REVISTA ESPAÑOLA DE FISIOLOGIA, 37, 91-96. 1981

Mechanism of Ossification. Calcium Uptake by the Bone Organic Matrix *

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(Received on December 4, 1979)

J. M.ª LLOBET, J. L. DOMINGO and B. PINTO. *Mechanism of Ossification. Calcium Uptake by the Bone Organic Matrix.* Rev. esp. Fisiol., 37, 91-96. 1981.

A protein in the bone matrix that retains calcium is described. This protein seems to start the ossification process. The reaction occurs in several steps from high energy phosphorylation to the formation of aggregates and calcium phosphate.

Deposit of calcium phosphate as apatite crystals appears to be the main mechanism of bone enlargement and growth (10).

Apatite seems to be formed inside of a specific vesicle existing in the organic matrix (1).

Calcium and phosphate have to be stored within the so called bone vesicles (2). In the first step the inorganic calcium is activated (4) while it is reacting with the phosphate (3). The resulting effect is the formation of calcium phosphate that precipitates in the vesicle as an insoluble product. Additionally, it remains attached to the organic material (5).

The present report shows evidence on the mechanism through which the organic material activates the calcium uptake as well as sheds light on the general mechanism of the reaction, that starts the ossification.

Materials and Methods

Assay of the calcium uptake by bone matrix activity. Triplicate polypropilene tubes contained in a final volume of 2 ml: 100 μmoles calcium chloride, 40 μmoles magnesium chloride, 2 μmoles ATP (sodium-5-adenosin triphosphate), approximate 50×10^3 cpm ^{45}Ca as calcium chloride, 200 μmoles Tris-HCl buffer (pH 9) and increasing amounts of bone extract (usually 2 mg protein). In blanks the ATP was omitted. The mixtures were incubated at 37° C for 1 hour. The reaction was stopped by freezing at —20° C for 10 minutes. After thawing, the mixtures were centrifuged at $52{,}000 \times g$ and 4° C for 1 hour. Precipitates were suspended in 2 ml of 0.1 H Tris-HCl buffer (pH 9.0)

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followed by centrifugation under the same conditions. Each pellet was dissolved in 0.5 ml of water. Suspension was poured on vials containing 10 ml of P.C.S. solution. Afterwards, the radioactivity was counted for 10 minutes in a scintillation counter.

Bone extract preparation. The bone organic material was obtained from rabbits of 1.5 kg body weight .The animals fasted for 24 h and were killed by decapitation. The bones were thoroughly cleansed of any soft organic material. The bones were then smashed until a paste was obtained. The paste was suspended an 1/10 of its weight (w/v) in 10 mM Tris-HCl buffer (pH 7.4) and 0.9 % NaCl. Afterwards, the suspension was adjusted to pH 7.4 with 0.1 M HCl, followed by filtration through a cheese cloth. Filtrates were centrifuged at 52,000 × *g* and 4° C for 1 h. Precipitates were dissolved on ten times its volume with 10 mM Tris-HCl (pH 7.4). This suspension was thoroughly homogenized, followed by freezing and thawing. This extract was centrifuged at 52,000 × *g* and 4° C for 1 h and the precipitates stored at —20° C until required.

Cellular localization. The nuclei were obtained by centrifugation at 500 × *g* for 30 min and 4° C. The supernatant was recentrifuged at 10,000 × *g* for 15 min to get the mitochondria. Finally, the microsomes were obtained by centrifuging the previous supernatant at 52,000 × *g* for 15 h. This supernatant (third) was the cytosol fraction. The buffer contained 10 mM Tris-HCl (pH 7.4) and 0.9 % NaCl. On each fraction the following enzymes were determined as markers: Malate dehydrogenase (7), lactate dehydrogenase (6), sodium ATPase (8) as well as the calcium uptake by bone matrix activity (c.t.b.m.).

Protein nature. The protein structure of the c.t.b.m. was investigated by heating and pronase digestion. The effect of high temperature on the c.t.b.m. was carried out by heating at 100° C for 5 minutes. After cooling the c.t.b.m. was assayed.

The pronase digestion was performed by adding in 1 ml final volume 10 μmoles of $CaCl_2$, 1 mg of protein from the bone matrix extract. The mixture was incubated for 8 h at 37° C. The reaction was stopped by freezing. After thawing, c.t.b.m. was determined.

Aggregates formation. It was investigated by carrying out the reaction of the c.t.b.m. as above described. The precipitates were dissolved with 1 ml of water in the control tubes and in the test with 1 ml of a solution of 50 mM $CaCl_2$, 20 mM $MgCl_2$ and 10 mM Tris-HCl (pH 9.0). The mixtures were then centrifuged at 52,000 × *g* for 1 h and 4° C. The precipitates were redissolved with 1 ml of water followed by protein quantification (11).

Reagents and apparatus. Sodium 5'-adenosine triphosphate, sodium pyruvate, reduced nicotin adenin nucleotide (NADH), sodium oxalacetate and pronase were bought from Merck. ^{45}Ca as $CaCl_2$ with a specific activity of 100 mCi/mmol and P.C.S. (Liquid phase combined system) came from Amersham Radiochemical Center. Rest of the reagents were the highest grade commercially available.

Centrifugations were performed on a Sorvall RC-2B refrigerated centrifuge and using the rotor SM-24. Spectrophotometric readings were carried out on a Beckman automatic recorder and Heto thermostat. Radioactive countings took place on a Nuclear Chicago, Scintillation counter, type Isocap-300.

Results

Each experiment was at least twice repeated.

Physicochemical properties of the cal-

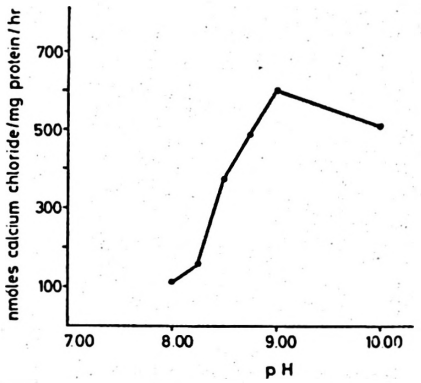

Fig. 1. *Optimum pH of the calcium uptake by bone matrix.*
The pH assay conditions varied from 6.5 to 10.

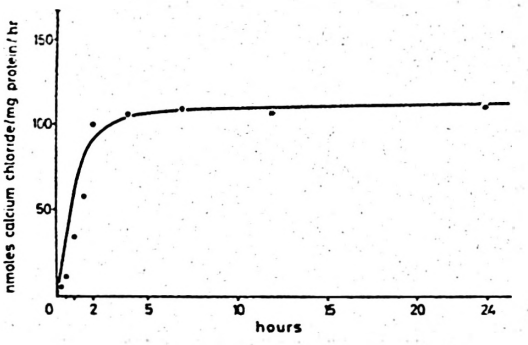

Fig. 3. *Effect of the equilibrium time on the calcium uptake by bone matrix activity.*
The reaction was stopped at 10, 30, 60, 90 and 120 min and 4, 7, 12 and 24 h.

cium uptake by bone matrix. The optimum pH was 9. It was determined by ranging the pH from 6.5 to 10 (fig. 1).

Maximum activity was obtained when the reaction was carried out at 37° C (fig. 2). The time of equilibrium was reached after 2 h. It was investigated by stopping the reaction at 10, 30, 60, 90 and 120 min and 4, 7, 12 and 24 h.

The ATP, magnesium and calcium effects were investigated by respectively varying the concentration of ATP from 0.1 to 10 μM, or MgCl from 0 up to 100 μM or $CaCl_2$ from 1 to 100 μM. These components were limiting factors of the reaction (figs. 4, 5, 6).

The biological activity responsible for the calcium uptake by bone matrix was

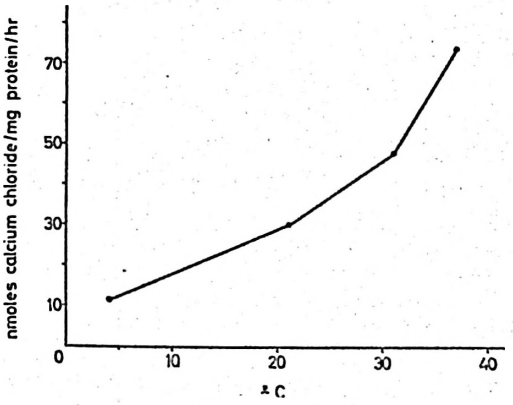

Fig. 2. *Effect of the incubation temperature on the calcium uptake by bone matrix.*
The reaction was carried out at 4, 21, 31 and 37° C.

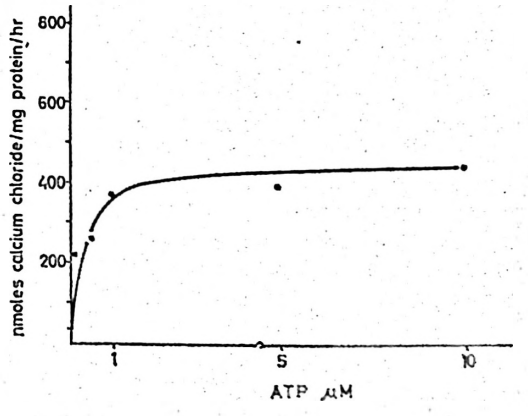

Fig. 4. *ATP concentration effect on the calcium uptake by bone matrix activity.*
The ATP concentrations ranged from 0.1 to 10 μM.

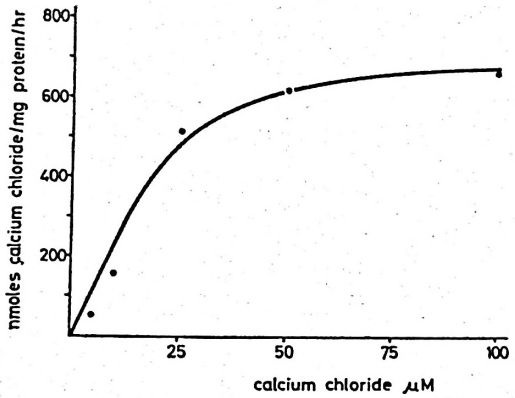

Fig. 5. *Effect of calcium concentration on the calcium uptake by bone matrix activity.* The calcium chloride concentration varied from 0 to 100 μM.

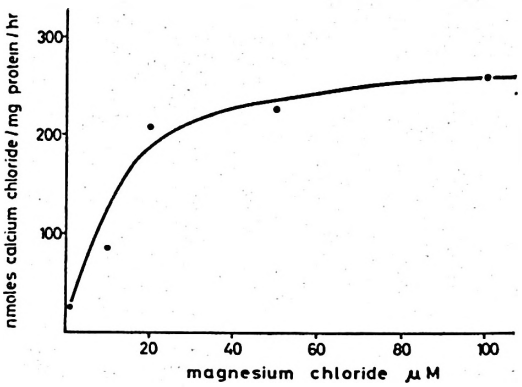

Fig. 6. *Magnesium concentration effect on the calcium uptake by bone matrix activity.* In the standard assay conditions the magnesium chloride varied from 0 to 100 μM.

localizated in the mitochondrial fraction of the cell although some activity was also present in the nuclear fraction (fig. 7).

The calcium uptake by bone matrix, in the presence of ATP and magnesium, lost the biological activity after heating at 100° C and pronase digestion (table I).

The calcium concentration affected the formation of precipitates while the bone matrix uptakes calcium, the amount of protein in the precipitates (aggregates) was higher, when calcium was present in the washes. In fact some disaggregation occurred when the calcium was removed from the buffer solution (table II).

Table I. *Heating and Pronase digestion.* Aliquots from bone matrix were either kept as control, heated at 100° C for 5 minutes or digested with pronase. The calcium uptake by bone matrix was then assayed as described in Methods. Each result is the mean of two experiments.

Sample	Calcium uptake by bone matrix	
	nmoles/mg protein	percentage
Control	904	100
After heating 100° C	112	12.4
After pronase digestion	72	7.9

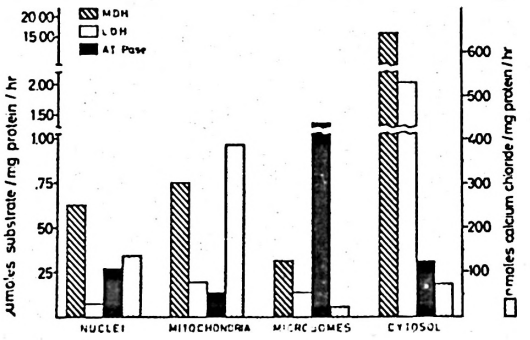

Fig. 7. *Cellular localization of the calcium uptake by bone matrix.* Nuclei, mitochondrial, microsomal and cytosol fractions from bone cells were obtained by centrifugation. Nuclei were morphologically identified. The mitochondrial microsomal and cytosol fractions were respectively identified by using as markers ATPase, malate dehydrogenase, and lactic dehydrogenase.

Table II. *Effect of calcium concentration on aggregates formation.*
The calcium uptake by bone matrix activity was determined in control and test tubes as described in Methods by adding 2.2 mg of protein to each assay tube. After the run, the precipitates were either dissolved with water (controls) or $CaCl_2$ (50 mM) plus $MgCl_2$ (20 mM) in the test. The pellets obtained by high speed centrifugation were dissolved with 1 ml of water in each tube followed by determination of protein. Results are mean of two experiments.

Sample	Amount per tube, mg	Percentage from original
Calcium plus magnesium	1.72	78
Water	0.44	20

Discussion

Ossification begins by the calcium uptake by a vesicle or body of similar density to the mitochondrial fraction of bone cells. These vesicles seem to be of bigger sizes than mitochondria as shown by the presence of the activity in the nuclear fraction.

The calcium uptake occurs in the presence of ATP and magnesium which appear to be limiting factors. The reaction seems to occur in several steps and catalyzed by a protein(s) as shown by the loss of the biological activity after heating and pronase digestion.

In the first step the bone matrix takes or binds calcium in the presence of ATP and magnesium. Such type of reaction implies the activation of the protein(s) by ATP through an energy mechanism. It can be suggested that the activation occurs by a high energy phosphorylated protein(s) that is capable to uptake calcium. The calcium is possibly incorporated to the protein through the liberation of inorganic phosphate (9). In such case, the calcium would be attached to the organic matrix by high energy bonds. Furthermore, the inorganic phosphate could react with additional amounts of calcium by forming calcium phosphate due to its water insolubility.

In the second step the calcium-protein complex monomer may react with additional monomers to form big aggregates that become insoluble and precipitate. The calcium concentration seems to be the controlling factor since the calcium removal led to some disaggregation and dissolution of the aggregates previously formed.

Resumen

Se identifica una proteína(s) en la matriz ósea que retiene calcio. Esta proteína parece ser el paso inicial de la osificación. La reacción se caracteriza por varios pasos que comienzan por la fosforilación de alta energía de la proteína seguida de la formación de agregados, así como de fosfato cálcico.

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