Methods have been developed for inducing chick chondrocytes to vesiculate in a defined medium that resembles the extracellular fluid of cartilage. In this medium, chondrocytes liberate alkaline phosphatase-rich membrane vesicles which are capable of calcium loading. For cultured cells, the rate of vesicle release is linear for 72 h. The vesiculation process is specific for viable chondrocytes and is stimulated by the presence of amino acids and glucose in the medium. Investigation of the composition of the inducing medium indicates that the high initial pH and buffer capacity contribute to the efficacy of this medium in promoting cell vesiculation. The biochemical and morphological characteristics of chondrocyte vesicles suggest that this novel induction system may be a useful model to study the biogenesis of matrix vesicles in endochondral ossification.

The pattern of mineralization of growth plate cartilage displays both spatial and temporal specificity. Calcification is related to the maturation of epiphyseal chondrocytes and is localized to a defined zone. Within this zone, cellular control of the ionic and macromolecular composition of the cartilage matrix regulates calcification. Recently, considerable interest has focused on the biochemical changes accompanying chondrocyte maturation and the initiation of mineralization.

Several hypotheses have been advanced to explain how the calcification process begins in the cartilage matrix. Most authorities consider that matrix components act either as promoters or inhibitors of mineralization. Both roles have become associated with the activities of MV' (1-3). These membrane-bound organelles, observed in the longitudinal septa, are intimately related to the deposition of apatite into the calcifying matrix (4). Several authorities believe that MV contain calcification initiator sites that serve to increase the local ion concentration (5, 6).

Previous investigations have focused on the morphology, composition, and enzymatic activities of MV (7-11). Compositional analysis has been carried out on vesicles isolated from enzymatic digests of cartilage. Prepared in this way, these vesicles appear to be rich in acidic phospholipids and contain high levels of alkaline phosphatase. From these studies, it has been concluded that MV originate from the plasma membrane of chondrocytes (12). However, these investigations have failed to provide information on factors controlling vesicle biogenesis. Moreover, it is unlikely that chemical analysis of preformed MV will resolve these issues.

In this report we describe a new method for studying vesicle formation. This method utilizes an induction system in which chondrocytes are stimulated to vesiculate in vitro. The enzymatic and biochemical characteristics of these vesicles are similar to MV. In addition, we show that vesiculation is a cell-specific process that requires healthy functional cells.

**MATERIALS AND METHODS**

**Freshly Isolated Cells**—Cartilage was removed from the resting and proliferative zones of the growth plate of 6-9-week-old chicks. The thinly sliced tissue was digested with bacterial collagenase (100,000 units/10 g of tissue, Sigma) in a medium containing 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, and 10 mM KCl supplemented with 10% fetal calf serum (Gibco). After 1.5 h, the cells were separated from the undigested material, washed with HBSS, pelleted, and resuspended in a vesiculation medium.

**Vesiculation of Freshly Isolated Cells**—Resuspended cells were incubated in SCL (13). We have previously shown that this solution will stimulate chondrocyte vesiculation (14). Other agents also tested for vesiculation promoting activity include formaldehyde (221 mM HCHO, 20 mM KPO4, pH 7.2, 150 mM NaCl, 2 mm diithiothreitol), lysozyme (20 mg/ml of hen egg white, 20 mM KPO4, pH 7.2, 150 mM NaCl), and an aqueous extract of cartilage (105,000 g supernatant of chick growth plate cartilage homogenate). After 1.5 h at 37 °C, the cells were then harvested by centrifugation at 900 × g for 5 min, and the cell supernatant was further clarified by centrifugation at 14,000 × g for 15 min. As this force is insufficient to sediment most of the vesicles, the suspended vesicles were permitted to load with Ca2+ by adjusting the ionic composition of the supernatant to 5 mM Ca2+, 3 mM Mg2+, and 5 mM ATP. Following incubation for 1.5 h at 37 °C, vesicles were sedimented by centrifugation at 14,000 × g for 15 min. Remaining membrane fragments and vesicles were isolated by centrifuging the supernatant at 105,000 × g for 18 h. For comparison purposes, conventional MV were also prepared from the cartilage digest using the method of Ali et al. (15).

**Preparation of Chondrocyte Cultures**—Sternal cartilage was dissected from chick embryos (15-16-days-old). The tissue was minced and digested at 37 °C in HBSS containing hyaluronidase (0.1%, bovine testis, Sigma), trypsin (0.2%, bovine pancreas, Sigma), and collagenase (0.5%). This digestion procedure was repeated three times. The cells were then pooled, washed, and resuspended in RPMI 1640 (Gibco) supplemented with 10% newborn calf serum (Gibco). The cell suspension (2.3 × 106 cells/ml) were plated onto plastic tissue culture dishes at a density of 3000-5000 cells/mm2 and incubated for 18 h at 37 °C in 5% CO2, 95% air.

**Vesiculation in Culture**—To study chondrocyte vesiculation, the culture medium was replaced with SCL, and vesicle production was measured for 0-3 days. Standard tissue culture media also tested for vesiculation-promoting activity include RPMI 1640, HBSS, complete minimal essential medium, Eagle’s basal medium, and Ham’s F-12. For vesiculation induction experiments, all media were used without serum but were supplemented with bovine serum albumin (0.1%). In most experiments, the medium was further supplemented with essential and nonessential amino acids (minimal essential medium), L-glutamine (2 mM), and glucose (0.1%). At the end of the vesiculation period, the supernatant was removed, and unattached cells were...
collected by centrifugation at 1,500 × g. Vesicles were isolated from the supernatant either by filtration through membrane filters (Nucleopore Corp., 0.1-μm pore size) or by centrifugation at 105,000 × g for 2.5 h. The adherent cells were removed from the dishes by mild trypsin-treatment and scraping. These cells were then combined with the unattached cells, resuspended in buffer (50 mM Tris-HCl, pH 7.2, 120 mM NaCl, 5 mM KCl, and 2 mM MgCl₂) and sonically disrupted. For comparison purposes, the vesiculation properties of fibroblasts and osteoblast-like cells were also studied. Fibroblasts were isolated from 8-day-old chick embryos and grown to confluence in culture using RPMI 1640+10% fetal calf serum. Osteoblast-like cells were isolated from 16-day-old chick embryo tibia by collagenase digestion and maintained in culture (Ham's F-12 + 10% serum) for 1 week before vesiculation testing.

**Results**

**Vesiculation-inducing Media**—Conditions favoring the release of vesicles from freshly isolated chondrocytes were studied by treating cells with several agents and tissue extracts (Fig. 1). Formaldehyde (HCHO) was tested because it has been reported to stimulate vesiculation and blebbing of cells (20). Lysozyme, SCL, and the aqueous cartilage extract were tested as possible in situ vesiculation promoters, while HBSS and Tris-Mg-sucrose (10 mM Tris-HCl, pH 7.4, 56 mM MgCl₂, 10% sucrose (TMS)) were included as control media. For these experiments, cells were incubated as shown for up to 3 h at 37 °C, and the cell supernatants were monitored for alkaline phosphatase activity. For both vesicle types, assays of NADPH-cytochrome c reductase and cytochrome oxidase activities were measured using ferrocytochrome c (Sigma) as substrate (18, 19).

Electron Microscopy—Vesicle morphology was examined using samples fixed in 2% glutaraldehyde in cacodylate buffer, pH 7.4. The sections were post-fixed with osmium tetroxide and stained with uranyl acetate.

In culture, the chondrocytes released 4-17% of their total alkaline phosphatase, while no more than 3% of the total lactate dehydrogenase activity was lost (Table I). If supernatant alkaline phosphatase activity arose from cell fragmentation, then a corresponding fraction of the total lactate dehydrogenase activity of the supernatant would also have been expected. This was not seen. Moreover, cell recovery and viability were greatest in SCL, while leakage of lactate dehydrogenase was lowest. We concluded, therefore, that the effectiveness of agents in promoting vesiculation could best be expressed by the ratio of the percentage of total alkaline phosphatase released to the percentage of total lactate dehydrogenase released. The vesiculation ratios of SCL and Ham's F-12 were approximately 6. As a high ratio is the result of a specific release of vesicles without cell damage, it is a stringent measure of vesiculation. When the effects of Ham's F-12 and SCL are compared, it can be seen that Ham's F-12 caused more alkaline phosphatase to be released from chondrocytes than SCL. However, as it also increased lactate dehydrogenase release, it is probable that some of the cells had become damaged. It was therefore concluded that SCL was the most appropriate medium for the further study of chondrocyte vesiculation.

Collection of Vesicles—Previous studies have shown that MV can accumulate calcium in the presence of ATP (6). It was reasoned that, if the alkaline phosphatase-rich particles released from chondrocytes by SCL were similar to MV, it should be possible to utilize this calcium-accumulating property to isolate the vesicles from the cell supernatants. Thus, vesicles isolated from chondrocytes were treated with Ca²⁺ and ATP (see “Materials and Methods”). The calcium-loaded vesicles were then harvested by centrifugation and analyzed for alkaline phosphatase activity and protein. The results of this experiment (Table II) clearly show that the calcium-loading protocol resulted in the sedimentation of high alkaline phosphatase activity particles. Thus, prior to loading, the alkaline phosphatase-specific activity of vesicles (14,000 × g pre) was 1.59 μmoles/min/mg of protein; after loading, the activity (14,000 × g post) increased 2-3-fold. Calcium analysis of the pellets also revealed an 8-fold increase in calcium content (0.098-0.74 μg of Ca/μg of protein) after calcium loading. When authentic MV isolated by conventional procedures were treated in a similar manner to vesicles, there was also a comparable increase in alkaline phosphatase-specific activity. For both vesicle types, assays of NADPH-cytochrome c reductase, a marker for endoplasmic reticulum, and cytochrome oxidase, an enzyme located in the inner mitochondrial membrane, indicated a low level of organellar contamination. However, the yield of vesicles prepared and collected this way was very low (0.25-10 μg of vesicle protein/g of cartilage, wet weight).

The morphology of the fraction (14,000 × g) is shown in Fig. 2. The electron micrograph reveals the vesicular nature of the particles released by SCL and sedimented after calcium loading. These vesicles resemble MV and range in diameter...
Induction of Chondrocyte Vesiculation in Vitro

Effect of culture media on chondrocyte vesiculation and viability

Sternal chondrocytes (9 × 10⁵ cells) were plated onto plastic tissue culture dishes in RPMI 1640 + 10% fetal calf serum. After 24 h, each medium was replaced with the appropriate vesiculation medium without serum, supplemented with bovine serum albumin (0.15%), essential and nonessential amino acids (minimal essential medium), L-glutamine (2 mM), and glucose (0.1%). After an additional 24 h, each medium was removed, centrifuged to remove detached cells, and assayed for alkaline phosphatase and lactate dehydrogenase activity. All the cells harvested from each dish were counted, stained for viability with trypan blue, homogenized, and assayed for alkaline phosphatase and lactate dehydrogenase. The vesiculation ratio is calculated as percentage of supernatant alkaline phosphatase to percentage of supernatant lactate dehydrogenase. MEM, minimal essential medium; EBM, Eagle’s basal medium.

| Medium     | Cell number | Viability | Alkaline phosphatase | Lactate dehydrogenase | Vesiculation ratio |
|------------|-------------|-----------|----------------------|-----------------------|--------------------|
|            | 10⁶ cells/culture | % | nmol/min/culture | % total | nmol/min/culture | % total |                      |
| SCL        | 7.0         | 100       | 5.31                  | 0.33       | 6.21               | 1.180       | 11.5                  | 0.99 | 6.27                 |
| RPMI       | 5.8         | 80        | 5.35                  | 0.30       | 5.61               | 1.050       | 26.7                  | 2.54 | 2.21                 |
| HBSS       | 4.6         | 80        | 4.08                  | 0.16       | 3.92               | 619        | 14.4                  | 2.33 | 1.68                 |
| MEM        | 5.8         | 80        | 7.05                  | 0.29       | 4.11               | 1.196       | 16.3                  | 1.36 | 3.02                 |
| EBM        | 5.8         | 100       | 4.27                  | 0.32       | 7.49               | 618        | 11.2                  | 1.80 | 4.16                 |
| Ham’s F-12 | 7.1         | 82        | 4.80                  | 0.81       | 16.9               | 1.781       | 50.2                  | 2.82 | 4.99                 |

TABLE II
Enzymatic characterization of vesicles

SCL was utilized to induce vesicle release from freshly isolated chondrocytes. A vesicle preparation was isolated by centrifuging the sample at 14,000 x g for 15 min (14,000 x g pre). The vesicles were incubated with Ca²⁺ (5 mM), Mg²⁺ (3 mM), and ATP (3 mM) and centrifuged at 14,000 x g for 15 min. The vesicle sediment (14,000 x g post) was collected. The supernatant (14,000 x g post) was centrifuged at 105,000 x g for 18 h and a final sample collected. Each sediment was analyzed for alkaline phosphatase, NADPH, cytochrome c reductase, and cytochrome oxidase.

For comparison, a MV preparation isolated from epiphyseal plate chondrocytes is also shown. Data are the means ± S.E.

| Fraction | Vesicles | Alkaline phosphatase | NADPH cytochrome c reductase vesicles | Cytochrome oxidase vesicles |
|----------|----------|----------------------|--------------------------------------|-----------------------------|
|          | MV       |                      |                                      |                             |
| Homogenate |         | 0.281 ± 0.046        | 0.018 ± 0.002                        | 0.020 ± 0.005               |
| 900 x g  |          | 0.156 ± 0.051        | 0.333 ± 0.066                        | 0.022 ± 0.001               |
| 14,000 x g pre | 1.591 ± 0.232 | 1.121 ± 0.102          | 0.029 ± 0.003                        | 0.008 ± 0.002               |
| 14,000 x g post | 4.320 ± 1.682 | 2.436 ± 0.384          | 0.021 ± 0.009                        |                             |
| 105,000 x g | 0.776 ± 0.123 | 0.835 ± 0.089          | 0.001 ± 0.000                        |                             |

from 0.02-2 µm. In most fractions examined, membranes and mitochondria were observed. When viewed at higher magnification, vesicles could be seen in a granular matrix. Fig. 2 shows that vesicles (V) have a complete outer membrane which surrounds an amorphous electron-dense material. "Empty" vesicles were also noted. The structures seen in this fraction are very similar to the microvesicles described by Trams et al. (21).

Time Course of Vesicle Release—The difficulty of studying freshly isolated cells for extended periods was overcome by development of the cultured chondrocyte vesiculation system. In these studies, embryonic chondrocytes were cultured for up to 3 days in SCL supplemented with bovine serum albumin, amino acids, and glucose. The data from such an experiment are shown in Fig. 3. The figure shows that the release of alkaline phosphatase is linear for 72 h. During this experiment, 90-100% of the cells excluded trypan blue and the total alkaline phosphatase did not change (0.5-0.6 nmol/min/10⁶ cells). In other experiments, maximum release was reached in 72-144 h, with as much as 45% of the cells’ alkaline phosphatase activity liberated. As shown in the figure, no more than 5% of the total lactate dehydrogenase activity was released although total enzyme activity increased from 100-600 nmol/min/10⁶ cells during the experiment. Finally, the vesiculation ratio (approximately 10) indicated that most of the enzyme release resulted from specific vesiculation.

Cell Specificity of Vesiculation—To determine whether vesiculation is chondrocyte-specific, vesiculation by fibroblasts was examined. Fibroblasts were treated with SCL containing bovine serum albumin in the presence and absence of amino acids and glucose. Fig. 4 shows that chondrocytes vesiculate to a much greater extent than fibroblasts. Supplementation of the medium with amino acids and glucose stimulated chondrocyte vesiculation more than fibroblast vesiculation. Since fibroblasts and chondrocytes have similar alkaline phosphatase contents (0.5-1.5 nmol/min/10⁶ cells), the difference between these cell types indicates a significant
vesiculation, modified SCL solutions were prepared. It was found that no single change in composition could completely eliminate the vesiculation induction property of SCL. However, significant differences also existed in the pH, bicarbonate and phosphate concentrations, Ca\(^{2+}\)/Mg\(^{2+}\) ratio, and ionic strength. To ascertain which of these factors were responsible for stimulation of chondrocyte vesiculation, modified SCL solutions were prepared. It was found that no single change in composition could completely eliminate the vesiculation induction property of SCL. However, a significant reduction in the vesiculation ratio was observed when the bicarbonate concentration was lowered.

Moreover, when experiments were performed for long periods, the medium pH was depressed when low bicarbonate SCL or HBSS was used in place of unmodified SCL. To study this phenomenon further, chondrocyte vesiculation by SCL solutions, pH 5.0-6.3, and unmodified SCL, pH 7.45, was compared. The results of this experiment show that the pH of the medium at the start of the experiment strongly influences the extent of vesiculation (Fig. 5). Thus, if the initial pH is low, total alkaline phosphatase release was decreased, while the liberation of lactate dehydrogenase and cell death were increased. An 8-10-fold increase in the vesiculation ratio was observed when the initial pH was varied from 5.0-7.45. Total alkaline phosphatase activity (0.7 nmol/min/10^6 cells) was not affected by pH, while the percentage of the total enzyme released varied from 9 to 16% and was correlated with the vesiculation ratio. Lactate dehydrogenase release was 36% at pH 5 and decreased to 5% in normal SCL.

Effect of Pharmacological Agents on Vesiculation—Earlier studies implicated mitochondrial activity, ion transport, and steroid and peptide hormones in the calcification process (22, 23). The interaction of the respiratory chain with vesiculation was studied using inhibitors of oxidative phosphorylation. In the presence of respiratory inhibitors (antimycin A) or uncouplers (2,4-dinitrophenol), specific vesiculation was inhibited (Table III). The effect of anoxia was also studied by culturing the cells in a sealed vessel containing an oxygen scavenger. As shown in Table III, anoxia also lead to a decrease in the alkaline phosphatase/lactate dehydrogenase ratio.

The relationship between transport and vesiculation was investigated utilizing monovalent (nigericin, monensin) and divalent cation (X537A) ionophores. Table III shows that low doses of ionophores did not affect vesiculation, while at higher doses there was cell death and massive release of both membraneous and cytoplasmic constituents (30-80% of total lactate dehydrogenase released). When cells were treated with the local anesthetic tetracaine, a calcium blocker, no effect on vesiculation was noted. Thus, specific involvement of ion transport in the vesiculation mechanism could not be documented. The high levels of alkaline phosphatase associated with high ionophore concentrations were clearly the result of cell death, and these data therefore support the use of the vesiculation ratio to assess specific vesicle release.

Treatment of osteoblastoma cells with dexamethasone has been reported to increase both total alkaline phosphatase activity and parathyroid hormone sensitivity (24). To determine whether corticosteroids modulate chondrocyte vesiculation, dexamethasone (0.02-1 μM) was added to the medium. No effect on either the total alkaline phosphatase activity or
the vesiculation ratio was noted. Furthermore, dibutryl-cAMP (1 mM) had no effect on alkaline phosphatase release from cultured chondrocytes. Finally, the consequences of protein synthesis inhibition on cell vesiculation were also studied. Cells treated with puromycin (2-5 mM) did not vesiculate, and, as might be expected, this treatment resulted in increased cell death.

**DISCUSSION**

This report describes a novel procedure for the production of vesicles from chondrocytes in vitro. These cell-derived particles resemble conventionally isolated MV. Thus, they are vesicular in shape and contain elevated levels of alkaline phosphatase, and they can load with calcium in the presence of MgATP. Furthermore, vesicle biogenesis is optimized by the use of a medium that has the same ionic characteristics as the extracellular fluid of growth plate cartilage. Finally, the vesicles are formed from viable cells, and interference with oxygen-dependent metabolic pathways seriously inhibits the vesiculation process. On the basis of their morphology, biogenesis, enzymatic composition, and calcium-loading activity, it is suggested that these particles are related to MV isolated from epiphyseal cartilage using the conventional collagenase digestion technique. Very recently, similar results have also been reported by Glaser and Conrad (25). These workers were able to produce alkaline phosphatase containing particles from cultured chondrocytes using serum-free media.

The observation that vesicles could load with calcium in vitro and could be sedimented by low centrifugal fields was used for the rapid isolation of particles and separation of "functional" from "nonfunctional" particles. Similar procedures have been utilized for the isolation of calcium-pumping vesicles from sarcoplasmic reticulum (26). The existence of alkaline phosphatase-containing vesicles which did not load with calcium and therefore did not sediment at low centrifugal force suggests that chondrocytes can be induced to release a heterogeneous group of membranous particles. This finding corroborates the observation by Trams et al. (21) who reported that particles with high ectoenzyme activity but of varying morphology can be released from vesiculating normal and tumor cells in culture. From a morphological and enzymatic viewpoint, these microvesicles and chondrocyte vesicles are very similar. In both cases, while the functional significance of these structures remains unknown, it is likely that they are formed by budding from the plasma membrane.

The mechanism by which the plasma membrane forms vesicles was not investigated. However, for vesiculation to occur, experiments with cultured cells indicate that there is minimal leakage of intracellular constituents, no loss of viability, and a requirement for normal cellular metabolism. Thus, supplementation of the defined medium with amino acids and glucose stimulated vesiculation while uncoupling of oxidative phosphorylation, anoxia, inhibition of protein synthesis, and cell death decreased cell-specific vesiculation. Experiments with ion transport effectors further emphasize the importance of normal metabolism in the vesiculation process. Thus, agents which could be expected to produce an increase in ion transport across the chondrocyte plasma membrane failed to stimulate vesiculation.

The efficacy of SCL in stimulating chondrocyte vesiculation is probably associated with the maintenance of cell viability and differentiation. Of the factors studied, the most important was considered to be the initial (high) pH of SCL and the elevated levels of phosphate and bicarbonate. These three factors serve to maintain the medium pH. Thus, SCL mimics the "ionic milieu of chondrocytes in vivo" and functions to enhance chondrocyte viability and thereby promotes cell vesiculation.

There is evidence to suggest that the oxygen tension in growth plate cartilage regulates extracellular mineralization by modulating the release of cell calcium and phosphate stores (22). The notion that changes in oxygen tension could also regulate the formation of MV has not previously been considered. If a similar mechanism controls MV formation in the growth plate, then vesiculation would be expected to occur in the normoxic resting and proliferative zones. In the hypertrophic zone, the combination of the low oxygen tension and the decrease in cellular pH, due to anaerobic glycolysis, would not only inhibit vesiculation, but would also hasten cell death. It is possible that this process would release both alkaline phosphatase and cell-derived membrane fragments that could also function as sites for mineral deposition. It remains to be determined what structural and functional differences exist between vesicles produced by viable cells and those produced as a result of cell death.

The high level of structural, temporal, and developmental organization of the growth plate requires complex cellular controls to provide the regulatory framework in which growth and calcification can proceed. Clearly, as MV provides the initial locus for mineral deposition, factors controlling their biogenesis must be carefully regulated. Until very recently, no system was available to study the mechanism and regulation of vesicle biosynthesis. The striking similarity between the vesicles produced by this new procedure and MV isolated from the growth plate suggests that this system may be a good model for the study of MV biogenesis in vivo.

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**TABLE III**

*Effects of modulators of cell metabolism and ion transport on vesiculation (expressed as the alkaline phosphatase/lactate dehydrogenase ratio)*

| Effectors        | Alkaline phosphatase/lactate dehydrogenase ± S.E. |
|------------------|--------------------------------------------------|
| Metabolic effectors |                                                |
| Control          | 6.43 ± 1.69                                     |
| 2.4-Dinitrophenol (0.5-2 mM) | 3.17 ± 0.56                                     |
| Anoxia           | 1.95 ± 0.25                                     |
| Antimycin A (30 μM) | 2.02 ± 1.21                                     |
| Dibutryl-cAMP (1 mM) | 6.3 ± 0.12                                      |
| Dexamethasone (0.02-1 μM) | 6.1 ± 0.29                                     |
| Ion transport effectors |                                                |
| Control          | 3.92 ± 0.07                                     |
| X537A (100 ng/ml) | 3.31 ± 0.07                                     |
| X537A (10 μg/ml)  | 0.3 ± 0.07                                      |
| Nigericin (100 ng/ml) | 1.33 ± 0.07                                    |
| Nigericin (10 μg/ml) | 0.24 ± 0.07                                    |
| Menadione (10-100 mM) | 3.25 ± 0.21                                    |
| Other effectors  |                                                |
| Control          | 6.43 ± 1.69                                     |
| Puromycin (2-5 mM) | 0.49 ± 0.21                                     |
| Tetracaine (0.01-1 μM) | 6.2 ± 1.6                                       |
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