

Changes in phosphoproteins of chicken bone matrix in vitamin D-deficient rickets

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Vitamin D-deficiency and rickets was produced in growing chicks. The resulting decrease in mineralization of whole bone and of fractions separated by density centrifugation was accompanied by a very significant decrease in the contents of *O*-phosphoserine and *O*-phosphothreonine. Likewise, the total amount of *O*-phosphoserine and *O*-phosphothreonine and the concentrations of these phosphoamino acids in EDTA extracts and in fractions obtained by molecular sieving was also reduced. These data provide the first *in vivo* evidence that phosphoproteins may be critically involved in the calcification of bone.

<i>Bone matrix</i>	<i>O</i> -phosphoserine	<i>Phosphoprotein</i>	<i>Vitamin-D</i>	<i>Rickets</i>
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1. INTRODUCTION

Phosphoproteins present in all of the normally and pathologically calcified vertebrate tissues have been postulated to play a significant role in the formation of a solid phase of calcium-phosphate in mineralized tissues [1–3]. To date, the only *in vivo* evidence in support of this thesis has been the finding that the concentration of organic phosphorous in the form of *O*-phosphoserine is reduced in the enamel proteins of rachitic, vitamin D-deficient rats [4], and the reported reduction in the amount of phosphoprotein found in human dentin in cases of dentinogenesis imperfecta, a condition in which dentin is less mineralized than normal [5].

To test this hypothesis in the case of bone, we have used the concentrations and amounts of *O*-phosphoserine and *O*-phosphothreonine in bone as an index of the content and extent of protein phosphorylation and have examined the changes in these parameters in normal and vitamin D-defi-

cient, rachitic chickens. Vitamin D-deficient rickets is accompanied by a significant decrease in the *O*-phosphoserine and *O*-phosphothreonine contents of bone matrix, and by a decrease in the total amount and concentrations of these protein-bound phosphoamino acids extracted in EDTA.

2. EXPERIMENTAL

For each experiment ~100 1-day-old white Leghorn male chicks obtained from Spafas Inc. (Norwalk CT) were kept in wire-bottom brooders with a constant heat source at 20°C. The experimental chicks were maintained on a special diet for 6 weeks [6]. Control chicks were fed a standard chick diet (no. 904603, ICN Pharmaceut.). Assessment of vitamin D-deficiency was done as in [6]. Results are reported from 7 separate expt.

2.1. Preparation of bone

The mid-diaphyses of the long bones and calvaria from 25 normal and rachitic chicks were separately pooled. The bones were frozen in liquid nitrogen, lyophilized and powdered in a liquid N₂ mill (Spex Industries, Metuchen NY) to a particle size of 1–10 µm. The bone powder was separated

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into fractions of different mineral content and density by a modification [6] of the procedure in [7].

2.2. Weight and composition of whole bone

Dry weight, wet weight, organic weight, total protein, collagen and non-collagenous protein, as well as the calcium magnesium and phosphorus concentrations of whole bone samples were determined as in [8].

2.3. Amino acid analysis

O-Phosphoserine and O-phosphothreonine were determined chromatographically [9] on a Beckman 121-M automatic amino acid analyzer (Beckman Instruments Inc.), after partial acid hydrolysis in triple-distilled constant boiling 6 N HCl at 106°C in vacuo [10]. Complete amino acid analyses were obtained on samples of protein hydrolyzed in triple-distilled constant boiling 6 N HCl, 106°C, in vacuo for 22 h, which were then chromatographed on the Beckman 121-M automatic amino acid analyzer.

2.4. Extraction of EDTA-soluble phosphoproteins of chicken bone

Bone powder was extracted at 4°C for 2 weeks by large volumes of 0.5 M EDTA (pH 7.4) containing the following protease inhibitors: phenylme-

thylsulfonyl fluoride (1 mM); ϵ -aminocaproic acid (50 mM); benzamidine hydrochloride (5 mM); and *p*-hydroxymercuribenzoic acid (1 mM) [6]. The pooled EDTA extracts were desalted by dialysis in Spectra-por I membrane tubing using water with the same inhibitors in the dialyzer reservoir, and were then lyophilized.

3. RESULTS AND DISCUSSION

Analyses of whole bone consistently demonstrated the following: rachitic bone contained less mineral and consequently more organic matrix and protein than normal bone; an elevated water content; a significant decrease in the O-phosphoserine and O-phosphothreonine contents (table 1). The decrease in total mineral deposited in bone was more clearly brought out by density centrifugation [6]. This also held true for the decrease in the O-phosphoserine contents (fig. 1). Table 1 is a typical experiment.

The decrease in the O-phosphoserine and O-phosphothreonine contents of vitamin D-deficient, rachitic bone was mirrored by a decrease (~40%) in the total amount of non-dialyzable, protein-bound O-phosphoserine and O-phosphothreonine which could be extracted from the vitamin D-deficient, rachitic bone by EDTA. This

Table 1

Composition of 6-week-old postnatal normal and vitamin D-deficient, rachitic chicken bone

Sample	Water (%)	Ash (%)	Organic (dry wt)	Ca (mg)	P (%)	Mg (dry wt)	O-phosphoserine		O-phosphothreonine	
							residues/ 10 ⁵ amino acids TP ^c	residues/ 10 ⁵ amino acids NCP ^d	residues/ 10 ⁵ amino acids TP ^c	residues/ 10 ⁵ amino acids NCP ^d
Calvaria										
C ^a	40.3	60.7	39.3	23.7	11.0	0.44	83	670	21	167
R ^b	49.2	50.1	49.9	19.5	9.3	0.29	42	305	10	73
Tibiae and femora										
C	26.3	67.3	32.7	25.8	12.0	0.51	81	640	—	—
R	42.1	61.3	38.7	23.2	11.1	0.38	57	423	—	—
Metatarsals										
C	35.1	63.6	36.4	24.6	11.5	0.47	85	798	—	—
R	46.2	53.3	46.7	20.5	9.7	0.33	62	543	—	—

^aC, control; ^bR, rickets; ^cTP, total proteins; ^dNCP, non-collagenous proteins

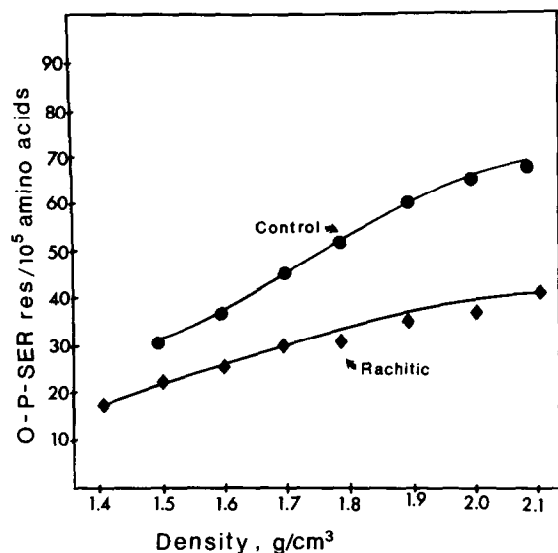


Fig. 1. The concentrations of *O*-phosphoserine in the density fractions of normal and rachitic chicken bone. Results of a typical experiment. Values represent average of two determinations which varied <5%.

was mirrored by the finding that the concentrations of *O*-phosphoserine and *O*-phosphothreonine in the crude EDTA extracts and in the two major G-100 fractions separated from the crude EDTA extracts [11] were also decreased by ~40% and ~60%, respectively, whether calculated as residues/10⁵ total residues or as percent serine phosphorylated. However, none of the phosphoprotein fractions in the EDTA extracts were taken to purity as reported in [11]. Thus, it is not possible to accurately determine the extent of phosphoprotein phosphorylation in the various phosphoprotein species present in bone matrix.

The modification of the non-collagenous phosphoproteins by the action of vitamin D is not unique. Vitamin D has been shown to affect other bone non-collagenous proteins such as osteocalcin [12,13] and osteonectin [13], bone collagen [14–16] and cartilage proteoglycans [17]. The results of the present experiments demonstrate that the decreased rate in the amount of mineral deposited in vitamin D-deficient rickets in chickens is accompanied by an overall decrease in the protein-bound *O*-phosphoserine and *O*-phosphothreonine contents of bone and in the non-collagenous, EDTA-extractable phosphoproteins. These ex-

periments provide the first *in vivo* evidence in bone to support the general hypothesis [1–3,18] that matrix-bound organic phosphorus plays a necessary role in the calcification of vertebrate tissues.

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