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Morphological Studies on the Epiphyseal Growth Plate Combined with Biochemical and X-Ray Microprobe Analyses

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Summary. The epiphyseal growth plate of the domestic pig was investigated topologically combining biochemical methods with electron microprobe microanalyses both correlated to histological controls. A lateral resolution of about 50 µm was reached. Highest nuclease activity was found in the lower columnar cell zone, while alkaline phosphatase showed maximal activity in the hypertrophic area, connected with maximal values for extractable, organically bound phosphorus, and extractable Ca and Mg. Acid phosphatase activity reached maximal values in the zone of the lower primary spongiosa, while the extractable P_i had maximal values at the end of the zone of bone remodelling. Microprobe analyses have shown that the extracellular Ca content (per dry mass) remained relatively constant at 0.7% (about 58 mM/kg wet weight for 66% tissue fluid) in all zones of the plate increasing to 1% in the vicinity of the first foci of mineralization. The intracellular P content (per dry mass) was about 4.5%, the extracellular 0.1–0.2% (about 10–20 mM/kg wet weight) increasing also to about 1% in the vicinity of the first foci of mineralization. Thus the $Ca \times P$ product was much higher than the ion-product of 2 mM^2 which is necessary for an in vitro mineralization of connective tissue. The extracellular S content (per dry mass) as a probable indicator of sulfated proteoglycans was relatively constant at about 3.5% in the different zones but decreased to about 0.3% in the fully mineralized regions. This indicates a loss of sulfur containing substances with mineralization which is not so high since the concentrations per dry mass must be normalized to a unit volume of equal density of mass.

Introduction

The epiphyseal growth plate is divided into different zones on the basis of morphological criteria (e.g. cell shape). While a good agreement exists on the definition of the different zones for the unipolar growth plate more complexity exists defining the zones of the bipolar growth plate (Schlüter 1978). The question arises whether the different morphological zones are also characterized by differ-

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ent chemical processes which lead to the primary mineralization in the hypertrophic zone. Some questions can be answered by topological analyses, i.e. by chemical and histochemical analyses carried out in direct correlation to the different zones of the growth plate (Wuthier 1969).

Boyde and Shapiro (1980) have carried out qualitative and Krefting et al. (1980) quantitative microprobe analyses intra- and extracellularly in the different zones of the growth plate of small laboratory animals. Gradients of the distribution of elements (e.g. Ca, P, Mg, Na, K) were received, which are fundamental for an understanding of the process of hard tissue formation. Nevertheless, microprobe analysis cannot distinguish between bound and unbound (ionic) elements and is not able to demonstrate the distribution of organic macromolecules (e.g. enzymes).

Alkaline phosphatase is an important enzyme for hard tissue formation and acid phosphatase for hard tissue remodelling (Reddi 1981). The Mg^{2+} ion is a cofactor of the alkaline phosphatase and probably has a regulating function for the process of mineralformation (Quint et al. 1980). We were interested to get more knowledge of the zonal distribution of components which may lead to a better understanding of hard tissue formation. Thus, we have cut continuous series of cryostate sections through the bipolar growth plate of the porcine proximal ulna, starting in the epiphyseal mineralized region ending in the metaphyseal zone of bone remodelling. In a repeating sequence sections were also chosen for lightmicroscopy in order to correlate the analyses directly with the morphological zones. We have also started to correlate the chemical analyses directly with electronprobe microanalyses. The results of these studies will be communicated.

Materials and Methods

2 mm thick longitudinal slices of the bipolar growth plate of the proximal ulna of ten-week old pigs (Sus domesticus) were frozen in supercooled liquid nitrogen at $-210°$ C. Under liquid nitrogen they were trimmed to blocks of $\sim 2 \times 2 \times 4$ mm. Serial 5 or 10 µm thick frozen sections were cut at $-25°$ C proceeding from the epiphyseal bone into the metaphysis (see: flow diagram, Fig. 1). The sections were distributed for the different analyses according to the following sequence: two 5 µm slices were taken for light microscopical control, five 5 µm sections for electronmicroprobe analysis, five 10 µm sections for the biochemical analyses of the buffer extracts, and five 10 µm sections for the chemical analyses.

Electronprobe Microanalysis. For electronprobe microanalysis the 5 µm thick cryostat sections were transferred to thin plastic films stretched over an 8 mm internal diameter aluminium cylinder which was painted with a carbon paint to reduce background. These sections were allowed to freeze dry for 24 h in the cryostat chamber at $-25°$ C; then they were coated with carbon by evaporation. The flattest sections were chosen for analysis after examination with a stereo binocular light-microscope. In the electronprobe the intra- and extracellular concentrations of Ca, P, S were measured separately in scanned fields of dimension 2.5×2.5 µm.

Quantitative results were calculated according to Hall's equation (Hall 1971); we used evaporated salt solutions as standards as described by Krefting et al. (1980). The characteristic, elementspecific radiation was recorded using a wave-length dispersive spectrometer system. White radiation was recorded to determine the dry mass using an energy dispersive X-ray analytical system. The probe current used was 10 nA.

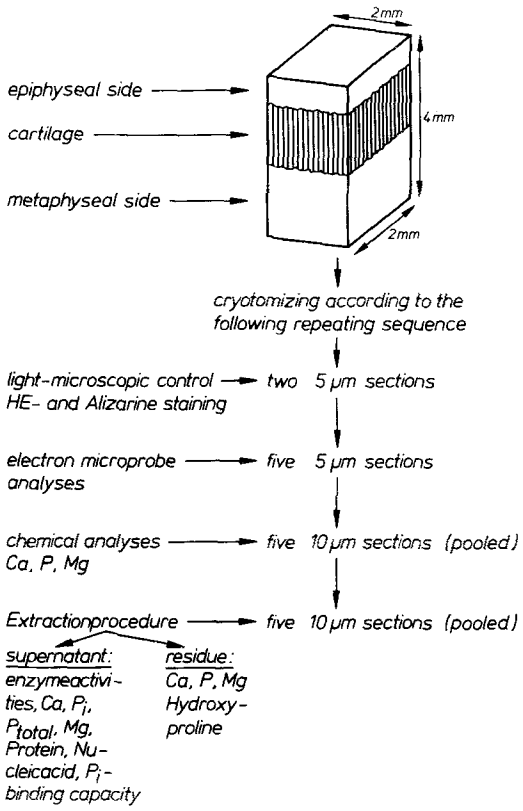

Fig. 1. Flowdiagram of the slicing procedure, demonstrating the distribution of the cross-sections for the different analyses

Chemical Analyses. Pooled frozen sections for biochemical analyses were transferred to an extraction solution containing 250 µl of 10^{-2} M glycine buffer, pH 8.0 in a 0.9% NaCl solution. After extraction periods of 10 min at 4° C as well as 24 h at 37° C, under slight shaking conditions, the tubes were centrifuged for 10 min at 20,000 g. The supernatants were separated from the sediments and the insoluble residues were washed briefly with water, dried, weighed and hydrolised under nitrogen using 6M HCl for 24 h in sealed tubes.

In the supernatants the following components were determined: Nuclease (Nase); acid phosphatase (acPase); alkaline phosphatase (APase); P_i-binding capacity (P_i-C); protein by the method of Lowry (1951); inorganic and organic phosphorus (P_i and P_o), according to Eibl and Lands (1970); and nucleic acid (NA) by measuring the extinction at 260 nm and 280 nm, according to Warburg and Christian (1941). Ca and Mg were measured in the supernatants and in the pooled sections after buffer extraction.

Enzyme Assays. Nuclease activity was measured by a modified method according to Althoff (1974): 20 µl of the supernatant was added to 1 ml of 0.05 M Na-acetate buffer, pH 5.5, containing 10^{-4} M adenylyl-(3′-5′)-adenosine(ApA) and 0.5 µl adenosine aminohydrolase [EC 3.5.4.4.] (Boehringer, Mannheim). The decrease of the extinction at 272 nm was measured. One unit was defined as the extinction difference of 0.01 per minute.

Acid phosphatase activity was determined by adding 10 µl of the extract to 200 µl 10^{-2} M p-nitrophenylphosphate (37° C, buffer: 5×10^{-3} Na-citrate, pH 4.5) with an incubation time of 30 min. After incubation 1.8 ml NaOH (1.0 M) was added. The extinction was measured at 405 nm against a blank.

Alkaline phosphatase activity was measured according to a modified method of Althoff et al. (1978): p-nitrophenylphosphate (10^{-2} M) in 1 M diethanolamine, pH 9.8 in the presence of 0.5 mM $MgCl_2$.

The phosphate (P_i) binding capacity (P_i-C) was defined as bound P_i (nmol per μg protein) after incubation for 15 min at 37° C. Experimental procedure: 50 μl of the supernatant were added to 500 μl 10^{-2} M glycine buffer pH 8.0, containing 30 nmol P_i. The remaining, unbound P_i was determined according to the method of Eibl and Lands (1970).

Ca and Mg were determined by a modified method according to Quint and Höhling (1979): After combustion of the samples with HNO_3/H_2O_2 the Ca content was determined by mechanized "injection method" of flame spectrometry and Mg was determined by AAS.

Results

A longitudinal section of the porcine proximal bipolar ulna growth plate cartilage is shown in Fig. 2, left. Higher magnification micrographs of transverse sections for different levels through this plate are located by lines to indicate the levels in the growth plate in which our topological analyses have been carried out, Fig. 2, right. They demonstrate the different characteristics of each zone. In this paper we shall describe only the results of the diaphyseal side of the bipolar plate. The epiphyseal side is smaller and gave analogous results.

Zone A

Zone of resting cells (germinative zone) contains relatively few cells which are small; it extends for some 50–80 μm in longitudinal direction.

Zone B

Proliferative zone contains cells which are still small but more numerous; they are arranged in pairs or in fours or eights proceeding in the direction of the diaphysis. The size of the cells increases continuously towards the end of the proliferative zone. In this region the cells are more densely packed and no group arrangement could be observed any more. Zone B extends for 200–250 μm.

Zone C

Upper columnar cell zone contains cells which have formed longitudinal rows which are of a relatively constant size: zone C extends for some 90–110 μm.

Zone D

We included in zone D cells which still have a typical *elliptical form*: zone D extends for some 100–130 μm.

Zone E

In this zone the *hypertrophy* of the cartilage cells increases rapidly, finally reaching a relatively *rounded* form. This zone extends for 160–190 μm.

Zone F

This zone contains cells of *maximal hypertrophy* which show already signs of degeneration; it extends for some 180–210 μm and may contain some *traces* of *calcified* cartilage, caused by the slightly arched structure of the growth plate.

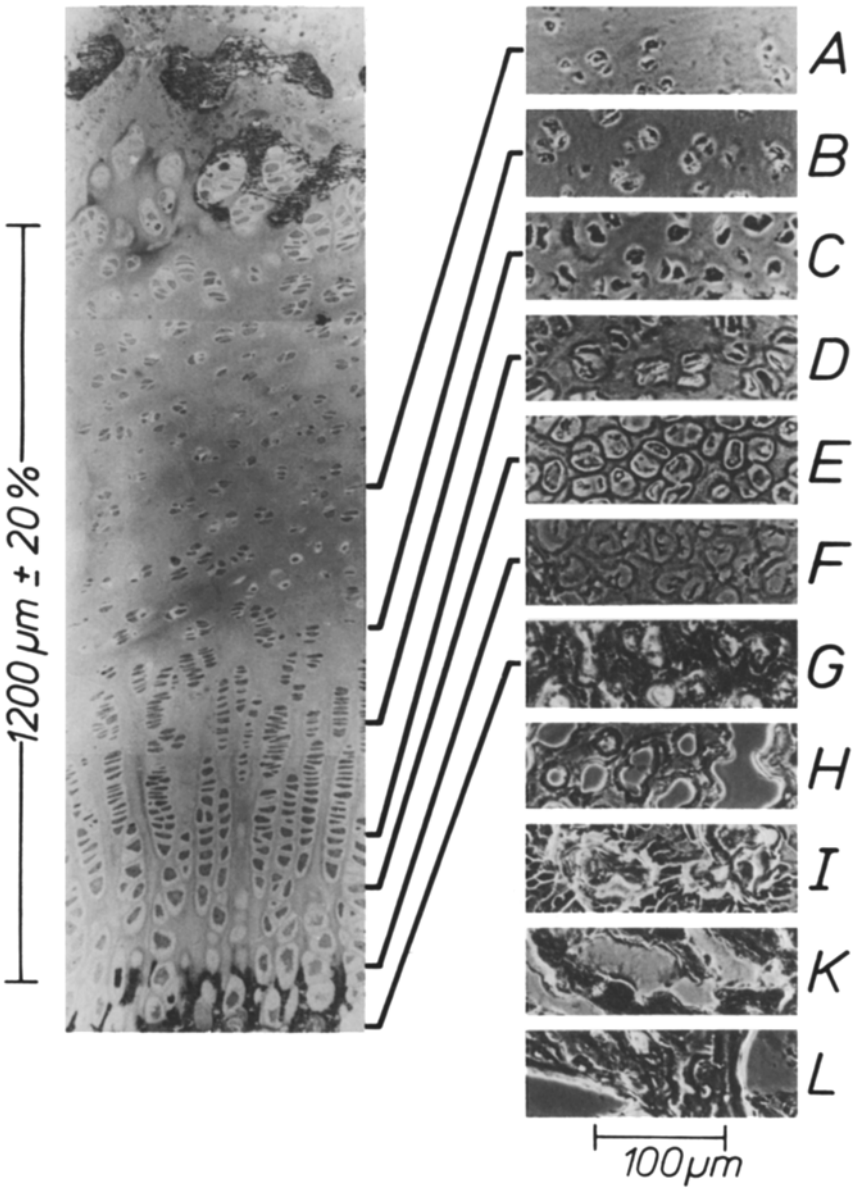

Fig. 2. Left side of the figure: Longitudinal section of a 10 week old porcine proximal ulna epiphyseal growth plate (mainly the unmineralized part of the bipolar growth plate). Right side of the figure: The coordinated cross-sections (metaphyseal part) of the different zones being analyzed

Zone G

Calcification was observed in this zone. The mineralized longitudinal septum between neighbouring longitudinal rows of cells can be seen. This zone extends for some 170–190 μm.

Zone H

In this zone the *calcification* of the longitudinal septum is *nearly complete*; the structure of the septum is altered by the incorporated mineral. Bone formation is commencing. The zone extends for 100–130 μm.

Zone I

In this zone a high cellular activity in the direction of bone-formation can be observed; the calcification has reached a high density. No structural elements of the longitudinal septum can be detected any more. The zone extends for 180–210 μm.

Zone K

In this zone *bone remodelling* occurs indicated by the formation of trabeculae, extending for 360–400 μm. At the end of this zone typical trabecular bone can be recognized.

Zone L

This zone contains pure trabecular bone; bone formation seems to be completed.

The *total* extent of the *diaphyseal side* of the plate which was analysed was about 2.3 mm, the extent of the *unmineralized* diaphyseal part being about *800 μm* while the extent of the *unmineralized* part on the epiphyseal side was about *400 μm*. The data given here are the average values calculated from 5 different animals and vary within ±20%.

Topological Distribution of the Extractable Compounds

Typical topochemical analyses averaged from five tissue blocks from 5 different animals are summarized in Fig. 3. The zones A–L correspond to those which have just been characterized morphologically.

The enzyme activities and the extracted electrolyte concentrations are given in relative values, taking the highest value found as 100%. The 100% values are summarized in Table 1.

The main results which can be derived from Fig. 3 (and Table 1) are the following: No remarkable enzyme activity could be extracted from the cartilage zone A (resting zone). At the end of this zone the *inorganic phosphate-binding capacity* showed a maximum; it decreased steeply to *zero* in zone *D*.

The *nucleic acid* content *increased* steeply in the *proliferative zone* (zone B) peaking at the beginning of zone C (upper columnar zone) and then falling continuously except for some variation in zone E and F (beginning and maximal hypertrophy), down to a constant level in the region of bone remodelling (zone I).

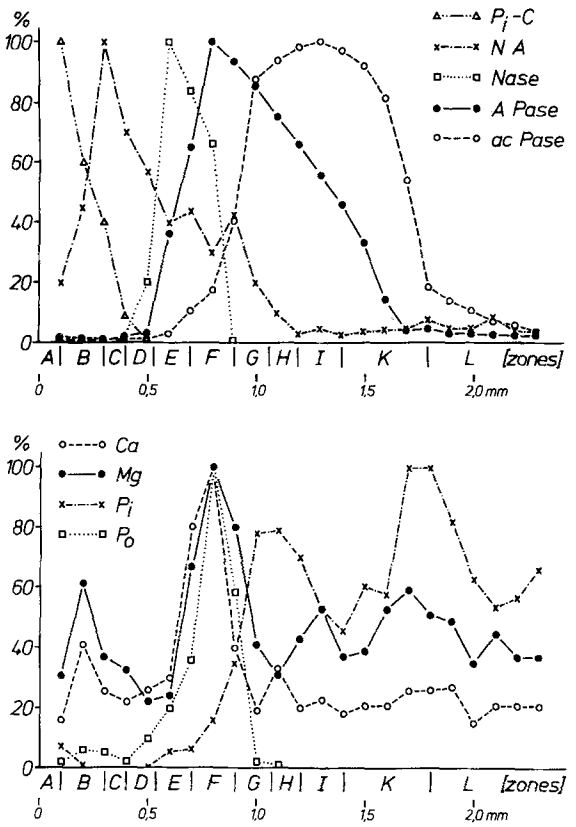

Fig. 3. Extracted compounds of the different zones of a 10 week old porcine proximal ulna epiphyseal growth plate (only the metaphyseal part). The values given are relative values, taking the highest value found as 100%. $P_i - C = P_i$-binding capacity; *NA* = nucleic acid; Nase = nuclease activity; *APase* = alkaline phosphatase activity; acPase = acid phosphatase activity; P_o = organic phosphorus

Table 1. Maximal values of the extracted compounds in the different zones of the growth plate

P_i-binding capacity [P_i-C][a]	6.1	Zone A
Nucleic acid content [NA][b]	0.18	Zone C
Nuclease activity [Nase][c]	12.0	Zone E
Alkaline phosphatase activity [APase][d]	23.3	Zone F
Organic phosphorus [P_o][e]	5.0	Zone F
Calcium [Ca][e]	5.1	Zone F
Magnesium [Mg][e]	0.25	Zone F
Acid phosphatase activity [acPase][d]	2.6	Zone I
Inorganic phosphate [P][e]	1.7	Zone K

These values given are means of five different growth plates. Each sample was analyzed twice. The relative error is 15–25%.

[a] nmol per μg protein; [b] mg per ml; [c] U per mg protein; [d] U per mg protein ($\times 10^{-3}$); [e] weight% per dry mass after extraction

Table 2. Total amounts of Ca, Mg and P in the different zones of the porcine proximal ulna growth plate

Zones	Ca	Mg	P
A (germinative)	0.26	0.055	0.20
B (proliferative)	0.94	0.075	0.65
C (palisades, upper)	2.20	0.15	1.23
D (palisades, lower)	1.88	0.15	1.20
E (hypertrophic)	4.21	0.15	2.35
F (degenerative)	6.85	0.20	3.34
G (primary calcification)	10.22	0.32	4.60
H (transition to primary spongiosa)	17.55	0.32	7.54
I (primary spongiosa)	19.75	0.51	8.80
K (remodelling)	13.12	0.32	6.56
L (trabecular bone)	22.10	0.45	9.16

The chemical data are given in weight% per dry mass. The coefficient of variation is 10–20%

Measurable amounts of a nuclease activity could be detected in zone D, where the first signs of cell hypertrophy can be observed, and peaked in zone E where the increase in cell size was very rapid.

A steep increase in alkaline phosphatase activity followed and overlapped with the nuclease activity. A maximal alkaline phosphatase activity was found in zone F, the zone of maximal hypertrophy and cell degeneration (erosion), just ahead of the mineralization front. It is possible that some slices may contain calcified material due to the slightly arched growth plates. The first increase in acid phosphatase activity was also measurable in zone F (maximal hypertrophy); this increase continued in zones G and H, peaking with a broad maximum in zone I.

The Ca and Mg content (probably mainly ions) which could be *extracted* from the sections with the buffer as well as the extracted *organic phosphorus* show strong *correlation* with the *alkaline phosphatase* activity since they all *peaked* together in zone F (maximal hypertrophy).

The maximal value for inorganic phosphorus was found in zone K (bone remodelling), but there was also a peak in zones G and H and increased in coincidence with the acPase activity.

Total Amount of Ca, P, Mg (Chemical Analysis) and the Intra- and Extracellular Distribution of Ca, P and S (Microprobe Analysis)

The total amounts of the elements Ca, P, Mg (integrating the intra- and extracellular distribution) were analyzed chemically and the intra- and extracellular distribution of Ca, P, Mg were analyzed by electron microprobe analysis. Since the freezing conditions in this project to avoid an intra-extra-cellular exchange of the mobile elements were not optimal the intracellular concentrations must be regarded with caution and are not included in Table 2. Meanwhile better freezing conditions could be achieved for the proximal growth plate of the tibia of rats, and results of an intra- and extracellular distribution for

Ca and P (and S) are reported by Krefting et al. (1980). It should be mentioned that a good agreement exists for the extracellular Ca, P and S contents between the tibia growth plate and the porcine growth plate of this project.

The main results summarized in Table 2 are the following: Taking the total amount of Ca of the germinative and proliferative zone (Zones A, B) (analyzed chemically) the concentration (per dry weight) lies in the range of 0.6%. This value lies in the same range of the extracellular Ca content of 0.7% analyzed by electronprobe microanalysis (corresponding to about 58 mM/kg wet weight, assuming a water content of 67% for the tissue).

While the total Ca content is steadily increasing up to about 7% in Zone F (zone of maximal hypertrophy) the extracellular Ca content analyzed by microprobe analysis remains relatively constant through all zones, increasing to about 1% just in the vicinity of the first mineralized islands. While this discrepancy in contents can be explained for Zone E by the fact that some foci of mineralized regions are included in the chemical analyses we have not yet clarified why the chemical Ca values are significant higher in Zone C and D.

Discussion

The reason for the extraction procedure must be discussed. The cryostat sections are 10 µm thick. Assuming an average cell diameter of about 20 µm, each cell would be cut by this procedure. Thus, the slicing is comparable with a mechanical homogenisation, and consequently the buffer treatment yielded an extract containing intra- and extracellular material. In order to keep the enzymes and the electrolytes in their physiological state, as far as possible, we did not use detergents but extracted under mild conditions with 0.9% NaCl solution. The slight alkaline medium of pH 8.0 was chosen to avoid a dissolution of apatite, which is slightly soluble at pH 7.0. Furthermore this pH value causes a reduction (about 20%) of the activity of the neutral proteases being present in these areas of the growth plate.

This electronprobe data showed that the extracellular Ca content remained relatively constant in the germinative and proliferative areas of the growth plate in the range 0.7% (58 mM/kg wet weight, for 67% water).

The extracellular P content in these zones lies in the range 0.1%–0.2% (10–20 mM/kg wet weight for 67% tissue water). The product of the contents of extracellular Ca and P lies in the range 580–1,160 mM^2. The ion product for Ca^{2+} and P_i which is necessary for an in vitro mineralization in a collagenrich matrix lies in the range 2 mM^2 (Fleisch and Neuman 1961). This means that the concentration of Ca and phosphate is much higher already in those zones which are far away from the mineralization front. On the one hand this would mean that a great reservoir, especially for Ca, exists already in the unmineralized regions, on the other hand we will have to explain in which way the system keeps Ca and phosphate in such a state that mineral nucleation is prevented till a stimulus for the nucleation is given in the hypertrophic zone.

Our quantitative microprobe data were in relatively good agreement with the results of microprobe analyses of Boyde and Shapiro (1980), except that

they could not detect extracellular P with their energy dispersive system already in the germinative and proliferative zone.

In the proliferative zone (Zone B, Fig. 3) we found high amounts of extractable Mg and Ca (probably mainly as ions), related to the remaining dry mass after extraction. However, this peak would disappear when the contents of extracted Ca and Mg would be related to the constant volume of the slices ($5 \times 10\ \mu m \times 2{,}000\ \mu m \times 2{,}000\ \mu m = 0.2\ mm^3$) and not to the remaining dry mass. A real maximum for extractable Ca and Mg occurs in Zone F (maximal hypertrophy) (Fig. 3).

The proliferating rate in the top region of the cell columns (Zone D) is still very high. The extracted nucleic acid content showed a maximal value here. These findings agree fairly well with those of Schmidt et al. (1978). A steep increase of nuclease activity in Zone E (hypertrophy) may be associated with a change in cell metabolism. Although the cells are strongly hypertrophying they are still very productive and several enzymes can be detected. Schmidt et al. (1978) found the highest lysozyme activity of the epiphyseal growth plate in this zone; and high activities of β-glucuronidase, cathepsin, alkaline phosphatase and acid phosphatase were found in this region by Granda and Posner (1971). Due to the relatively high resolution of our analyses, we were able to differentiate between the zonal distribution of the enzymes.

The present results demonstrate clearly that the nuclease activity occurs first and is followed shortly, and being overlapped, by an increase in the alkaline phosphatase activity; the acid phosphatase increase occurs at a later stage. If the resolution of our analytical technique would have been lower, these enzyme activities would have been detected together.

With regard to the sequence of the enzyme activities there is good reason to assume that the nuclease activity has to do with the splitting of phosphodiester linkages in DNA and RNA when they are released into the extracellular space in connection with the beginning degeneration of the cells. Thus, the nuclease activity would produce phosphomonoesters. Such monoesters are well-known to be inhibitors of the calcification process (Blumenthal et al. 1977).

In the following region of maximal hypertrophy and cell erosion (zone F) alkaline phosphatase activity showed a sharp maximum accompanied by a peak of buffer extractable organically bound phosphorus, Mg and Ca (Fig. 3). The extracted Ca^{2+} in this region obviously does not originate from dissoluted apatite, otherwise a peak for extractable P_i has to be present at the same place.

Mg^{2+} and Ca^{2+} can form soluble 1:1 complexes with the phosphoesters. Especially the Mg-phosphomonoester complexes are good substrates for the alkaline phosphatases of mineralizing collagenrich systems and can be split in this region (Althoff et al. 1978). The controlling function of Mg during the calcification process was discussed recently (Quint et al. 1980).

With regard to the special slope of the alkaline phosphatase activity in the calcified regions of the growth plate, it is possible that a second alkaline phosphatase activity exists indicated by the shoulder of the curve in the zones H, I and K (Fig. 3).

At the end of Zone F (maximal hypertrophy) organically bound phosphorus

has disappeared; the intercolumnar cartilage matrix becomes more and more calcified. We conclude that the alkaline phosphatase causes the liberation of orthophosphate in the right locations resulting in mineralization.

The increase in acid phosphatase activity and the parallel increase of extractable inorganic phosphate indicate the first signs of bone remodelling (Reddi 1981). It is interesting to note that the total Ca and P content is high in the zones H, I and L (cartilage mineralization → bone formation). In the zone of bone remodelling (Zone K) however, Ca is decreased to 13% and P to 7%.

With regard to the formation of trabecular bone, a process in which osteoclastic activities are involved, the mineralized intercolumnar cartilage matrix has to be catabolized. This mechanism is, of course, different from the process of primary cartilage mineralization and will not be discussed.

The aim of these studies was to analyze the early stages of mineralization by high topological resolution. Comparing the present results with the distribution and activity of the matrix vesicles it seems important to note that the zone in which most of the matrix vesicles appear (Anderson 1967; Bonucci 1967; Ali 1976) and become mineralized, coincides with zone F (zone of maximal cell hypertrophy) in which the alkaline phosphatase activity, the buffer extractable organically bound phosphate, the extractable Ca and Mg have a maximum. Thus, these data show that topological analyses with relatively high resolution may reflect the chronology of the cartilage mineralization.

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