III. A Simple Method for the Isolation of Matrix Vesicles

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INTRODUCTION

Recently we reported ultrastructural observations which indicated that matrix vesicles were actual constituents of the intercellular organic matrix during embryonic tooth development (14). It is assumed that many of these matrix vesicles are involved in formative aspects of dentinogenesis or amelogenesis, two primary extracellular processes that occur during embryonic tooth formation.

The fractionation of matrix vesicles from cartilage by means of demineralization followed by collagenase digestion of the organic matrix and subsequent differential centrifugation has been reported (1) and recently reviewed with respect...
to the function of matrix vesicles during initial mineralization and calcification (3). Matrix vesicles within the intercellular matrix associated with epithelial-mesenchymal interactions during odontogenesis may have functions comparable to those suggested for cartilage, or may possibly mediate the transfer of developmental information between heterotypic cells (11).

This report describes a simple procedure for the isolation of matrix vesicles from embryonic tooth primordia by collagenase digestion of the intercellular organic matrix and subsequent gel filtration column chromatography. Ultrastructural criteria indicate that isolated matrix vesicles are limited by a triaminar unit membrane and are morphologically similar to those observed in situ.

**MATERIALS AND METHODS**

*Preparation of Organ Cultures and Radioactive Labeling*

New Zealand white rabbits, 24 days pregnant, were killed by injecting 10 ml of air into the left ear vein. The embryos were removed, decapitated, and stored on ice. Maxillary and mandibular incisor tooth primordia were extracted as previously reported (12). Each organ culture group consisted of 90 tooth primordia placed into a 17 X 100 mm (16 ml) test tube containing 4 ml A buffer (NaCl, 125 mM; KCl, 3 mM; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid [HEPES; CalBioChem, La Jolla, Calif.], 20 mM; dextrose, 5 mM; Gibco antibiotic-antimycotic mixture [Grand Island Biological Co., Berkeley, Calif.], 0.05 ml/100 ml, pH 7.4). All solutions used in these experiments were sterilized by filtration (0.22 µm pore size) before use. Sterile, disposable plastic labware was used throughout all procedures.

Radioactive choline, previously shown to be incorporated into membrane phosphatidylcholine (5), was selected as a possible precursor for the limiting membrane of matrix vesicles. Each organ culture group (4 ml) received 50 µCi choline-methyl-3H chloride (specific activity, 1.0 Ci/mmol, New England Nuclear Corp., Boston, Mass.). The organ culture tubes were sealed and incubated at 37°C for 4 hr. Radioactive buffer was removed and primordia were washed twice with ice-cold B buffer (NaCl, 5 mM; HEPES, 10 mM; 2-mercaptoethanol, 0.02% [v/v]; Gibco antibiotic-antimycotic mixture, 0.05 ml/100 ml, pH 8.2).

*Isolation of Intercellular Matrices*

The entire experimental design is outlined in Fig. 1. The procedure for isolating the intercellular organic matrix is a modification of that previously described (15). After the dental papilla was removed from each tooth, the germinative one-third of each intercellular organic matrix was isolated and transferred into a 17 X 100 mm test tube containing 5 ml of B buffer. Vibratory shaking was selected to remove adherent...
Light microscopy was employed to evaluate steps during the isolation and enzymatic digestion of the intercellular matrix. (A) After removal of the dental papilla from tooth primordia, the lower one-third of the intercellular matrix (im) was isolated. Note the inner enamel epithelium (iee) and adjacent preodontoblasts (po) adherent to the organic matrix. (B) The vibratory shaking procedures removed all adherent cells from the matrix. (C) The residual matrix after collagenase digestion. Under these conditions the matrix was not totally digested. × 400.

cells from the isolated matrices. The test tube was shaken at 60 cycles/sec for 1 min with an electromagnetic vibrator. This step was repeated three times, using 5 ml of fresh B buffer each time. Any residual cellular debris in suspension with the matrices was removed by filtering the entire mixture through a stainless steel screen (No. 4578-B10, Arthur H Thomas Co., Philadelphia Pa.) having grid openings 0.4 mm square. The matrices were washed three times with 2 ml of B buffer in this way.

Matrix Vesicle Isolation

The matrices were removed from the filter assembly and placed into a 12 × 75 mm (6 ml) test tube containing 1.2 ml B buffer plus 500 units/ml collagenase (CLSPA grade, Worthington Biochemical Corp., Freehold, N. J.). The test tube was rotated slowly for 4 hr in a 37°C water bath. The resulting enzyme digest was briefly shaken (5 sec) to disperse aggregated material and then centrifuged at 500 g for 1 min to sediment large debris. The upper 1.0 ml of the supernatant was pipetted out and then eluted with B buffer through a column 0.8 cm in diameter filled with Bio-Gel A-15m to a height of 27 cm.

Each fraction collected was 0.37 ml. Effluent absorbance at 260 nm was continuously monitored with a Gilford flow cell and recording spectrophotometer (Gilford Instrument Laboratories, Inc, Oberlin, Ohio). Radioactivity was determined by counting 0.05 ml of each fraction in 10 ml of Aquasol (New England Nuclear Corp) in a Tri-Carb liquid scintillation spectrophotometer (Packard Instrument Co., Downers Grove, Ill.).

Light and Electron Microscopy

Intact tooth primordia, isolated intercellular organic matrices before and after vibratory shaking...
procedures, matrices after collagenase digestion, and pelleted material from the excluded volume of the Bio-Gel A-15m column were processed for light and electron microscopy. The pellets were obtained by placing 0.5 ml of the maximally 260 nm-absorbing void volume eluant into conical BEEM capsules (130-SPC, Ted Pella Co., Tustin, Calif). The capsules were loaded into plastic adaptors cast to fit 0.5 X 2 inch polyallomer tubes and centrifuged in a SW-65 swinging bucket rotor for 40 min at 40,000 g.

Procedures for fixation, dehydration, embedding, sectioning, and staining were essentially those reported by Hay and Revel (7). Primary fixation was carried out for 20 min in formaldehyde-glutaraldehyde buffered with 0.2 M cacodylate at pH 7.2. Tissues were postfixed in 1.33% osmium tetroxide and stained in the block with 2% uranyl acetate. After rapid dehydration through a graded series of alcohol, specimens were embedded in Epon 812 (8).

All tooth primordia and isolated matrices were sectioned in a plane parallel to their long axes. Thick sections (0.5 μm) for light microscopic observations were stained with 1% toluidine blue. Pellets of isolated matrix vesicles were sectioned perpendicular to the direction of centrifugal force. Thin sections with silver-to-gray interference colors were cut with a diamond knife on a Sorvall MT-2 ultramicrotome and stained with lead citrate (10). Electron microscope observations were performed on a Zeiss EM-9S instrument.

**Cytochemical Demonstrations of ATPase, Alkaline Phosphatase, and Acid Phosphatase**

Specimens of intact tooth primordia and isolated matrix vesicle preparations were incubated for cytochemical demonstration of adenosine triphosphatase (ATPase) activity and alkaline and acid phosphatase activities as specified by Matsuzawa and Anderson (9); substrate-free media were used as enzyme controls. All solutions were prepared before use and maintained at 37°C throughout the experiment. Incubation times were extended to 30 min. All sections were observed unstained.

**Observations**

**Light and Electron Microscopy of Intercellular Organic Matrices**

Immediately after the mechanical removal of the dental papilla from the intact tooth primordia, the lower or germinative one-third of the intercellular matrix (progenitor predentine) was isolated and examined for adherent cellular contamination (Fig. 2 A). The region of isolated organic matrix ranged from 10 to 20 μm in thickness. Subsequently, vibratory shaking procedures were used to efficiently remove adherent cells. Light microscope observations of the intercellular matrices immediately after vibratory shaking indicated that adherent mesenchymal (preodontoblast) and epithelial (inner enamel epithelium) cells were removed (Fig. 2 B). Under the conditions of these experiments collagenase digestion of isolated, acellular matrices did not totally digest this material (Fig. 2 C).

Ultrasound observations of the secretory regions of both inner enamel epithelium and adjacent preodontoblast cells in situ indicated numerous membrane-limited structures within the intercellular organic matrix (Fig. 3 A). Serial sectioning of this material was employed in order to discern whether the matrix vesicles were discrete extracellular structures (outside of the plasma membranes associated with cell processes or cell extensions) or were continuous with cell processes. Since the matrix vesicles were 0.05-0.1 μm in diameter, thin sections (circa 500-700 Å thick) could easily be reconstructed to determine which structures were cell processes and which were matrix vesicles. Within the forming organic matrix, numerous matrix vesicles of varying sizes (0.05-0.1 μm) and electron opacities were observed.

After removal of the dental papilla from each of the extracted tooth primordia and subsequent procedures to remove adherent cells from the...
matrix, light and electron microscope examination did not demonstrate any cellular contamination. Ultrastructural observations of isolated matrices after vibratory shaking indicated the inclusion of numerous matrix vesicles (Fig. 3 B) and other membrane-limited structures. The matrix vesicles within the isolated matrix preparation were comparable to those observed in situ. Ultrastructural examinations of the remaining organic matrix after collagenase digestion procedures did not indicate retained vesicles. Most of the matrix vesicles were removed from the intercellular matrix after the collagenase digestion (Fig. 3 C).

**Isolation of Matrix Vesicles by Gel Filtration Chromatography**

Fig 4 represents the gel filtration chromatographic separation of the collagenase digest with Bio-Gel A-15M. The elution profile of the tritiated radioactivity is compared with the 260 nm ultraviolet absorption pattern throughout the chromatographic separation. The cross-hatched region in the void volume represents the eluant volume pelletted by centrifugation and subsequently prepared for electron microscope observations. This isolation procedure resulted in a preparation which contained numerous matrix vesicles ranging in size from 0.05 μm to 0.1 μm (Fig. 5 A). The majority of the membrane-limited structures within the isolated fraction were comparable to the matrix vesicles observed in situ. Many of the isolated matrix vesicles were limited by a discrete trilaminar membrane (Figs. 5 B-G, arrows). In addition to matrix vesicles isolated from the intercellular organic matrix, other vesiculated structures were discernable (Fig. 5 A).

**ATPase, Acid Phosphatase, and Alkaline Phosphatase Activities in Matrix Vesicles**

In specimens of tooth primordia incubated with ATPase, reaction product was consistently found on the cell membranes of both inner enamel epithelium and preodontoblast cells and on the limiting membrane of matrix vesicles. Isolated matrix vesicles showed ATPase activity on the limiting trilaminar membranes. Acid phosphatase activity was abundant in lysosomes, Golgi cisternae, and intracytoplasmic vesicles within both cell types. Reaction product was found in matrix vesicles in situ and in many of the isolated vesicles. Alkaline phosphatase activity was not present in the isolated matrix region or in the resulting

![Figure 4](image-url)  
**Figure 4** Radioactivity-3H (closed circles) and 260 nm absorbance patterns of the collagenase digest supernatant after elution on a Bio-Gel A-15M column (fractions of 0.87 ml). Tooth explants were cultured in a medium containing choline-methyl-3H-chloride for 4 hr. India ink (used to establish void volume), transfer RNA, and uridine were used as column markers. Most of the absorbance in fractions 30–40 is attributed to collagenase. The hatched region of the excluded volume was selected to centrifuge into a pellet for electron microscope examination.
matrix vesicle preparations. However, alkaline phosphatase activity was demonstrable in the regions of the dentine matrix associated with calcification in situ.

**DISCUSSION**

The isolation of fractions containing high yields of matrix vesicles should be considered prerequisite to studies of biochemical characterization and
biological function. In the present research we describe a procedure for the isolation from an intercellular organic matrix (10-20 μm in thickness) of morphologically recognizable matrix vesicles. These extracellular structures have been termed matrix vesicles within cartilage matrix formation (2) and have been implicated as a mediator for the local concentration of ions prerequisite for initial mineralization (3, 4, 17). Recently, matrix vesicles have been isolated from calf cartilage and found to contain ATPase, alkaline phosphatase, and acid phosphatase enzymes (1). This isolation procedure for obtaining a matrix vesicle fraction depended upon demineralization of dissected cartilage, exhaustive collagenase digestion of the organic matrix (18 hr), and differential centrifugation which pelleted the matrix vesicles in a 150,000 g fraction. In our studies, varying ATPase and acid phosphatase activities were present in many but not all matrix vesicles. No alkaline phosphatase activity was detected in our preparations. The essential features of the simple, reproducible method described in this report are mechanical isolation of the odontogenic organic matrix, relatively short collagenase digestion (4 hr), and gel filtration column chromatography (Fig. 1). This methodological approach was designed to minimize cellular destruction during the isolation of the intercellular matrix, to limit artificial membrane vesiculation inherent in such procedures, and to retain the integrity of the limiting membrane of the matrix vesicles. Electron microscopy of the matrix vesicle pellets obtained by centrifugation showed them to be free of microchondria and other easily recognizable intracytoplasmic structures (Fig. 5 A). Light and electron microscopy were employed in preliminary studies to determine the actual volume of the pelleted material. Direct counting of thin sections indicated a yield of approximately 3 × 10^5 matrix vesicles per 900 μg isolated matrices (dry weight) starting material.

Previously, we reported that isolated intercellular organic matrix enhanced cytodifferentiation within homotypic cells in vitro (16). Subsequently, the isolated matrix was found to contain several species of methylated RNAs (13, 15). During these experiments radioautographic and radiochemical analyses after organ culture experiments with radioactive thymidine indicated that no radioactivity was incorporated into the matrix material. However, both adjacent cell populations, inner enamel epithelia and predentinoblasts, incorporated appreciable amounts of labeled thymidine.

Might the matrix vesicle fraction isolated from progenitor predentin material contain the RNAs previously reported to be components of the intercellular organic matrix? Preliminary experiments indicate that a "liporibonucleoprotein complex" is associated with what has been referred to as matrix vesicles (6). Might the absence of alkaline phosphatase in this germinal organic matrix imply that these matrix vesicles have functions other than calcification? Continued experimentation along these lines is currently in progress.

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