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Lipid and Bone Matrix Calcification *in Vitro*[1] (38019)

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The purpose of this study was to determine whether lipid was responsible for bone matrix calcification. Irving (1) has cited an abundance of indirect evidence linking lipid and bone calcification. However, as pointed out by Shapiro (2), ". . . there is as yet no clear experimental proof that lipids are necessary for the . . . mineralization of the matrix of hard tissue."

A series of studies, recently reviewed (3), has established that apatite formation in the microorganism *Bacterionema matruchotii* is lipid dependent. The same was found with human dentin and dental calculus (4). An investigation of bone matrix calcification using the methods of the microbiologic studies would serve two purposes. It would provide experimental data not previously available and it would examine the validity of a microbiologic model for vertebrate calcification.

Materials and Methods. Calcifiable bone matrix was prepared from marmoset femurs. Legs were obtained from adult animals of unknown age immediately after death. The bones were cleaned of soft tissue by *Dermestes maculatus* larvae and stored at −15°. Approximately 2.5 g of femur shafts were split and the marrow removed. The bone segments were washed in several changes of deionized water, dried under nitrogen at 45°, and pulverized to 20 mesh in a liquid-nitrogen-cooled laboratory mill. The coarse bone powder was decalcified by dialysis against 2 *N* formic acid. Decalcification was estimated to be complete when the diffusate gave three successive zero calcium readings by atomic absorption spectrophotometry. The decalcified matrix was washed by dialysis against denionized water. The material was considered to be acid free when the electrical conductivity of the diffusate equaled that of deionized water.

Calcifiability of the matrix was tested by shaking 15 mg at room temperature in an air-tight 50-ml centrifuge tube filled with a metastable calcium phosphate solution (4). The solution was changed daily for 7 days. The matrix was recovered, water washed, air dried at 45°, and radiofrequency ashed at approximately 100° for 2 hr. The residue was analyzed by X-ray diffraction. Instrumentation and techniques have been detailed previously (5).

The matrix was lipid extracted by stirring 500 mg in 150 ml of chloroform–methanol–conc. hydrochloric acid (2:1:0.01, v/v/v) for 24 hr. The particulates were collected by filtration on a solvent-resistant micropore membrane (Millipore UH), washed with an additional volume of solvent, nitrogen dried at 45°, and examined for calcification as the intact matrix had been.

The crude phospholipid fraction was separated from the total lipid extract essentially as had been done with *B. matruchotii* (6). An exception was that the lipid extract was neutralized and washed with 0.2 vol of 0.2 *N* sodium bicarbonate at 4° for 16 hr.

The two lipid fractions were examined for calcifiability by shaking 0.5 mg of each in air-tight 10-ml centrifuge tubes filled with the metastable calcium phosphate solution. All other procedures were the same as those used with the matrix and the lipid-extracted matrix.

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Results. Decalcification of 2.5 g of bone powder took between 9 and 10 days and required from 17 to 20 changes of formic acid. Water washing by dialysis usually took 6–7 days. The typical yield of matrix approximated 600 mg.

Radiofrequency-ashed residues of matrix and of the phospholipid fraction gave X-ray diffraction patterns for apatite (Fig. 1) after 7-day exposures to the metastable calcium phosphate solution. No evidence for crystallinity was found with either the lipid-extracted matrix or the acetone-soluble lipid.

Discussion. Lipid was required for calcification of the matrix prepared from marmoset femurs. This indicates the probability that lipid is involved in bone calcification. The result is in accord with Irving's observation (7) that calcifying sites in bone react to lipid stains. Bone matrix calcification involved the phospholipid fraction rather than acetone-soluble lipid. This correlates with several ultrastructural studies (8) indicating bone mineral nucleation in extracellular vesicles of epiphyseal cartilage. Since the vesicles are membrane bound, they must be phospholipid rich. In "preliminary electron-microscopic studies" (9), isolated vesicles formed apatite crystals after incubation in a mineral-supplemented medium.

The choice of test tissue, marmoset femurs, was arbitrary. The choice of decalcifier, however, was based on prior experience with *B. matruchotii.* The calcified organism recalcified after decalcification with formic acid but not after decalcification with EDTA. Dealcification was complete with each agent. The reasons for this disparity have not yet been clarified.

The reliability of *in vitro* calcification studies involving matrices prepared by decalcification of tissue hinges on the completeness of decalcification. In this study, decalcification was determined by atomic absorption spectrophotometry. However, technical limits are such that only an estimate of decalcification was possible. On the other hand, subsequent to lipid extraction, the matrix did not calcify. If decalcification had not been complete, crystal growth would have occurred and the extracted matrix would have calcified during the 7-day incubation in the metastable calcium phosphate solution.

Acidified chloroform–methanol was selected for lipid extraction of the bone matrix

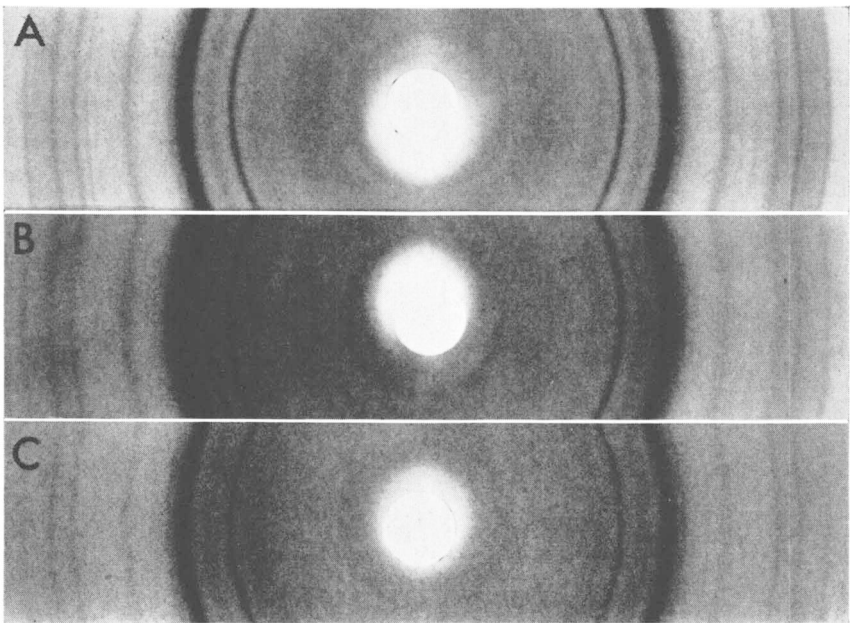

FIG. 1. Positive prints of X-ray diffraction patterns. (A) Original bone; (B) calcified matrix; (C) calcified phospholipid fraction of matrix.

because of earlier findings (unpublished) with decalcified dentin and elephant tusk. Neutral chloroform–methanol extraction of these collagen-containing tissues did not yield calcifiable lipids and the matrices retained calcifiability. After extraction with acidified solvent, calcifiable lipids were recovered and the extracted matrices lost calcifiability. The subject of lipids in collagen is concisely covered by Nikkari and Heikkinen (10). Their data suggest that *in vitro* calcification by collagen preparations may be due to retained lipid.

Composition of the crude phospholipid fraction extracted from marmoset bone matrix is not known. That of *B. matruchotii* has been defined partially (11) and a major component is a basic protein–acidic phospholipid complex. A similar complex, synthetically prepared, formed membrane-bound vesicles and nucleated apatite upon suspension in a metastable calcium phosphate solution (12). Whether a protein–lipid complex is responsible for microbial calcification or bone matrix calcification remains to be determined.

Summary. A calcifiable matrix was prepared by decalcification of marmoset femurs. Lipid extraction rendered the matrix noncalcifiable. The crude phospholipid fraction of the lipid extract induced apatite crystallinity in a metastable calcium phosphate solution. The acetone-soluble fraction did not. The results show lipid is involved in calcification of a bone matrix, *in vitro,* and that the nucleator is in the crude phospholipid fraction.

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1. Irving, J. T., "Calcium and Phosphorus Metabolism." Academic Press, New York (1973).

2. Shapiro, I. M., *in* "Biological Mineralization" (I. Zipkin, ed.), p. 117. Wiley–Interscience, New York (1973).

3. Ennever, J., and Takazoe, I., *in* "Biological Mineralization" (I. Zipkin, ed.), p. 629. Wiley–Interscience, New York (1973).

4. Ennever, J., Vogel, J. J., and Benson, L. A., J. Dent. Res. **52**, 1056 (1973).

5. Ennever, J., Vogel, J. J., and Streckfuss, J. L., J. Dent. Res. **50**, 1327 (1971).

6. Takazoe, I., Vogel, J. J., and Ennever, J., J. Dent. Res. **49**, 395 (1970).

7. Irving, J. T., Nature **181**, 704 (1958).

8. Anderson, H. C., Matsuzawa, T., Sajdera, S. W., and Ali, S. Y., Trans. NY Acad. Sci. **32**, 619 (1970).

9. Ali, S. Y., and Evans, L., Biochem. J. **134**, 647 (1973).

10. Nikkari, T., and Heikkinen, E., Acta Chem. Scand. **22**, 3047 (1968).

11. Vogel, J. J., and Ennever, J., Clin. Orthop. Related Res. **78**, 218 (1971).

12. Vogel, J. J., Campbell, M. M., and Ennever, J., Proc. Soc. Exp. Biol. Med. **143**, 677 (1973).

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