The Role of a Lipoprotein in the Intracellular Hydroxyapatite Formation in *Bacterionema Matruchotii*

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Bacterionema matruchotii, a large filamentous oral microorganism, has been shown to acquire hydroxyapatite intracellularly.[4, 13, 15] Takazoe[16] demonstrated that the initial event in this calcification was the concentrating of calcium within the cell by organic binding. Takazoe, Vogel and Ennever[18] found that the factor(s) responsible for Ca^{++} binding and for subsequent hyroxyapatite nucleation could be removed from the cells with a total lipid extraction. Further fractionation of the total lipid revealed that a crude phospholipid component could induce hydroxyapatite nucleation in a metastable calcium phosphate solution. In this report we will elaborate on the nature of the nucleating component and on its probable morphologic location within the cell. Certain correlations can be drawn between our observations on *B. matruchotii* calcification and some current concepts concerning vertebrate calcification.

MATERIALS AND METHODS

Cells to be used for the extraction procedures and for determination of calcium binding or hydroxyapatite formation were obtained by growing the Richardson #13 strain of *Bacterionema matruchotii* aerobically in brain-heart infusion (Difco Labs, Detroit) for 7 days at 37 C. The cells were harvested by centrifugation, water-washed and freeze-dried.

The total lipid extraction and fractionation procedures were those described previously[18] and are outlined in Figure 1. The method for determining calcium binding and hydroxyapatite nucleation have also been described.[5, 18]

The amino acid content of the crude phospholipid fraction was analyzed after acid hydrolysis. 100 mg of dried crude phospholipid were refluxed in 25 ml of 6N HCl for 48 hours. The hydrolysate was extracted 3 times with 50-ml volumes of ethyl ether, decolorized with activated charcoal and the excess acid removed by evaporating to dryness in vacuo several times. The residue was dissolved in redistilled water and diluted to 10 ml. The amino acid content of the hydrolysate was determined using a Phoenix Model K-8000 amino acid analyzer (Phoenix Precision Instrument Co., Philadelphia).

Intracellular calcification and related cell ultrastructure were studied by electron microscopy. Fresh cells were fixed with osmium tetroxide and glutaraldehyde and then embedded in Epon 812. After sectioning, those preparations for ultrastructure study were poststained with lead citrate and uranyl acetate.[17] Cells examined for calcification were not poststained. The preparations were examined in a model HU-11B Hitachi electron microscope (Hitachi, Japan).

RESULTS

The numerical values following the fractions outlined in Figure 1 indicate the amount of calcium bound. The crude phospholipid fraction (CPF) had the greatest

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relative Ca^{++} binding capacity, indicating that the binding component was concentrated into this fraction. The CPF constituted about 2.5 per cent of the freeze-dried cell weight.

A positive sign (+) denotes that a particular fraction induced hydroxyapatite formation in a metastable calcium phosphate solution. Crystallinity was established by electron microscopy, and hydroxyapatite was identified by electron diffraction.[18] Crystallinity was most pronounced in the CPF.

Thin-layer chromatography on silicic acid[14] showed that the CPF contained the following phospholipids: phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol, and a diphosphoinositide. Small quantities of cholesterol and cholesterol esters were carried over into the phospholipid fraction, but further purification showed that these compounds were not involved in Ca^{++} binding. A large quantity of material which was characteristic of a lipoprotein was also present. This material was biuret-positive and soluble in moist chloroform. Because of the presence of lipoprotein the CPF was subjected to acid hydrolysis and the amino acid content of the hydrolysate was determined. Chromatography of the CPF prior to acid hydrolysis showed that no free amino acids were present. The amino acid composition of the hydrolysate is given in Table 1. A portion of the serine is derived from its phosphatide. The most prominent feature is the relatively high lysine content, suggesting that the protein moiety is basic. An approximate calculation indicates that the lipoprotein represents about 25 per cent of the CPF dry weight.

The electron micrograph of an unstained section (Fig. 2 A) shows intracellular calcification in *B. matruchotii*. The hydroxyapatite crystals appear as needles. Clusters of crystals give a brushy appearance similar to that described for early endochondral calcification. Double staining reveals more of the cell ultrastructure as shown in Figure 2 B. The cell features are somewhat different than those of noncalcified cells[17]; however, mesosomes are still present.

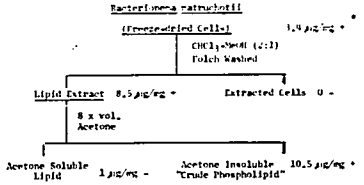

FIG. 1. Outline of the lipid fractionation of *Bacterionema matruchotii* cells.

* Numerical values indicate micrograms Ca^{++} bound per milligram dry weight.
Plus sign (+) indicates that fraction induced hydroxyapatite nucleation.

DISCUSSION

A crude phospholipid fraction, obtained from *Bacterionema matruchotii*, can induce hydroxyapatite formation in a metastable calcium phosphate solution. One of the

TABLE 1. Amino Acid Composition of Crude Phospholipid Hydrolysate

Amino Acid	*μ Moles/100 mg Phospholipid*
Alanine	13.6
Arginine	2.8
Aspartic acid	8.8
Glycine	17.1
Glutamic acid	7.2
Histidine	11.0
Isoleucine	8.9
Leucine	18.7
Lysine	39.6
Phenylalanine	2.0
Proline	5.2
Serine	11.6
Threonine	6.0
Valine	4.5

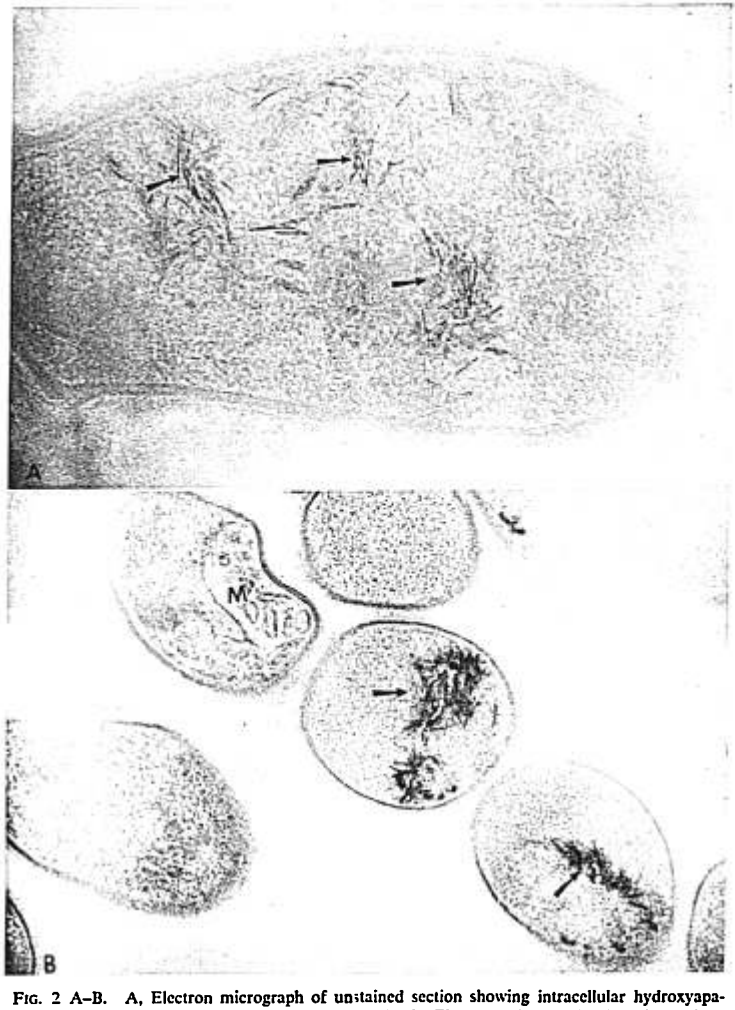

FIG. 2 A–B. A, Electron micrograph of unstained section showing intracellular hydroxyapatite deposition in *B. matruchotii* (arrows) (×57,000). B. Electron micrograph of double stained section. Note mesosomes (M) and persistence of some crystallinity after staining (×31,000).

major components of this fraction is a lipoprotein. Preliminary analysis suggests that it is a complex between a basic "lysyl" protein and the acidic phospholipids, phosphatidyl serine, and the inositides. It was classified as a lipoprotein because of its reaction with biuret and its solubility in moist chloroform. The complex is similar to basic protein-phospholipid complexes which have been described for myelin membranes[3, 12] and for cytochrome C[2]. The calcium-binding capabilities of these lipoproteins have been discussed by a number of investigators.[3, 7, 10, 12] An attempt to isolate the lipoprotein and test it for nucleation is in progress.

Although it has not been established that the initial hydroxyapatite nucleation occurs on or within the mesosomes, the data suggest that the membranes play a role in the process. The bulk of bacterial phospholipids occur in the membranes[11] and lipoproteins are generally components of membranous structures. In addition, the distribution of apatite crystals within the cytoplasm suggests an association with the mesosomes.

A study using the osteolathyrogen, beta aminopropionitrile (BAPN), provides indirect evidence for the involvement of the mesosomes. Calcification is inhibited when *B. matruchotii* is grown in the presence of BAPN, and an ultrastructure study showed that the mesosomes are markedly affected by this agent while other cell features appear normal.[18] The mesosomes are reduced in both number and complexity. It is hoped that in the near future the separation of cellular components by zonal centrifugation will permit a study of the mesosomes separately.

Two current concepts pertaining to vertebrate calcification are relevant to our observations on *B. matruchotii*. Irving and Wuthier[9] have re-emphasized the role of lipids in the initial stages of calcification. Histologic studies by Irving[8] have shown that lipids are dominant just prior to the onset of calcification. Wuthier[21] has identified these lipids as being mostly acidic phospholipids. Travis[19] postulated that a lipid or lipoprotein was involved in the transport of Ca^{++} to the calcification site during skeletal deposition in the crustacean. Urist[20] also mentioned a "muco-lipoprotein" complex in his *lost mold* theory for calcification. The other concept concerns the involvement of membrane-like structures in early calcification. Recently, Anderson[1] reported finding vesicles surrounded by trilaminar membranes in endochondral calcification. Some of the earliest detectable crystals were seen within the vesicles. The membranous nature of the vesicles is analogous to that of the mesosomes seen in *B. matruchotii*.

The ideas that lipoproteins and membranous structures are involved in calcification are consistent with each other since, generally, lipoprotein complexes are associated with membranes. What remains to be determined is what actual function these have in the calcification process and, if they do, how specific is the protein-phospholipid association in order for nucleation to occur. One of our objectives is to try to use *B. matruchotii* calcification as a model system in which possible mechanisms can be evaluated.

SUMMARY

A crude phospholipid fraction was involved in the intracellular calcification in *Bacterionema matruchotii*. One of the major components of this fraction is a lipoprotein consisting of a complex between a basic protein and acidic phospholipids. The lipoprotein is most likely part of the intracytoplasmic membranes or mesosomes. Electron microscopy showed that the early deposition of hydroxyapatite crystals was closely associated with the mesosomes. The findings are analogous to the involvement of lipids and membranous structures described in some types of vertebrate calcification.

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