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ALKALINE PHOSPHATASES IN ENDOCHONDRAL OSSIFICATION OF RATS LOW IN CALCIUM AND VITAMIN D DEFICIENT

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Young rats fed a low calcium and vitamin D deficient diet for 2 weeks developed hypocalcemia and increased alkaline phosphatase activity in serum. The serum alkaline phosphatase activity (pNPPase) was found to be of skeletal origin. In accordance, the total non-specific alkaline phosphatase (pNNPase) activity in the microsomal fraction of tibial epiphyseal cartilage and metaphysis increased in the deficiently fed group when compared to the normal group. An increased activity in the microsomal fraction of tibial epiphyseal cartilage and metaphysis was shown both for inorganic pyrophosphatase and total ATP-degrading enzyme activity in the deficient group. This was also found in the presence of R 8231, indicating an increased activity of Ca^{2+}-ATPase, shown to be present in both the epiphyseal plate and the metaphysis. These increased enzyme activities were consistent with the known effects of hypocalcemia and/or parathyroid hormone (PTH) on bone alkaline phosphatase activity. The increase in Ca^{2+}-ATPase might, however, be a direct response to the hypocalcemia present in the deficient animals. Furthermore, the findings in the present study support the view that the same alkaline phosphatase iso-enzyme is present at different calcification loci.

Key words: alkaline phosphatases; endochondral ossification; hypocalcemia and vitamin D deficiency

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Non-specific alkaline phosphatases (APase) of bone and cartilage have been shown to release inorganic phosphate from a number of different substrates, among them ATP and inorganic pyrophosphate (Eaton & Moss 1967, Majeska & Wuthier 1975). Apart from the ATP degradation performed by APase, Felix & Fleisch (1974) and Messer (1975) have described a Ca^{2+} and Mg^{2+} activated ATPase in bone. In the dentinogenically active odontoblast, Granström et al. (1979) have separated a specific Ca^{2+}-ATPase from APase, which was shown to be involved in the cellular calcium transport by Linde & Granström 1981). A close resemblance between the alkaline phosphatase iso-enzyme pattern in odontoblasts and epiphyseal cartilage has previously been shown by Granström & Linde (1977), but data on the presence of a Ca^{2+}-ATPase at endochondral ossification sites are still lacking. In the odontoblasts-predentin region of young rats low in calcium and vitamin D deficient, the activities of APase, inorganic pyrophosphatase (PP_iase), total ATP-degrading enzyme activity and Ca^{2+}-ATPase were found to be increased (Engström et al. 1977a, Engström et al. 1977b). Also, in tibial metaphysis of vitamin D deficient hypocalcemic rats an increased activity of APase was reported by Wergedal (1969). In the epiphyseal cartilage and the cartilage portion of the intermaxillary suture of rats low in calcium

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and vitamin D deficient, Engström et al. (1980) found an increase in alkaline phosphatase activity, when compared to normal rats, using histochemical methods. This overall increase in APase found at different hard tissue forming loci of low calcium and vitamin D deficient animals was shown to be reflected by increased serum APase values (Motzok 1950, DeLuca & Steenbock 1956, Wergedal 1969). In low calcium and vitamin D deficient rats there is a stimulation of the parathyroids (Engström 1980). In response to parathyroid hormone stimulation, increased activities of ATPase and PP_iase have been reported (Morgan et al. 1973, Ramp 1975, Lieberherr et al. 1977, Lieberherr et al. 1979). These studies on parathyroid-stimulated changes in alkaline phosphate activities are *in vitro* studies and their results are not directly applicable to the previously mentioned *in vivo* low calcium and vitamin D deficient studies, although the findings are not contradictory.

Thus the aim of the present study was twofold: first to investigate if it is possible to identify a specific Ca^{2+}-ATPase in epiphyseal cartilage and metaphyseal bone, and secondly to find possible changes in activities of APase iso-enzymes in the epiphyseal and metaphyseal region of young rats fed a low calcium and vitamin D deficient diet.

MATERIAL AND METHODS

Animals. Young male Sprague-Dawley rats (body weight 75 g) were used. The rats were divided into two groups. One group was given a vitamin D deficient, low calcium diet (R 25) and a second group a control diet (R 47). Both groups were fed their respective diets for 2 weeks. The experimental groups were held in a dark room with no daylight. The Ca and P content of the diets are shown in Table 1. The animals had free access to tap water, with negligible mineral content.

Blood analyses. At the termination of the experimental period, the animals were anesthetized by ether inhalation. Blood was withdrawn by intra-cardiac puncture and analyzed for calcium and phosphate content at the laboratory for clinical chemistry at the local university hospital (Sahlgrenska sjukhuset, Göteborg, Sweden). Serum alkaline phosphatase activity measured as para-nitro-phenyl phosphatase (pNPPase) was determined according to a colorimetric method described by Granström & Linde (1972).

Table 1. Content of P and Ca in the diets. Values are expressed as g per kg content of diet

Diet code	R 25	R 47
Total Ca (%, w/w)	0.04	1.2
Total P (%, w/w)	0.7	0.7
Vitamin D	–	+

Preparation of tissue. The tibia from each animal was dissected out and immediately placed in a 0.15 M NaCl solution. The tibia metaphysis was prepared by a modification of the method of Borle et al. (1960). After freeing the bone from muscle and tendinous attachments, the epiphyseal cartilage was removed. Under a stereo-microscope each tibia was cut longitudinally into two pieces, the marrow carefully removed and the metaphysis region carefully dissected out. The epiphyseal cartilage and metaphysis were transferred to different Sorvall homogenizers containing 500 µl 0.32 M sucrose in 0.01 M Tris-HC buffer pH 7.4. After crushing with a glass rod the sample was homogenized for 15 min in the cold. The homogenates were centrifuged at 40,000 × *g* for 30 min. The sediment was resuspended in the sucrose solution and recentrifuged at 40,000 × *g* for 30 min. The pellet, containing cellular debris, extracellular material, nuclei and mitochondria was discarded. The supernatants from the two centrifugations were combined and the microsomal fraction was pelleted at 151,000 ×*g* for 60 min. The pellets were suspended in 2 ml glycine-NaOH buffer pH 9.8. In each group, the total enzyme activity in 10 different homogenates was measured. The enzyme analysis was run in triplicate for each homogenate.

Enzyme assays

pNPPase. The enzyme assay was performed according to Granström & Linde (1977). The incubation buffer was 0.1 m glycine-NaOH, pH 10.2, containing 3 mM $MgCl_2$ and a substrate concentration of 3 mM p-NPP. From the microsomal fraction, a 10 µl portion was taken for the enzyme assay.

PP_iase, total ATP degradation and Ca^{2+}-ATPase. The colorimetric methods used for the assay of these enzymes were modified after Wöltgens & Ahsman (1970) (PP_iase) and described by Fredén et al. (1975) (ATPase degradation) and Granström & Linde (1976) (Ca^{2+}-ATPase) respectively. The pH of the incubation buffer (0.1 M glycine-NaOH) was 8.8 in the PP_iase assay and 9.8 in the assay of total ATP degradation and Ca^{2+}-ATPase. The substrate content was 8 mM with regard to $Na_4P_2O_7$ and Na_2ATP, and 3 mM $CaCl_2$ was added in the Ca^{2+}-ATPase assay and 3 mM $MgCl_2$ in

the other assays. The reaction mixture for the determination of Ca^{2+}-ATPase also contained 0.1 mM R 8231 (generously donated by Dr M. Bergers, Janssen Pharmaceutica, Belgium), a specific inhibitor of APase.

The calculated total activity of the enzymes was related to tissue amount assayed as DNA according to Kissane & Robins (1958) and protein according to the method by Lowry et al. (1951) using albumin as a standard.

Characterization. To partially characterize the enzymes, the following tests were performed. The pH-optima were determined in 0.1 M glycine-NaOH buffer. Maximal substrate concentrations were determined. The influence of concentrations up to 24 mM of Mg^{2+} and Ca^{2+} ions on the enzymes were tested. Incubations were followed for up to 5 h to study the linearity of the enzyme reaction. The effect of 0.1 mM R 8231 was also tested.

The effect of heat on pNPPase activity was studied. After heat-treatment of the microsomal fraction at 56°C for different time intervals, the activity was subsequently determined according to the method above.

In order to determine if the major part of serum alkaline phosphatase activity was of skeletal origin, the heat-inactivation patterns of sera and metaphysis from animals fed the different diets were studied. Each sample was divided into two aliquots one of which was kept in the cold. The remaining aliquot was transferred to a water bath, temperature 56°C. After heat-treatment for 0, 5, 10, 15, 20 or 25 minutes, the aliquot was transferred to an ice-water bath for 10 minutes. The aliquots were removed from the ice-water bath and APase activity determined when they had reached room temperature.

RESULTS

Characterization of enzyme activities

The characteristics recorded below did not differ between the epiphyseal cartilage and the metaphysis; therefore, no distinction between the sources of enzyme was made when presented.

Optimal reactions were obtained at pH 8.8 (PP_iase), 9.8 (total ATP degradation and Ca^{2+}-ATPase) and 10.2 (pNPPase). The p-NPPase reaction showed a normal *substrate saturation* up to at least 24 mM. Enzyme activities with PP_i and ATP as substrates were optimal at 3 mM substrate concentration whereas higher concentrations of substrate were slightly inhibitory. *Mg^{2+}* added to the incubation medium in concentrations up to 3 mM activated all enzyme activities when the substrate concentration was 3 mM. Higher concentrations inhibited all enzymes.

Ca^{2+} added to the medium slightly activated the Ca^{2+}-ATPase and total ATP-degrading enzyme activity in concentrations up to 3 mM. Higher concentrations inhibited the enzymes. Both p-NPPase and PP_iase were slightly inhibited by Ca^{2+} at all concentrations.

The pNPPase activity was linear with *incubation time* during the first 30 min whereas PP_iase and total ATP-degrading enzyme activity were linear with incubation time during the first 45 min. Ca^{2+}-ATPase still showed a linear reaction after 5 h.

The effect of 0.1 mM *R 8231* is shown in Table 2. The remaining enzyme activities after 30 min and 2 h are compared to the enzyme activities unaffected by R 8231. As can be seen from the Table, after 30 min, R 8231 inhibits all enzyme activities. Remaining activities vary between 2.5 and 14 percent, ATPase activity being least affected. After 2 h, however, there is a higher proportion of ATP-degrading enzyme (39–42 percent) than of the other enzymes.

The pattern of *heat-inactivation* is seen in Figure 1. The alkaline phosphatases (APase) in the

Table 2. Effect on enzyme activities of R 8231. Values expressed as percent of enzyme activity without R 8231 present. Mean values of five experiments

Source of enzyme	Substrate	Incubation time	Activity remaining after 0.1 mM R 8231 was included (%)
Metaphysis	ATP	30 min	14
,,	ATP	2 h	42
,,	PP_i	30 min	5
,,	PP_i	2 h	11.2
,,	p-NPP	30 min	2.5
,,	p-NPP	2 h	9.7
Epiphyseal cartilage	ATP	30 min	13
,,	ATP	2 h	39
,,	PP_i	30 min	5.8
,,	PP_i	2 h	11.8
,,	p-NPP	30 min	3
,,	p-NPP	2 h	8.3

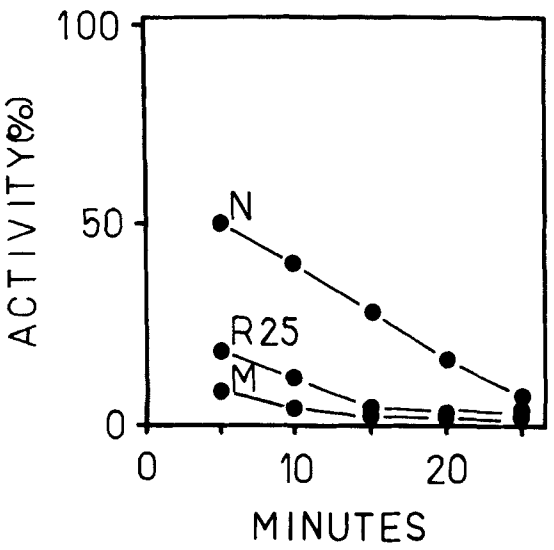

Figure 1. Effect on heating serum and the microsomal fraction of metaphysis at 56°C for different times. Remaining APase activity expressed as percent of maximal activity. N = Serum APase from animals fed an adequate diet. R 25 = Serum from animals fed the low calcium and vitamin D deficient diet. M = APase from microsomal fraction of metaphysis of animals fed the low calcium and vitamin D deficient diet as well as of animals fed the adequate diet. The S.D. was 7.3 percent of the activity or lower at each point.

metaphysis region of both the normal and the deficient animals showed a great sensitivity to heat. After 5 minutes of heat-treatment, the activity was reduced to 10 percent of the non-treated activity. The APase activity in normal serum showed greater resistance to heat and decreased to 15 percent of non-treated serum after 25 minutes of heat-treatment. The pattern of heat-inactivation of APase in serum from animals fed the deficient diet (R 25) when compared to the inactivation patterns of APase in tibia metaphysis and serum from the control group, fed the normal diet (R 47), resembled the pattern of the metaphysis. The activity decreased to 15 percent of the non-treated value after a heat-treatment of 5 minutes. The increased serum APase in the experimental group seemed to be of skeletal origin. This is consistent with the reported finding of increased activity of the metaphysis in the deficiently fed group.

Total activity determinations

The phosphatase activities recorded from the epiphyseal cartilage and metaphysis in the groups of animals fed the two diets are shown in Tables 3 and 4. The values are related both to DNA and protein content in the microsomal fractions. As can be seen from the Tables, all enzyme activities showed a significant increase in the R 25 group when related to DNA. When related to protein, the increase was less in the epiphyseal plate and the Ca^{2+}-ATPase activity was not significantly raised. In the metaphysis, only pNPPase in the R 25 group showed significantly increased activity when related to protein.

DISCUSSION

It is well-known that APase exists in bone and cartilage, and that it is a non-specific enzyme

Table 3. Phosphatase activities in epiphyseal cartilage of rats fed a low calcium, vitamin D deficient diet (R 25) and rats fed an adequate diet (R 47). Activities (mean values ± S.D. n = 10) are given as nmol liberated phosphate per min and μg DNA or μg protein. The Ca^{2+}-ATPase activity was assayed in the presence of 0.1 mM R 8231

	Activity per DNA			Activity per protein		
	R 25	R 47	<P	R 25	R 47	<P
p-NPPase	1.26±0.27 (n=10)	0.32±0.13 (n=10)	0.001	0.17±0.04 (n=10)	0.12±0.04 (n=10)	n.s.
PP_iase	2.18±0.38 (n=10)	0.12±0.06 (n=10)	0.001	0.30±0.06 (n=10)	0.03±0.02 (n=10)	n.s.
Total ATP-degradation	1.64±0.38 (n=10)	0.47±0.24 (n=10)	0.001	0.28±0.05 (n=10)	0.17±0.03 (n=10)	n.s.
Ca^{2+}-ATPase	0.33±0.06 (n=10)	0.13±0.05 (n=10)	0.001	0.05±0.01 (n=10)	0.05±0.01 (n=10)	n.s.

Table 4. Phosphatase activities in metaphysis of rats fed a low calcium, vitamin D deficient diet (R 25) and rats fed an adequate diet (R 47). Activities (mean values ± S.D. n = 10) are given as nmol liberated phosphate per min and μg DNA or μg protein. The Ca^{2+}-ATPase activity was assayed in the presence of 0.1 mM R 8231

	Activity per DNA			Activity per protein		
	R 25	R 47	<P	R 25	R 47	<P
p-NPPase	0.29±0.10 (n=10)	0.08±0.01 (n=10)	0.001	0.04±0.01 (n=10)	0.01±0.006 (n=10)	n.s.
PP_iase	0.65±0.22 (n=10)	0.43±0.08 (n=10)	0.001	0.09±0.03 (n=10)	0.10±0.03 (n=10)	n.s.
Total ATP degradation	0.61±0.24 (n=10)	0.36±0.08 (n=10)	0.001	0.08±0.01 (n=10)	0.08±0.03 (n=10)	n.s.
Ca^{2+}-ATPase	0.19±0.08 (n=10)	0.09±0.02 (n=10)	0.001	0.03±0.01 (n=10)	0.02±0.005 (n=10)	n.s.

which hydrolyzes mono-, di- and triphosphatase (Eaton & Moss 1967, 1968, Fleisch & Neuman 1961, Majeska & Wuthier 1975). In epiphyseal cartilage, a Ca^{2+}-Mg^{2+}-ATPase with characteristics different from APase in bone has been reported by Felix & Fleisch (1974), Messer (1975) and Messer et al. (1975).

In odontblasts, the view is that at least two different phosphatases are active in the presence of an alkaline pH. One is a non-specific alkaline phosphatase (APase) which is active against p-NPP, PP_i and ATP, among other substrates. The other is a specific Ca^{2+}-ATPase which prefers ATP as substrate, but to a lesser degree hydrolyzes ADP, GTP and some other nucleotides (Granström & Linde 1976).

The findings from this study support earlier findings that there exist at least two enzymes capable of degrading ATP at alkaline pH in bone and cartilage. The APase of bone and cartilage show many characteristics similar to the APase of dentin and enamel organ (Granström & Linde 1977). The activity remaining after total ATP degradation in the presence of R 8231 shows characteristics similar to the Ca^{2+}-ATPase reported in odontoblasts (Granström & Linde 1976) and bone (Felix & Fleisch 1974). In the light of the conclusions discussed in the paper of Granström & Linde (1976), we consider that the remaining activity of ATP degradation, in the presence of R 8231, is due to a separate enzyme, here tentatively named "Ca^{2+}-ATPase". Majeska & Wuthier (1975) recorded the same inhibition pattern with R 8231 for phosphate activities in cartilage, but did not discuss this finding in relation to a specific Ca^{2+}-ATPase.

In a previous biochemical study of alkaline phosphatases in odontoblasts or rats deficient in vitamin D and low in calcium, we found a considerable increase in all alkaline phosphatase activities from the deficient animals (Engström et al. 1977b).

In an earlier histochemical study on epiphyseal cartilage and the cartilage portion of the intermaxillary suture of rats fed a low calcium, vitamin D free diet, a slight increase in activity of alkaline phosphatases was observed (Engström et al. 1981). It is our opinion that the biochemical techniques employed in this study are superior to the histochemical techniques when comparing enzyme activities. The increase in enzyme activities found in this study was highest when the activity was related to DNA as compared to when it was related to protein. It is reported that osteoid increased considerably when feeding young rats a low calcium, vitamin D free diet (Engström et al. 1981). As a large amount of the osteoid is included when the tissues are dissected out the increased protein content might explain the smaller increase in enzymatic activity, as compared to activity expressed in relation to the DNA content.

An increase in APase activity in bone has been previously shown in vitamin D deficient and hypocalcemic rats (Wergedal 1969) and chicks (Motzok 1950). These reports and our findings demonstrate a local response in hard tissue forming cells to diets deficient in calcium and vit-

amin D. The influence of vitamin D on enzymatic activities in bone was excluded by Wergedal & Baylink (1971) and Wergedal et al. (1973). The increase might be a direct response to the hypocalcemia. Ca^{2+}-ATPase has been suggested to be related to the calcium transport of the cell (Ali et al. 1970). The increase in Ca^{2+}-ATPase might thus be due to a decreased supply of calcium ions in the deficient animal.

The increase in pNPPase, PP_iase and total ATP-degrading enzyme activity in the deficient animal might be a response to the increased level of circulating immunoreactive parathyroid hormone in serum noted with the present deficient diet (Engström 1980). In previous studies on the odontoblasts in low calcium and vitamin D deficient rats, indications of an increased metabolism were found (Engström et al. 1977b, Engström et al. 1978, Engström 1980). The parathyroid hormone has been reported to stimulate the matrix-production of bone (Vaes & Nichols 1962, Kalu et al. 1970, Walker 1971, McGuire & Marks 1974). It is not known at present whether the changes in alkaline phosphatase activities are due to the hypocalcemia *per se* or the increased parathyroid hormone level.

In conclusion, this study has shown the presence of two alkaline phosphatases in epiphyseal cartilage and metaphyseal bone, one a calcium active ATPase and the other a non-specific APase active against pNPP, PP_i and ATP, among other substrates. Both these alkaline phosphatases were found to have increased activity in epiphyseal and metaphyseal bone of low calcium and vitamin D deficient rats. This increase is consistent with the known effects of hypocalcemia and/or high PTH on bone alkaline phosphatase activities.

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