Calcification of a Lysozyme-Inositol Phosphatide Complex *in Vitro*[1]

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The ability of the microorganism, *Bacterionema matruchotii*, to acquire hydroxyapatite intracellularly has been well documented (1). Cellular lipid was found to be essential for this microbial calcification (2). Further, polar lipid extracted from *B. matruchotii* induced apatite formation *in vitro* (2). The polar lipid component associated with apatite nucleation was a lipoprotein composed of basic protein and acidic phospholipid (3). An investigation into the possible function of lipoproteins in apatite formation was initiated from these observations on microbial calcification. This report describes *in vitro* apatite nucleation obtained with a synthetically prepared basic protein–acidic phospholipid complex.

Materials and Methods. Commercially available egg-white lysozyme (Nutritional Biochemicals) was selected as a basic protein. The acidic phospholipid used was beef-brain inositol phosphatide (Nutritional Biochemicals) which by chromatographic analysis consisted chiefly of the diphosphoinositide with smaller amounts of phosphatidyl inositol, phosphatidyl serine and triphosphoinositide. The complex was prepared using a procedure similar to one described by Palmer and Dawson (4). Lysozyme and the inositol phosphatides were dissolved separately in 0.12 *N* aq. NaCl at concentrations of 1 mg/ml. The solutions were filtered through sterile Swinnex-25 units with type HA filters (Millipore Corporation) and the lysozyme solution was titrated with the phospholipid solution until precipitation was complete. The volume ratio of protein to phospholipid was 2.0. The preparation was allowed to stand at 25° for 30 min then centrifuged at 12,000*g* for 5 min at 5°. The precipitate was resuspended and washed 2× with deionized water. The complex was tested for apatite nucleation as follows: Five-milligram portions of the precipitate were transferred to air-tight 50-ml screw-top polycarbonate centrifuge tubes. The precipitates were washed 1× with carbonate buffered metastable calcium phosphate solution[2] (MCPS) and suspended in the same solution for either 5 or 7 days at 25°. The solutions were changed daily. The complex suspended for 7 days was washed 2× with deionized water, 1× with CH_3OH, 1× with $CHCl_3$–CH_3OH (2:1) and dried *in vacuo* at 50°. The dried residue was finely powdered and examined by X-ray diffraction using a powder camera film technique with Ni-filtered Cu radiation generated at 30 kV, 20 mA anode current for 5 hr (Phillips Electronics). Untreated precipitate and the 5 day sample were washed 2× with deionized water and fixed in 1% OsO_4 for 1 hr. Sections (60–90 nm) were post stained with uranyl acetate and examined in a Hitachi 11E electron microscope.

Results. The residue from the 7-day sample gave an X-ray diffraction pattern typical for biologic apatite (Fig. 1). Electron microscopy showed that lysozyme plus inositol phosphatides precipitated as a lamellar liquid-crystalline phase (Fig. 2a, aa) similar to that obtained by precipitating cytochrome C

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[2] The composition of the metastable calcium phosphate solution in g/liter was: Na_2HPO_4, 0.276; NaCl, 4.090; $NaHCO_3$, 1.850; KCl, 0.370 and Ca^{2+}, 0.080 (added from a stock solution). The solution was acidified with CO_2 for Ca^{2+} addition and storage. A working pH of 7.2 was obtained by aeration.

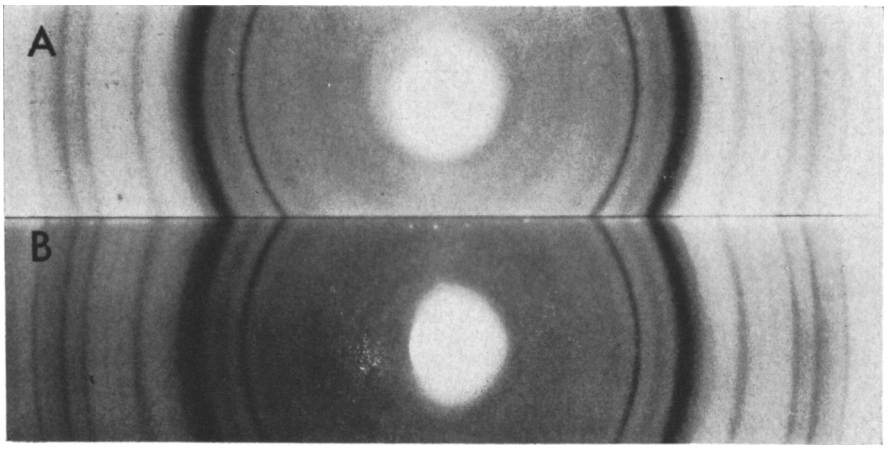

FIG. 1. X-ray diffraction patterns for (A) residue obtained after suspending the lysozyme–inositol phosphatide complex in metastable calcium phosphate solution for 7 days and (B) powdered human dentin as a biologic apatite reference.

with brain phospholipid (5). Vesicles bound by multi-layered membranes formed when the complex was suspended in MCPS (Fig. 2bb). Mineral deposition was found in association with vesicle membranes. In addition to a granular amorphous appearance, needle-like crystals characteristic for apatite were present (Fig. 2b). Apatite formation did not occur when either lysozyme or the inositol phosphatide was suspended in MCPS for 7 days.

Discussion. The results show that a synthetically prepared lipoprotein could induce apatite nucleation, *in vitro,* and that the integrity of the complex was essential. Equally significant was the formation of membrane-bound vesicles during suspension of the complex in MCPS.

A number of investigators have observed membrane-bound vesicles associated with the initial stages of calcification in bone (6), cartilage (7), dentin (8) and human aortic valves (9). Apatite crystals and granular amorphous mineral deposits were seen on or within the vesicles. According to Anderson *et al.* (10) these extracellular matrix vesicles may promote calcification by increasing the local concentrations of calcium and/or phosphate. The findings reported here with the synthetic lipoprotein substantially strengthen a membrane-related calcification concept. The results also correlate with the observations that acidic phospholipids are associated with the onset of calcification in vertebrate tissues (11).

Although the results at this point are primarily observational, certain properties of protein–phospholipid complexes indicate that the nature of calcium binding is relevant to apatite formation. Ca^{2+} binding by acidic phospholipid was shown to be the initial step in *B. matruchotti* calcification (12). In addition, the Ca^{2+} binding properties of acidic phospholipids both free and protein-bound have been well documented (13–16). Recently, Cotmore *et al.* (17) demonstrated that incorporation of Ca^{2+} into acidic phospholipid micelles was enhanced by inorganic phosphate and was due to the formation of a phospholipid–amorphous calcium phosphate complex. The binding of calcium by membrane lipoprotein can also result in a decrease in net charge and loss of water of hydration (18). Thus, through a process of desolvation with the simultaneous accumulation of Ca^{2+} and HPO_4^{2-}, the intermembrane structure can provide for the concentrating of ions necessary for calcification with minimal energy expenditure. Protein–phospholipid complexes such as the one described in this paper might provide a tool for studying calcification mechanisms.

Summary. A synthetic basic protein–acidic phospholipid complex was prepared from ly-

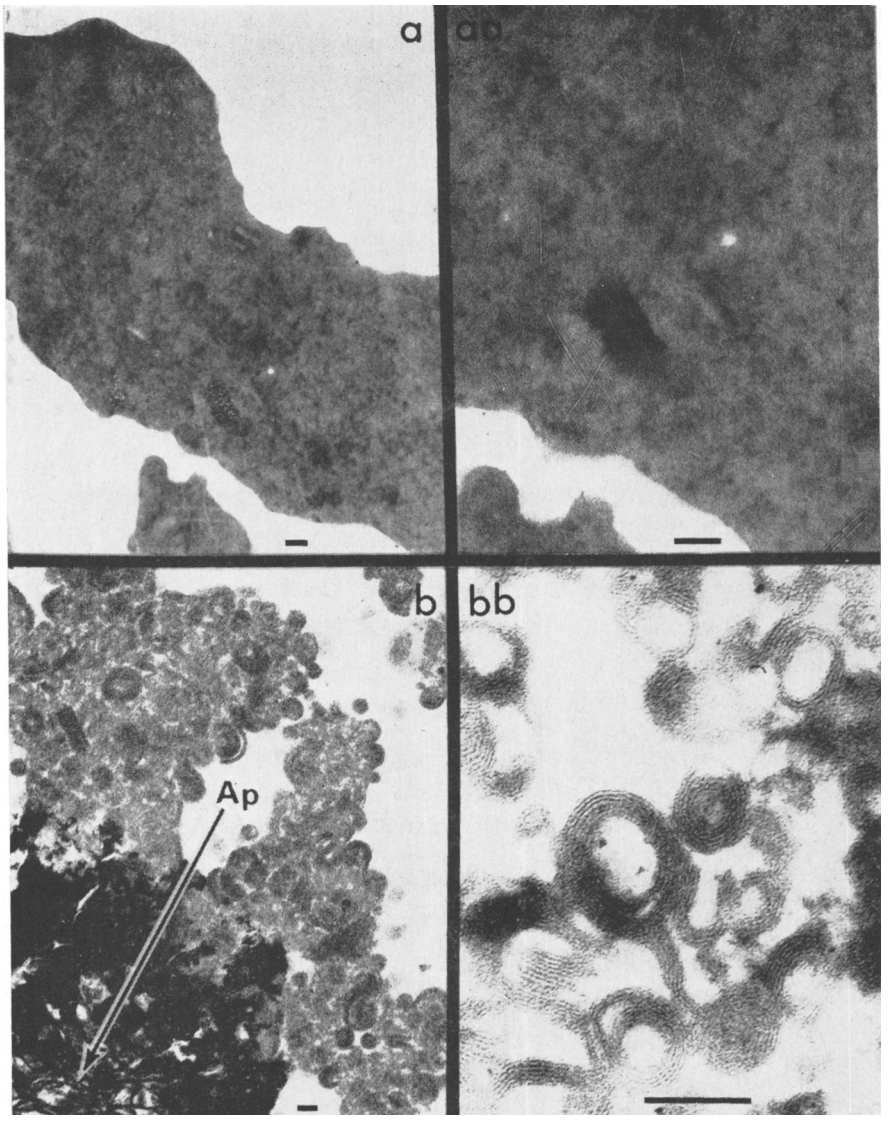

FIG. 2. Electron micrographs of the initial lysozyme–inositol phosphatide complex (a, × 28,000) with higher magnification (aa, × 67,000) to show lamellar liquid-crystalline structure and of the complex after suspension in the calcium phosphate solution showing mineralization associated with vesicles (b, × 18,000). Note needle-like apatite crystals (Ap.). Multilayer membranes of vesicles can be seen with higher magnification (bb, × 144,000). Lengths of bars equal 100 nm.

sozyme and inositol phosphatides. Suspension of the complex in metastable calcium phosphate solutions resulted in formation of membrane-bound vesicles and apatite nucleation. The results suggest that calcium binding lipoproteins might have a function in calcification.

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