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# Lipid and Calculus Matrix Calcification In Vitro

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*Lipid is necessary for calcification of a calculus matrix. Matrix was prepared by decalcification of dental calculus. The matrix calcified when it was exposed to a metastable calcium phosphate solution. After extraction with chloroform-methanol, the matrix lost the capacity to calcify. The lipid extract was calcifiable.*

The hypothesis that lipid is responsible for calcification of a matrix prepared from dental calculus was tested. The assumption was based on three facts. First, a form of lipid initiates calcification in *Bacterionema matruchotti*,<sup>1-3</sup> the most thoroughly studied example of microbiologic calcification.<sup>4-7</sup> Second, microbiologic calcification, although not universal, occurs in organisms other than *B matruchotii* in a chemically defined medium.<sup>8</sup> Finally, calculus matrix contains masses of microorganisms, some of which are spatially associated with early deposits of mineral.<sup>9-10</sup>

Proof of the hypothesis will serve two purposes. It will provide information potentially applicable to dental calculus. And it will strengthen the idea of a common denominator among phosphatic calcifications.

## Materials and Methods

To prepare calculus matrix, 0.5 gm water-washed, air-dried, pooled human dental calculus was stirred in 250 ml 2 M formic acid. The acid was changed daily until it gave three consecutive zero calcium readings by atomic absorption spectrophotometry.<sup>a</sup> The

decalcified material then was washed repeatedly with deionized water until the wash water had the same electric resistance as unused deionized water.<sup>b</sup> The resultant matrix, decalcified and acid-free, was dried under nitrogen at 45 C. Yields approximated 60 mg.

The matrix was checked for calcifiability by shaking 2.5 mg at room temperature in an air-tight, 10 ml centrifuge tube filled with a metastable calcium phosphate solution (Table). The solution was changed daily for six days. The matrix then was recovered, water-washed, air-dried at 45 C, and examined by X-ray diffraction. The instrumentation and techniques have been reported previously.<sup>11</sup>

To extract lipid, 40 mg of matrix was suspended in 100 ml chloroform-methanol (2:1, v/v) for 48 hours. The suspension was filtered through a solvent-resistant membrane.<sup>c</sup> The extracted matrix was washed on the filter surface with an additional 500 ml solvent and was air-dried. The filtrate was washed with 0.12 M sodium chloride to reduce nonlipid contaminants.<sup>12</sup> The lipid-containing phase was drawn off and evaporated to dryness at 45 C in vacuo. The lipid was washed with acetone to decrease the nonpolar, hydrophobic content and was redried at 45 C under nitrogen.

The lipid extract and the extracted matrix were tested for calcifiability. The procedure used was essentially the same as that used for the intact matrix. Two exceptions were that the lipid was suspended in the metastable calcium phosphate solution for only 40 hours, and the solution was not changed.

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<sup>a</sup> SP-90 atomic absorption spectrophotometer, Unicam Instruments, Cambridge, Eng.

<sup>b</sup> Purity Meter PM-5, Barnstead Still & Sterilizer Co., Boston, Mass.

<sup>c</sup> Filter Type UH, Millipore Corporation, Bedford, Mass.

TABLE  
COMPOSITION AND PREPARATION OF METASTABLE  
CALCIUM PHOSPHATE SOLUTION

Na <sub>2</sub> HPO <sub>4</sub>	0.276 gm
NaCl	4.090 gm
NaHCO <sub>3</sub>	1.850 gm
KCl	0.370 gm
Ca <sup>++</sup>	0.080 gm
Water	qs 1 liter*

Note: All salts except calcium were dissolved in 800 ml deionized water. The pH was adjusted to approximately 6.6 with carbon dioxide. The calcium was added from a stock solution and the volume was brought to 1 liter. The solution was filtered (Filter Type HA, Millipore Corp., Bedford, Mass), and stored in air-tight bottles at 4 C. The pH was adjusted to 7.20 by aeration just before use.

\* Sufficient quantity to make 1 liter.

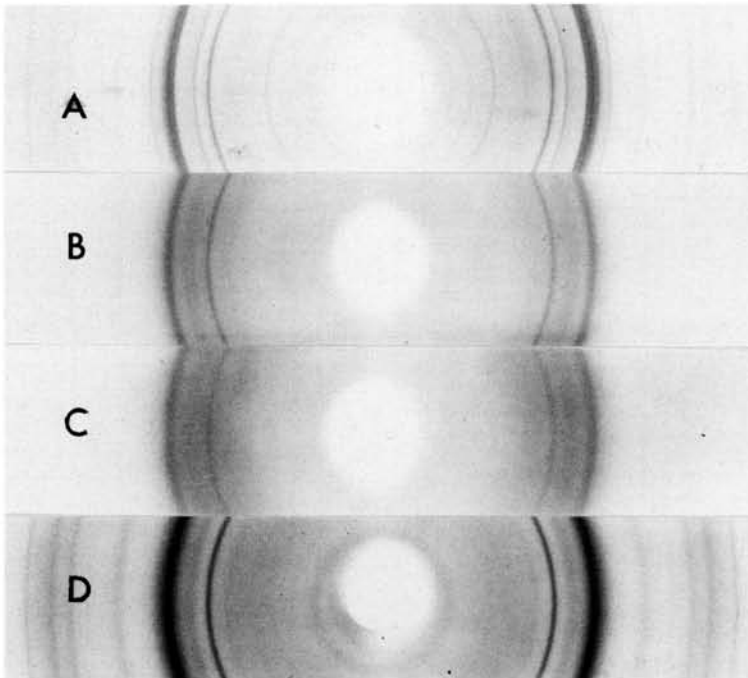
### Results

The decalcified calculus matrix calcified during a six-day exposure to the metastable calcium phosphate solution, which was fresh daily. The lipid extracted from the matrix calcified within a 40 hour period in the same type of solution. The lipidfree matrix failed to calcify under the same conditions used with the decalcified, nonextracted matrix.

Both matrix calcification and lipid calcification were apatite. Typical X-ray diffraction patterns are shown in the illustration. For comparison, diffraction patterns of dental calculus and root dentin are included in the illustration. The calculus pattern contains evidence of whitlockite and apatite. Whitlockite was never detected in either matrix or lipid calcifications. No pattern was obtained by X-ray diffraction of lipid-extracted matrix after exposure to metastable calcium phosphate.

### Discussion

Lipid was required for calcification of a matrix prepared from dental calculus. This indicates the probability, suggested several years ago by Mandel and Levy,<sup>13</sup> that lipids are involved in dental calculus calcification. If so, calculus calcification most likely is mediated by microorganisms, because calculus matrix lipid is primarily of microbial origin.<sup>14</sup> This does not conflict with observations<sup>9,10</sup> that initial calculus calcification is between microorganisms, rather than within them. The extracellular portion of



Positive prints of X-ray diffraction patterns: A, original calculus; B, calcified calculus matrix; C, calcified lipid fraction; D, human dentin (unashed).

calculus matrix could be at least partly of microbial origin as a result of cell death and autolysis. The time lag that occurs between plaque formation and the beginning of calcification is in accord with this view.<sup>9,15</sup>

The matrix and lipid calcifications consisted of small apatite crystals. This is evident by the relatively broad bands of the X-ray diffraction patterns. Crystal size may be due to a size-limiting agent present in the calcifiable substrates, or incubation time in the metastable calcium phosphate solution. Extending incubation time could help resolve this question.

Whitlockite, which was apparent in intact calculus, was not detected in matrix calcification. Whitlockite in calculus is believed to be the result of magnesium influence.<sup>16,17</sup> The formic acid used for matrix preparation removed magnesium. Calcification of matrix, therefore, was unaffected by magnesium.

The possibility must be considered that the lipid-extracted matrix acquired amorphous calcium phosphate, which would not be detected by X-ray diffraction. This is unlikely. When kept in contact with the preparative solution, amorphous calcium phosphate readily converts to apatite.<sup>18</sup> Furthermore, in preliminary trials lipid extraction virtually eliminated the calcium-binding capacity of the matrix.

A pattern is beginning to emerge among experimental phosphatic calcifications. The *B. matruchotii* calcification factor initially was localized in the lipid component of the cell.<sup>1</sup> Subsequently, the crude phospholipid fraction of the organism was found to contain the nucleator.<sup>2</sup> Lipid also was shown to be involved in calcification of a matrix made from root dentin. Here too, calcification proved to be a function of the crude phospholipid.<sup>19</sup> Work is in progress to determine whether the phospholipid fraction regulates calculus matrix calcification.

### Conclusions

Lipid was shown to be responsible for calcification of a calculus matrix. Matrix was prepared from pooled human calculus by formic acid decalcification. The matrix nucleated apatite crystallinity during a six-day exposure to a metastable calcium phosphate solution that was changed daily. Extraction of matrix with chloroform-methanol

(2:1, v/v) before the metastable calcium phosphate exposure eliminated the capacity of the matrix to calcify. The recovered lipid extract induced apatite formation within a 40 hour period in the metastable calcium phosphate solution.

The findings indicate the probability that lipid is involved in calcification of dental calculus. If so, calculus calcification is most likely a function of microorganisms, because calculus matrix lipid is primarily of microbial origin. The results also point to a common denominator among experimental phosphatic calcifications.

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