. This becomes clear from the fact that about 50% of the films made, cracked spontaneously during drying. Further a large part of the films which survived the drying cracked while transferring the samples onto them. This cracking might be caused by tension in the film caused by nonuniform thickness. This nonuniform shows up from the light reflected by the film (the reflected light shows a rainbow pattern). Non of the films made by the first technique cracked during drying and only some cracked by transferring the samples onto the foil. The difference in time needed to produce the films is more than compensated by the time delay and problems resulting from cracked films during sample preparation, vacuum desiccation and beam analysis.

First trials using a steel punch to make the holes in the sample slide resulted in burrs on the edge of the hole. These burrs can rupture the backing foil causing the sample to be lost for beam analysis. To minimize the probability of rupture, the edge must be as
smooth as possible. Although it is possible to buy/make a dedicated but expensive punch for this type of plastic sheet, an other approach is used. A tool is made which is capable of holding the piece of plastic sheet and which can be clamped in the chuck of a lathe. By using a scalpel instead of a chisel, holes can be cut in the plastic sheet. This approach gives a burr free result and is also able to cut holes with different diameters which is not possible with a punch of fixed diameter. So the diameter of the hole can be matched to the size of the sample, this is important because the smaller the hole, the lower the risk of rupture of the foil. From the trials to make Formvar foils in a reproducible way it has become clear that the microscope slide has to be extremely clean and dry. The best way to prepare the slides is by putting them in a soap solution ("Dubro citroen" works the best) for at least a couple of hours and than washing them with demineralized water and finally drying them with tissue paper. Once a microscope slide is clean the Formvar foils easily detach from the glass. After two foils (one from each side of the glass) the microscope slide has to be cleaned again but the washing with soap solution can be limited to a few seconds. After the first few foils it appears that the glass becomes cleaner and cleaner because the foils detach more easily.

Analysis of the first foils with light transmission/reflection microscopy shows small fragments of glass and dust on the surface. The glass splinters are the result of the scoring with the scalpel. These fragments will also show up in beam analysis due to their large areal density and should be avoided. It has shown that the best way to avoid glass splitters is to cut the foils instead of scoring the edges. Contamination by dust in the air is avoided by drying the foils in a vacuum desiccator instead of drying in the fume-hood. After making the foils, the microscope slide is best to be stored in the soap solution until the next time keeping it clean.
C Data

Table 5: Fit results for differential scattering cross section from literature [27, 28, 29] and the production cross section from Paneut [30] valid for proton energies between 2800 keV and 3000 keV.

<table>
<thead>
<tr>
<th></th>
<th>H</th>
<th>C</th>
<th>N</th>
<th>O</th>
<th>P</th>
<th>S</th>
<th>Cl</th>
<th>K</th>
<th>Ca</th>
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</thead>
<tbody>
<tr>
<td>a</td>
<td>-0.0707</td>
<td>-0.0095</td>
<td>-0.0241</td>
<td>0.0023</td>
<td>0.1194</td>
<td>0.1379</td>
<td>0.1473</td>
<td>0.1476</td>
<td>0.1425</td>
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<tr>
<td>b</td>
<td>614.35</td>
<td>101.39</td>
<td>152.36</td>
<td>71.619</td>
<td>582.49</td>
<td>405.87</td>
<td>266.02</td>
<td>74.497</td>
<td>15.138</td>
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</table>


Table 6: Mass attenuation coefficients [cm²/g] of target elements for different X-ray energies from Paneut [30].

<table>
<thead>
<tr>
<th></th>
<th>H</th>
<th>C</th>
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<th>Cl</th>
<th>K</th>
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<tbody>
<tr>
<td>P-Kα 2013 eV</td>
<td>3,450</td>
<td>303.1</td>
<td>470.0</td>
<td>664.7</td>
<td>283.6</td>
<td>352.8</td>
<td>426.2</td>
<td>635.1</td>
<td>769.5</td>
<td>67.38</td>
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<tr>
<td>S-Kα 2307 eV</td>
<td>2,543</td>
<td>198.8</td>
<td>315.9</td>
<td>457.8</td>
<td>2323</td>
<td>242.2</td>
<td>293.2</td>
<td>437.7</td>
<td>530.4</td>
<td>44.58</td>
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<tr>
<td>Cl-Kα 2622 eV</td>
<td>1,909</td>
<td>133.8</td>
<td>213.4</td>
<td>319.3</td>
<td>1637</td>
<td>1934</td>
<td>206.3</td>
<td>308.7</td>
<td>374.0</td>
<td>30.25</td>
</tr>
<tr>
<td>K-Kα 3313 eV</td>
<td>1,131</td>
<td>64.88</td>
<td>104.2</td>
<td>156.8</td>
<td>863.5</td>
<td>1020</td>
<td>1193</td>
<td>211.2</td>
<td>197.5</td>
<td>14.89</td>
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<tr>
<td>Ca-Kα 3691 eV</td>
<td>0.897</td>
<td>46.45</td>
<td>74.78</td>
<td>112.9</td>
<td>642.7</td>
<td>759.2</td>
<td>888.0</td>
<td>1184</td>
<td>147.0</td>
<td>10.73</td>
</tr>
</tbody>
</table>

The number of digits is reduced to 4.

Table 7: Stopping powers [eV/1e15 atoms/cm²] of target elements for protons from SRIM 2003 [15].

<table>
<thead>
<tr>
<th></th>
<th>H</th>
<th>C</th>
<th>N</th>
<th>O</th>
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<th>S</th>
<th>Cl</th>
<th>K</th>
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</tr>
</thead>
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<tr>
<td>2750 keV</td>
<td>0.5155</td>
<td>2.239</td>
<td>2.579</td>
<td>2.842</td>
<td>4.523</td>
<td>4.735</td>
<td>5.137</td>
<td>5.567</td>
<td>5.758</td>
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<tr>
<td>3000 keV</td>
<td>0.4785</td>
<td>2.097</td>
<td>2.409</td>
<td>2.664</td>
<td>4.252</td>
<td>4.454</td>
<td>4.830</td>
<td>5.237</td>
<td>5.419</td>
</tr>
</tbody>
</table>
D  Fitting routines of WinAxil

This section contains the brief information about the background fitting routines found in the WinAxil helpfile. The exact working methods of these fitting routines are not clear.

- **The linear continuum function**
  The modeling background function, called linear can be defined as:

  \[
  Y_{\text{background}}(i) = \sum_{l=0}^{L} a_l (E - E_0)^l
  \]

  where \(a_l\) are the fitting parameters. Since they are linear, they do not have to be initialized. \(E_0\) is often chosen to be in the middle of the fitting region.

  Wide regions often exhibit too much curvature to be described by a linear polynomial. If \(a_l\) equals zero, the background is described as a constant. If \(a_l\) equals 1, a straight line will be used. This background can be used in small energy regions (up to 3 keV). If wider regions have to be fitted, or curvature is present, the use of this background is not recommended.

- **The Exponential continuum function**
  If one wants to fit the entire spectrum, or a part of the spectrum where high curvature is present, an exponential polynomial background model is often used.

  \[
  Y_{\text{background}}(i) = e^{\sum_{l=0}^{L} a_l (E - E_0)^l}
  \]

  This function is nonlinear in the parameters \(a_l\). This means that start values (for fitting) should be established. In order to achieve a converging background fit, this is done by estimating the background by means of the SNIP (Statistical Non-linear Iterative Peak clipping) algorithm. After obtaining the background, a linear fit of the logarithm of the obtained background to the is performed, obtaining the initial start values.

- **The Smooth Filter continuum function**
  As a good alternative to an algebraic function to describe the background, one can calculate the background first, and strip it from the measured spectrum before applying the least-squares fit.

  In order to do this, one compares the content of channel \(i\) with the mean of the two neighboring channels:

  \[
  m_i = \frac{Y_{i-1} + Y_{i+1}}{2}
  \]

  If the value of \(m_i\) is smaller than the channel content \(Y_i\), the content of channel \(i\) is replaced by the mean \(m_i\). Repetition of this process for the whole spectrum, gradually causes the peak to be stripped away.