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Calcium–Phospholipid–Phosphate Complexes in Mineralizing Tissue[1] (40103)

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Past work has shown that the acidic phospholipids are involved in tissue mineralization (1–3). Calcium–phospholipid–phosphate (Ca–PL–PO_4) complexes, containing the acidic phospholipids, phosphatidyl inositol (PI) and phosphatidyl serine (PS), were isolated from the long bones of developing animals (4). These complexes induced hydroxyapatite (HA) formation in metastable calcium phosphate solutions (5). The same study also showed that the uncomplexed acidic phospholipids when placed in metastable calcium phosphate solution, first formed a Ca–PL–PO_4 complex and then nucleated HA. The fact that more complexed lipids were found in the bones of developing as opposed to more mature animals further supports the thesis that such compounds are related to the onset of mineralization. The purpose of this study was to analyze further for complexed lipids in a number of hard and soft tissues in order to enlarge our view of the role of the Ca–PL–PO_4 complexes in mineralization.

Materials and methods. This investigaton was carried out on white New Zealand rabbits of ages 1–4 days (50–70 g), 4–6 weeks (0.9–1.4 kg), 12–16 weeks (1.5–1.8 kg), and older than 100 weeks (3.6–5.4 kg). The tissues studied were: cortex of long bones, epiphyseal regions of long bones (including articular cartilage, growth plate, and secondary ossification centers), skull bones (cranium and mandible), scapulae, marrow, adipose tissue, heart muscle, pooled leg muscles, ear cartilage, and incisors. All bones were scraped to remove the periosteum and marrow, ground in a liquid nitrogen-cooled colloid mill, washed in 0.05 M tris-hydroxy-methyl amino methane-HCl (Tris) buffer at pH 7.4 to remove blood, and then were lyophilized. Noncalcified tissues were frozen and minced with dry ice and then lyophilized. The ash weight (after heating to equilibrium weight at 600°), was determined for samples of each of the lyophilized tissues. Tissues of at least three different rabbits in each age (weight) group were analyzed separately; the analyses on each tissue specimen were performed a minimum of 3×.

Lipids were extracted from weighed dried tissue specimens using, (A) an earlier published method (4) and, (B) a new enzymedependent method described here for the first time. In method A each sample of tissue was sonicated in the presence of 2:1:1.5 chloroform:methanol:Tris buffer (30 ml/g). Total lipids, obtained after repeated sonications, were washed in dilute buffer, then dried under a stream of nitrogen and stored in vacuum. Repeated treatment of the total lipids with 2:1 ethanol:ether (v/v) solubilized the noncomplexed lipids leaving the complexed lipids as the residue.

In order to make certain that the sonication procedure (A) removed all lipids, certain tissue residues, remaining after the sonication-lipid extractions, were decalcified (5% EDTA) and then reextracted for lipid (6). The organic phase was then washed with water, dried under a stream of N_2 gas and stored in vacuum.

In method B the specimen was decalcified by dialysis against a 0.1 *M* pH 5 phthalate buffer (3 liter/g/day) for a 5-day period. The complex had previously been shown to be stable in acid above pH 4 (5). The remaining matrix, containing less than 10% mineral, was washed in water, lyophilized, and digested for 16 h at 37° in a Tris-buffered, pH 7.4 solution containing 1000 units each of collagenase and hyaluronidase per g of tissue (7). The lipids were then extracted from the remaining solid and aqueous phases by the addition of 2:1 chloroform:methanol so that the final volume ratio was 2:1:1 chloroform:methanol:buffer. After washing the organic phase with buffer, the total lipids were dried under a N_2 stream and the non-complexed lipids were isolated from the com-

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plexed by ethanol:ether extraction as in method A, above.

All isolated complexes were washed in 10^{-5} *N* HCl to remove any traces of mineral. This procedure did not change the composition of the complexed lipids to any significant extent. Complexed lipids were then separated from any non-complexed lipid impurities by two-dimensional thin layer chromatography (8) in which the complexed lipids remained at the origin while the noncomplexed lipids migrated. The noncomplexed lipids were purified by chromatography on Sephadex G-25 (9), dried under a nitrogen stream and weighed. Aliquots of the complexed lipids were then dissociated in formic acid and partitioned in 2:1:1 chloroform:methanol: 1N HCl (10). The Ca (11) and inorganic PO_4 (12) content of the organic phase were analyzed colorimetrically. The phospholipid content of the complexed and noncomplexed lipids was analyzed by two-dimensional silica gel chromatography (8) followed by analysis of the organic P content of the individual spots (13). Organic P content of lipids extracted after demineralization was also determined, but the individual phospholipids in these very small fractions were not identified.

Samples (0.3 mg) of each of the complexed lipids isolated from all of the tissues, as well as certain noncomplexed lipid samples, were each suspended in 10 ml of a metastable calcium phosphate solution (5) and the suspensions mixed for 7 days at room temperature. Any solid formed after 7 days was separated by centrifugation (10 min at 2000 rpm) and the Ca and PO_4 concentration of the supernates measured by atomic absorption spectrophotometry (14) and colorimetry (12), respectively, for comparison with the composition of the original metastable solution. The apatitic nature of all precipitates was confirmed by X-ray diffraction and chemical analysis (5).

Results. Table I shows that for all ages of animals studied, a Ca–PL–PO_4 complex can be extracted from mineralized tissues, but not from those tissues which do not normally calcify. In the table the weight percentage of complexed lipid in the tissues is expressed in several ways: (a) per total lipid (CL/TL), (b) per demineralized (i.e., organic) dry weight (CL/ORG), and (c) per dry tissue weight (CL/T). Also listed is the percentage of lipid P in the complex: (d) per total lipid P (CL-P/TL-P), and (e) per total acidic phospholipid P (CL-P/APL-P). The acidic phospholipids were phosphatidyl inositol, phosphatidyl serine, phosphatidic acid, and lysophosphatidyl inositol. Expressed in this manner it is evident that the complexed lipids were present in greatest amount in the most actively calcifying tissues (long bone, skull and scapula), and were very low in noncalcifying tissues (marrow, adipose, muscle, heart and ear cartilage).

There was no significant difference between the yield of complexed lipids obtained by the sonication method (A) and by the enzyme method (B). The yield of complexed lipid per total lipid in table I is the average yield for both extraction methods. In addition, independent of the method used, no additional phospholipid was released following EDTA decalcification of the extracted tissues.

The composition of several of the isolated complexes is shown in Fig. 1. Corroborating our earlier studies (4), all complexes isolated, in addition to those shown in the figure, have a Ca to total phosphate molar ratio of 1:1 and contained variable amounts of the acidic phospholipids and inorganic phosphate. In addition, each of the isolated complexes when placed in the metastable calcium phosphate solution yielded hydroxyapatite at the same rate. Figure 2 shows that the rate of change in composition of the metastable solution resulting from exposure to each of a variety of complexed lipid substrates was the same after the same time interval. The noncomplexed lipid fraction caused no change in the composition of these metastable solutions, (not shown), in the time period studied.

Discussion. Ca–PL–PO_4 complexes were absent from all nonmineralized tissues, even those rich in both lipid and Ca. This fact, plus the observation that complexed lipids are present in all mineralizing tissues, reaffirms the view that the complexes play some role in the calcification process. We have extended our earlier study (4) to show, with a greater number of tissue types and greater age range, that the highest proportion of complexed lipids, relative to a number of tissue parameters, is found in the younger, actively growing animals.

It was previously suggested (4, 5) that the

TABLE I. COMPLEXED LIPIDS OF MINERALIZED AND NON-MINERALIZED RABBIT TISSUES.

Tissue	Age[a]	CL/TL[b]	CL/ORG[c]	CL/T[d]	CL-P/TL-P[e]	CL-P/APL-P[f]
Long bone	1–4 day	16 ± 2 $n = 9$	1.02 ± .09 $n = 9$	0.65 ± .07 $n = 9$	45 ± 5 $n = 6$	73 ± 8 $n = 3$
	1 Mo	11.5 ± .2 $n = 12$	0.81 ± .03 $n = 12$	0.34 ± .03 $n = 12$	22.1 ± .7 $n = 6$	45 ± 5 $n = 3$
	4 Mo	7.6 ± .7 $n = 12$	0.27 ± .03 $n = 12$	0.087 ± .008 $n = 12$	12 ± 2 $n = 6$	14 ± 3 $n = 3$
	Adult	4 ± 1 $n = 12$	0.25 ± .04 $n = 12$	0.078 ± .01 $n = 12$	10 ± 2 $n = 5$	10 ± 3 $n = 3$
Skull bone	1–4 day	18 ± 2 $n = 9$	0.37 ± .07 $n = 9$	0.32 ± .09 $n = 9$	33 ± 5 $n = 6$	58 ± 6 $n = 3$
	4 Mo	14 ± 2 $n = 9$	0.20 ± .02 $n = 9$	0.54 ± .05 $n = 9$	21 ± 3 $n = 8$	27 ± 3 $n = 3$
	Adult	11 ± 2 $n = 11$	0.12 ± .02 $n = 11$	0.50 ± .04 $n = 11$	15.4 ± 0.5 $n = 4$	23 ± 1 $n = 3$
Incisor	1–4 day	16.5 ± .2 $n = 8$	0.44 ± .09 $n = 8$	0.072 ± .005 $n = 8$	6.8 ± .8 $n = 3$	52 ± 4 $n = 3$
	4 Mo	4 ± 3 $n = 6$	0.19 ± .09 $n = 6$	0.02 ± .01 $n = 6$	9.9 ± .8 $n = 3$	10 ± 2 $n = 4$
	Adult	3 ± 1 $n = 12$	0.1 ± .1 $n = 12$	0.002 ± .001 $n = 12$	6 ± 1 $n = 3$	7 ± 1 $n = 3$
Scapula	1–4 day	20.2 ± .3 $n = 9$	0.59 ± .03 $n = 9$	0.4 ± .1 $n = 9$	47 ± 6 $n = 5$	77 ± 5 $n = 3$
	4 Mo	7.13 ± .06 $n = 9$	0.6 ± .1 $n = 9$	0.080 ± .007 $n = 9$	23 ± 6 $n = 5$	27 ± 3 $n = 3$
	Adult	1.5 ± .4 $n = 9$	0.3 ± .2 $n = 9$	0.049 ± .009 $n = 9$	6.2 ± .7 $n = 3$	6 ± 2 $n = 3$
Epiphyseal cartilage	1–4 day	13.9 ± .7 $n = 10$	0.72 ± .03 $n = 10$	0.20 ± .1 $n = 10$	9 ± 1 $n = 3$	10 ± 1 $n = 5$
	4 Mo	6 ± 1 $n = 9$	0.32 ± .09 $n = 9$	0.14 ± .03 $n = 9$	0.6 ± 1 $n = 4$	1 ± 1 $n = 5$
Marrow	Adult	0.020 ± .06 $n = 9$	0.058 ± .004 $n = 9$	0.058 ± .004 $n = 9$	0 $n = 3$	—
Adipose	Adult	0.021 ± .02 $n = 9$	0.04 ± .02 $n = 9$	0.04 ± .02 $n = 9$	0 $n = 3$	—
Leg muscle	4 Mo	0.03 ± .02 $n = 9$	0.0022 ± .0005 $n = 9$	0.0022 ± .0005 $n = 9$	0 $n = 3$	—
Heart	Adult	0.020 ± .005 $n = 9$	0.002 ± .001 $n = 9$	0.0022 ± .0005 $n = 9$	0 $n = 3$	—
Ear cartilage	1–4 day	0.00 $n = 6$	0.00 $n = 6$	0.00 $n = 6$	0 $n = 3$	—
	Adult	0.00 $n = 6$	0.00 $n = 6$	0.00 $n = 6$	0 $n = 3$	—

[a] Rabbit age; 1–4 days = 50–70 g, 1 Mo = 1.0–1.4 kg, 4 Mo = 1.5–1.8 kg, Adult = greater than 3.5 kg.
[b] CL/TL, wt. % complexed lipid per total lipid ±SE of measurement for *n* extractions.
[c] CL/ORG, wt. % complexed lipids per demineralized (i.e., organic) dry wt. ±SE for *n* extractions.
[d] CL/T, wt. % complexed lipids per dry tissue wt., ±SE for *n* extractions.
[e] CL-P/TL-P, % organic phosphorus in complexed lipids fraction relative to organic phosphorus in total lipid fraction, ±SE for the analysis of *n* complexes.
[f] CL-P/APL-P[f], % organic phosphorus in complexed lipids relative to organic phosphorus in total acidic phospholipids ±SE for the analyses of *n* complexes.

Ca–PL–PO_4 complexes may be components of the extracellular matrix vesicles known to be present in all mineralized tissues (15) and/or components of the membranes of calcifying cells (16). Wuthier has recently reported the isolation of such Ca–PL–PO_4 complexes from epiphyseal cartilage matrix vesicles in amounts constant per unit vesicle (17). Since the presence of extracellular matrix vesicles is associated with the onset of mineralization (15) it is not surprising that our greatest yields of complex were obtained from those tissues which are most actively forming new mineral.

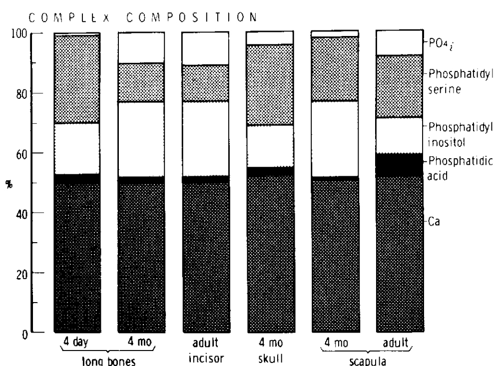

FIG. 1. The mean chemical composition (mole %) of Ca–PL–PO_4 complexes extracted from a variety of calcified tissues.

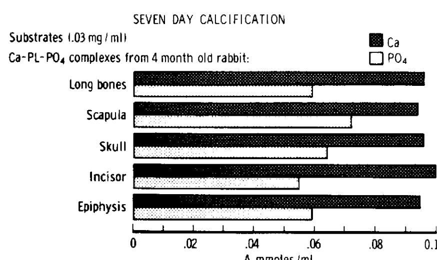

FIG. 2. The effect, after 7 days (25°), of a series of complexed lipid substrates, extracted from different tissues, on the level of Ca and PO_4 in a metastable Ca–PO_4 solution. The composition of the original metastable solution was 1 m*M* Ca, 1 m*M* HPO_4, pH 7.5, 0.05 *M* Tris-HCl.

Studies are underway in our laboratory to determine the distribution and the concentration of the complex in the zones of the epiphyseal growth plate. In addition, we are seeking to determine whether Ca-PL-PO_4 complexes are components of the proteolipids which others (18–20) have isolated from mineralizing tissues and which have been shown to induce *in vitro* hydroxyapatite formation.

Summary. Calcium-phospholipid-phosphate complexes have been isolated from mineralized tissues using a sonication-dependent method described previously and a new method which depends on the use of enzymes to remove the nonlipid matrix from tissues which were decalcified under mild conditions. Both methods gave comparable results. The complexes were found to be constituents of all mineralized rabbit tissues; bone, calcified cartilage, and tooth. No such complexes could be isolated from the nonmineralized tissues; muscle, ear cartilage, bone marrow, adipose tissue. The proportion of Ca–PL–PO_4 is highest in those tissues which are involved in active mineralization, i.e., the younger tissues. All complexes isolated from mineralized tissues contain 50 mole % Ca, and variable amounts of the acidic phospholipids and inorganic phosphate. However, the Ca:total P molar ratio was always unity. All isolated complexes were shown to induce *in vitro* hydroxyapatite formation from metastable calcium phosphate solution and, thus, are believed to be associated with *in vivo* calcification.

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