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CHANGES OF FEMORAL ALKALINE PHOSPHATASE ACTIVITY 
IN ADULT RATS TREATED BY SORBITOL ENRICHED OR 
VITAMIN D₃ DEFICIENT DIET

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The effect of vitamin D₃-deficiency and dietary sorbitol on serum calcium level,
the activity and alkaline phosphatase (AP) pattern in femoral epiphysis were studied.
Rats fed a diet supplemented with sorbitol or vitamin D₃ showed the same serum
calcium concentration and AP activity in serum and femur. Rats fed a vitamin
D₃-deficient diet displayed decreased serum calcium concentration and increased AP
activity both in serum and femur.

Four forms of AP were isolated from the femur of these rat groups: of M, 100 000,
110 000, 130 000 and 165 000. Rats receiving the diet supplemented with sorbitol
showed a marked rise in the activity of the M, 165 000 form, and appearance of a new
monomer of 100 000, never formed in two remaining groups.

The effect of sugars on alkaline phosphatase (AP) activity and calcium
absorption is well known [1, 2].

The correlation between enzyme activity, vitamin D₃ and calcium
absorption in adult rat intestine as well as in cartilage cells of rachitic chicks
has been reported [4, 5].

The results of our previous study showed also stimulating effect of vitamin
D₃ on intestinal alkaline phosphatase, especially in rat duodenum [6].
Moreover, we demonstrated that vitamin D₃ affects only alkaline phosphatase
F₃ built up of two identical subunits of M, 90 000. Vitamin D₃ fails to influence
the activity of the homodimer F₁ built of the M, 65 000 monomers [6].
The aim of the present study was to determine whether a vitamin D₃-deficient or sorbitol-enriched diet does influence the activity of individual forms of AP in adult rat femur.

MATERIALS AND METHODS

All experiments were performed on male Wistar rats. Just after weaning, the animals were divided into three groups. Group C was fed a diet containing (g/100 g): starch 69.4; purified casein 18; peanut oil 8; vitamin A 500 UI; KH₂PO₄ 0.9; salt mixture 2.7 and vitamin B mixture 1. This diet contained a normal level of calcium and phosphorus but was completely free of vitamin D₃. The second group (D) was fed the same diet supplemented with vitamin D₃ 5 UI daily, and the third group (S), with 12% sorbitol (without the addition of vitamin D₃). After 4 weeks, the animals were killed. The part of femur containing the metaphysis, epiphyseal cartilage and epiphysis, was removed, liberated from bone marrow, washed with cold water and pressed as described in the previous paper [7].

Enzyme activity assay. Total AP activity was measured using p-nitrophenylphosphate (PNPP) as substrate, according to Bessey et al. [8]. AP activity was located on the gel using α-naphthylphosphate.

Phosphorylation procedure. Native AP forms in femur homogenate, aqueous supernatant and DOC extract of pellet were incubated for 30 s at 0°C in 100 μl of a solution containing: 15 mM imidazole, pH 6.8, 50 mM KCl, 0.125 mM EGTA, 6.25 mM EDTA, 0.5 μCi [³²P]ATP (specific activity 3000 Ci/mmol). The protein content was 50 μg per assay. The reaction was stopped by addition of 50 μl of ice-cooled 10% trichloroacetic acid, containing 50 mM H₃PO₄ and 0.5 mM ATP. The precipitate was washed with the latter solution and then dissolved in a mixture containing: 5% glycerol, 50 mM Tris/HCl buffer, pH 7.8, 2% SDS, 50 mM dithiothreitol.

Polyacrylamide gel electrophoresis. Native and denatured samples (3 μg of protein) were loaded on minigel slab of 5 or 6.75% acrylamide for electrophoresis [9]. Electrophoresis was carried out at 10 mA per gel for 1.5 h. Autoradiography of dried slab gel was performed using X-Omat Kodak films.

Identification of AP monomers. It is possible to detect AP monomers in nonpurified material: femur homogenate, aqueous supernatant and pellet extract because the enzyme in the presence of [γ-³²P]ATP forms a radioactive phospho-intermediate of alkaline phosphatase. OH groups of serine in the active centre in each of AP subunits are the site of phosphate binding; each of them after denaturation is labelled by ³²P. Therefore the separated monomers can be detected on autoradiograms after gel electrophoresis. They can be identified as phospho-intermediate forms of AP on the basis of the following criteria: resistance during the phosphorylation to alkaline treatment, and complete inhibition by excess of an other substrate, such as inorganic phosphate or glycerophosphate [10, 11].
RESULTS

The level of calcium in serum and total alkaline phosphatase activity in serum and femur of adult rat as well as the distribution of enzyme forms and their monomers in the three groups of animal fed different diets were studied (Table 1).

Table 1
Total alkaline phosphatase activity and calcium level in serum and femur of adult rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Ca (mg/l)</th>
<th>Alkaline phosphatase (UI/l)</th>
<th>(UI/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D₃-deficient (C)</td>
<td>58 ± 3.4</td>
<td>402.7 ± 15</td>
<td>657 ± 84</td>
</tr>
<tr>
<td>Vitamin D₃-enriched (D)</td>
<td>103 ± 2.1</td>
<td>180.2 ± 6.8</td>
<td>338 ± 35</td>
</tr>
<tr>
<td>Sorbitol enriched (S)</td>
<td>99 ± 0.2</td>
<td>192.5 ± 7.2</td>
<td>344 ± 26</td>
</tr>
</tbody>
</table>

The rats receiving vitamin D₃ showed a normal serum calcium concentration and normal AP activity in serum and femur. The rats fed a vitamin D₃-deficient diet displayed a decrease in serum calcium concentration and a rise in AP activity both in serum and femur. The rats fed diet supplemented with sorbitol exhibited similar levels of calcium and enzyme activity as the rats fed vitamin D₃ (Table 1).

The distribution of different alkaline phosphatase forms and their monomers in femur of adult rats fed different diets were obtained by polyacrylamide gel electrophoresis. In rat femur of all three groups of animals, four alkaline phosphatase forms of \( M_r 100000, 110000, 130000 \) and 165000 were present (Fig. 1 AB): two forms of \( M_r 110000, 165000 \) in the water supernatant (soluble) and three of \( M_r 130000, 110000 - 115000 \) and 100000 in DOC extracts were observed (Fig. 1B). The major form of \( M_r 110000 - 115000 \) found in extract I and extract II was less strongly bound to membranes as compared with the two remaining forms of \( M_r 130000 \) and 100000, which were released only after DOC extraction. With ATP as substrate the native forms of AP gave enzyme-phosphate intermediates.

On SDS polyacrylamide gel electrophoresis AP dissociated into the intermediate-containing subunits of \( M_r 50000, 55000, 65000 \) and 75000 — 80000 (Fig. 2).

In vitamin D₃-deficient rats (Fig. 1C), the activity of all femoral AP forms was increased especially that of \( M_r 110000 \) as compared with rats receiving vitamin D₃ (Table 1, Fig. 1A, lane D). In group C an increase of radioactivity of \(^{32}\)P in the monomer of \( M_r 55000 \), corresponding to the native form of \( M_r 110000 \), was observed (Fig. 2C).
Fig. 1. Distribution of alkaline phosphatase activity in adult rat femur. Polyacrylamide gel electrophoresis as in Methods. AP activity was detected on gel in presence of α-naphthyl phosphate as substrate. Diet: C, vitamin D₃-deficient; D, vitamin D₃ supplemented; S, 12% sorbitol added (without the addition of vitamin D₃). 1, crude homogenate; 2, extract I; 3, extract II.

Fig. 2. Autoradiography of ³²P-labelled monomers of alkaline phosphatase in adult rat femur. The samples after phosphorylation and denaturation were dissolved in a mixture containing 5% glycerol, 2% SDS, 5 mM dithiothreitol and 50 mM Tris/HCl buffer, pH 7.8. The ³²P-labelled monomers of AP were separated by polyacrylamide gel electrophoresis. Autoradiography of dried slab gel was performed using X-Omat Kodak film. Diet: C, vitamin D₃-deficient; D, vitamin D₃ supplemented; S, 12% sorbitol added (without the addition of vitamin D₃).
Rats receiving the diet supplemented with sorbitol always showed a substantial rise of the activity of the $M_r 165,000$ form (Fig. 1S). Frequently the appearance of a monomer of $M_r 100,000$, never found in groups C and D, was observed (Fig. 2S).

DISCUSSION

The effect of vitamin D$_3$ and sorbitol on calcium absorption is well known [12, 13]. The bone is the most important target organ for this vitamin. There is also extensive evidence for participation of alkaline phosphatase in bone calcification [14, 15].

The present studies demonstrated that vitamin D$_3$ deficiency causes a decrease in Ca concentration in serum, paralleled by well established increase in the total AP activity in femur and serum. Moreover, in vitamin D$_3$-deficient rats, the activity of all femoral AP forms was always increased, especially that of $M_r 110,000$ form, as well as the $^{32}$P-radioactivity of its monomer of $M_r 55,000$.

It seems of interest that the effect of vitamin D$_3$ on bone AP differs from its effect on the intestinal enzyme. The dietary deficiency of vitamin D$_3$ is responsible for a selective rise of activity of the $M_r 110,000$ form in bone and a decrease in the $M_r 180,000$ form in intestine. In contrast, the presence of vitamin D$_3$ in the diet diminishes the activity of femoral phosphatase of $M_r 110,000$ and considerably raises the level of the $M_r 180,000$ form in rat duodenum [6].

In animals kept on a sorbitol enriched diet the activity of the soluble form of $M_r 165,000$ in bone was enhanced. Moreover, sorbitol was responsible for the appearance of a new monomer of $M_r 100,000$ never found in the remaining animal groups. The mechanism of these changes is so far unknown. It has been reported, however, that various sugars and sorbitol activate some AP forms and may facilitate Ca absorption [16]. It is also known that long term dietary administration of sugars may lead to calcification disorders and osteopetrosis [17].

The influence of vitamin D$_3$ and sorbitol on AP forms and their role in calcium absorption and bone calcification requires further studies.

REFERENCES


