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Nucleation of Microbiologic Calcification by Proteolipid (39348)

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The objective of this investigation was to isolate the component of crude phospholipid responsible for *Bacterionema matruchotii* calcification. The microorganism, an actinomycete of the human oral biota, forms intracellular calcium phosphate deposits which give diffraction maxima characteristic of apatite (1). *B. matruchotii* calcification is lipid dependent (2) with activity restricted to the crude phospholipid (3). A complex assumed to be a lipoprotein (4) is present in *B. matruchotii* crude phospholipid. Calcium binding by phospholipid–protein complexes has been reported (5). The possibility that such complexes participate in vertebrate calcification has been considered (6). These data, in addition to calcification of a synthetically prepared lipoprotein (7), emphasized the probable importance of the protein-containing portion of *B. matruchotii* crude phospholipid.

Materials and methods. Crude phospholipid was obtained from *B. matruchotii,* ATCC 14266. The organism was grown 7 days at 37° in 250-ml vol of a chemically defined medium (Table 1). Twenty-liter batches were harvested by centrifugation (30,000 *g*, 5 min, 5°). Cells were water washed and freeze-dried without protective colloid. Total lipid was extracted by suspending the full yield of dried cells, usually about 5 g, in 300 ml of chloroform–methanol 2:1, v/v (CM) for 18–20 hr at room temperature. The extract was filtered (Millipore UH) and nonlipids were removed by a 0.2 vol, 0.12 *M* sodium chloride wash (8). The chloroform phase was drawn off and concentrated to $^1/_8$ vol under vacuum at 45°. Volume was restored with acetone and the extract held at 5° for 24–36 hr. The crude phospholipid precipitate was recovered by centrifugation, acetone-washed, and immediately dissolved in 10 ml of CM.

Crude phospholipid was fractionated by column chromatography with hydroxypropylated dextran gel (Sephadex LH-20). The CM solution was applied in two 5-ml vol to a 2.5 × 51.0-cm column equilibrated with CM. To detect protein, each 320-ml elution was monitored with 280 nm ultraviolet. Individual fractions and various recombinations were stored at −15° until dried at 45° under nitrogen flow.

Samples selected for calcifiability tests were: (a) all fractions recombined, (b) the single, protein-containing fraction, and (c) all fractions recombined except the protein-containing fraction. Fifteen-milligram quantities of each were incubated at 37° in air-tight 50-ml centrifuge tubes filled with carbonate-buffered pH 7.20 metastable calcium phosphate solution (9) containing 0.02% thymol. At 24-hr intervals pH determinations were made, the tubes were centrifuged, and the solutions changed. Incubation periods ranged from 96 to 192 hr. At termination the samples were examined for microbial contamination, water-washed, air-dried, and heated to 700°. Residues were examined by X-ray diffraction using a powder-camera film technique with nickel-filtered copper radiation generated at 30 kV, 20 mA anode current for 5 hr.

The protein-containing fraction was partially characterized. Amino acid composition was determined (Phoenix K-800 Autoanalyzer) from an acid hydrolysate. General phospholipid identification was done with thin-layer chromatography (10).

Results. The fortuitous occurrence of two separated ultraviolet absorbances (Fig. 1) during elution permitted division of the crude phospholipid into five fractions. The solid phase obtained after incubation of either fraction 2 or the recombined five fractions in the metastable calcium phosphate solution, when ashed, gave X-ray diffraction patterns for apatite (Fig. 2). Fraction 2 calcified within 96 hr. The recombined fractions required 168 hr. No evidence for apa-

TABLE I. MEDIUM FOR *Bacterionema matruchotii*.

	Per Liter
Glucose	2.00 g
Casein hydrolysate, vitamin-free	4.00 g
Salts	
$NaHCO_3$	1.85 g
Na_2HPO_4	550 mg
CH_3COONa	147 mg
$CaCl_2 \cdot 2H_2O$	14.5 mg
$MgSO_4 \cdot 7H_2O$	35 mg
$FeSO_4 \cdot 7H_2O$	4 mg
$MnSO_4 \cdot H_2O$	0.15 mg
$NaMoO_4 \cdot 2H_2O$	0.15 mg
Vitamins	
Para-aminobenzoic acid	2.0 mg
Riboflavin	2.0 mg
Calcium pantothenate	2.0 mg
Inositol	2.0 mg
Thiamine	2.0 mg
Nicotinic acid	1.0 mg
Pyridoxine HCl	1.0 mg
Biotin	0.1 mg
Folic acid	0.1 mg
Growth Factors	
Pimelic acid	0.1 mg
Thioctic acid	0.1 mg

Nitrogen bases: 20 mg of each of adenine, guanine, thymine, uracil, xanthine dissolved in 100 ml of 0.1 *M* KOH.

Medium is made up in 0.15 *M* N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid adjusted to pH 7.4 with NaOH. Filter-sterilization is required.

tite formation was obtained with the recombined fractions minus fraction 2 exposed to the calcium phosphate solution for 192 hr.

The solutions containing either fraction 2 or recombined fractions gave daily postincubation pH readings between 6.90 and 6.98. With recombined fractions minus fraction 2 the readings were consistently 7.20. At termination none of the samples was contaminated.

Preliminary analyses indicated approximately 38% of fraction 2 was protein. The amino acid data obtained are given in Table 2. The remainder of the fraction consisted of cardiolipin, inositides, and possibly other acidic phospholipids. Dried fraction 2 was a light brown, granular powder. It was insoluble in water and salt solutions and soluble in chloroform–methanol mixtures. Each of the other dried fractions was a lipidlike semisolid with no protein detectable by biuret or ninhydrin.

Discussion. The component of *B. matruchotii* crude phospholipid responsible for calcification is a protein–lipid complex with lipidlike solubilities, a proteolipid. Proteolipids, first noted in brain tissue, are found in a variety of biologic membranes (11). Proteolipid involvement in microbial calcification is additional evidence that initiation of *B. matruchotii* calcification is membrane-associated (12). More important than spatial considerations, the finding is the first instance in which a specific chemical entity has been shown experimentally to be essential for a form of biologic calcification. Further, the proteolipid was isolated from a fastidious organism cultivated in a chemically defined medium. This provides for readily controllable investigation of biosynthesis related to biologic calcification.

The incubation time for fraction 2 calcification is relatively unimportant at this stage of investigation. The calcification procedure used was a closed system which, while preventing contamination and pH elevation, underwent progressive ion depletion between solution changes. Greater calcification efficiency would be shown with a continuous-flow procedure. A more meaningful measure of efficiency, however, would be to determine the minimum calcium × phosphate product required for nucleation.

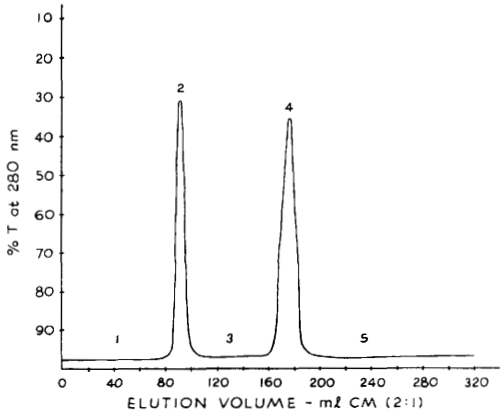

FIG. 1. Hydroxypropylated dextran gel (Sephadex LH-20) column chromatography of *B. matruchotii* crude phospholipid. Five-milliliter aliquots of sample in chloroform methanol 2:1, v/v (CM), were applied to 2.5 × 51-cm column equilibrated with CM. Elution volumes of 4 ml were pooled to yield five fractions.

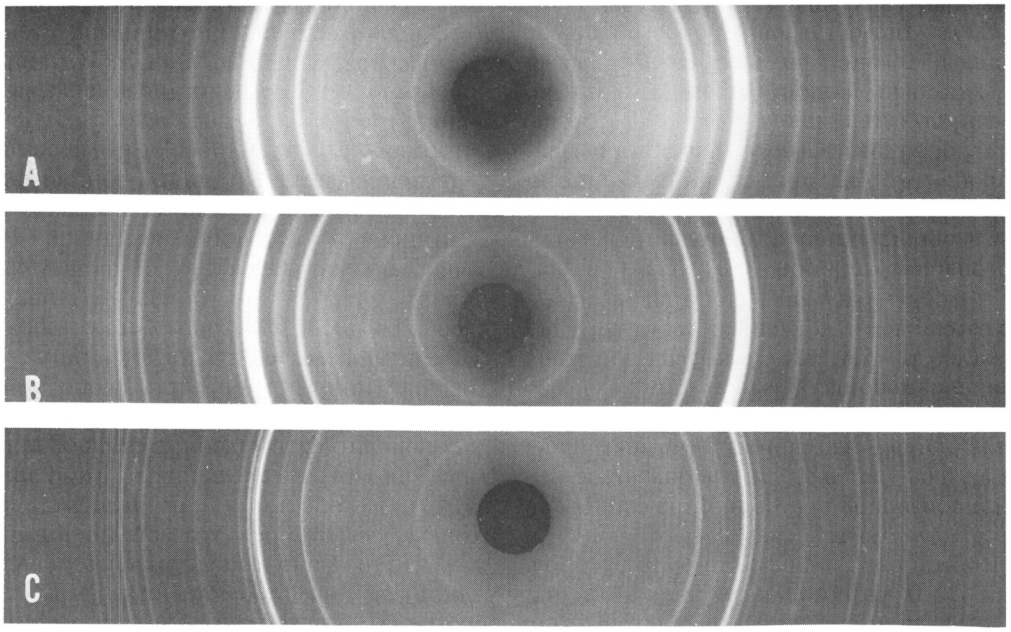

FIG. 2. X-ray diffraction patterns of ashed residues from fraction 2 (A) and recombined five fractions (B) of *B. matruchotii* crude phospholipid after incubation in metastable calcium phosphate solution. A pattern from human dentin (C) is a biologic apatite reference.

TABLE II. AMINO ACID COMPOSITION OF *Bacterionema matruchotii* CRUDE PHOSPHOLIPID FRACTION 2.

Amino acid	Protein (m*M*/mg)
Aspartic acid	0.75
Glutamic acid + proline	0.89
Serine	0.80
Threonine	0.40
Valine	0.14
Methionine	0.19
Tyrosine	0.05
Phenylalanine	0.25
Glycine	1.29
Alanine + cysteine	1.16
Leucine	1.27
Isoleucine	0.43
Lysine	0.55
Histidine	0.11
Arginine	0.68
NH_3	0.80

The preliminary amino acid data do not permit an accurate determination of whether the protein is acidic or basic. Therefore the type of protein–acidic phospholipid interaction cannot be clarified. However, the presence of both hydrophobic and basic amino acids would provide binding sites for acidic phospholipids. Irrespective of the nature of the relationship, the acidic phospholipids, except for cardiolipin, are difficult to dissociate from the protein.

Crude phospholipid is necessary for calcification, *in vitro,* of matrices prepared from bone (13) and elephant tusk (14). The critical issue is whether proteolipid is the active fraction in bone and tusk calcification. If so, *B. matruchotii* can be considered a microbiologic analogue for nucleation of these forms of vertebrate calcification.

Summary. The component of crude phospholipid responsible for *B. matruchotii* calcification was isolated. Crude phospholipid, extracted from the microorganism, was separated into five fractions by column chromatography. A single, protein-containing fraction catalyzed apatite formation in a metastable calcium phosphate solution. The nucleating fraction was identified as a proteolipid.

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