Isolation and Characterization of Calcium-accumulating Matrix Vesicles from Chondrocytes of Chicken Epiphyseal Growth Plate Cartilage in Primary Culture*

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Matrix vesicles (MV) can be readily isolated from culture media of chicken growth plate hypertrophic chondrocytes grown in primary culture. The chondrocytes maintain normal morphology and synthesize type II collagen throughout the culture period. The culture-derived MV are morphologically indistinguishable from MV seen in situ and are rich in alkaline phosphatase. Formation of alkaline phosphatase-rich MV is strongly influenced by the stage of culture: large numbers are released shortly after cell seeding; marked decrease is seen during cell spreading and rapid cell division; notable resurgency in alkaline phosphatase-rich MV production occurs as the cells attain confluency. Increasing the initial chondrocyte seeding density proportionately increases MV production. Cells derived from the hypertrophic region are much more capable of forming alkaline phosphatase-rich MV than those from the proliferating zone, indicating that MV formation is dependent on cellular differentiation.

MV released by the cultured chondrocytes were compared in protein and phospholipid composition and in their ability to accumulate mineral ions, with plasma membrane fractions and collagenase-released MV obtained from the same tissue. Electrophoretic patterns of proteins, and the phospholipid profiles, suggest that significant modification of the plasma membrane occurs during MV formation. The vesicles are capable of accumulating large amounts of mineral ions from a metastable synthetic cartilage lymph when supplied with alkaline phosphatase substrates. This culture system thus appears to be a useful model for isolating native MV and characterizing factors required for vesicle formation and mineralization.

Matrix vesicles (MV) are known to be associated with the earliest detectable mineralization in cartilage (1, 2), intramembranous bone (3, 4), mantle dentin (5, 6), and alveolar bone (7, 8). Yet despite numerous studies of calcification induced by MV in vitro (9–13), there is as yet no concensus as to the mechanism by which the vesicles induce this important biological process. One of the fundamental problems which has hindered progress in this area has been the lack of suitable methods for MV isolation. While the original collagenase method (14) has been used in numerous studies on MV-induced calcification (9–11, 15), there has been a continuing question whether the nonspecific proteases present in crude collagenase (16) alter the vesicle proteins and thereby affect the results obtained (12, 17). Other methods of vesicle isolation which do not involve protein digestion have been used (12–13). While these preparations have the ability to rapidly induce mineralization, they generally suffer from lack of homogeneity. Furthermore, there have been notable differences between MV preparations in the requirements for organic phosphate substrates and in the kinetics of the mineralization.

One approach that offers promise for enabling the isolation of native-type MV is the use of cultured chondrocytes. There have now been two reports on the isolation of MV from primary cultures of chondrocytes (18, 19); however, because of differences in culture conditions, the mechanisms by which these culture-derived vesicles were formed, and whether the isolated MV were capable of inducing calcification are unknown. Although other cell culture studies have reported the presence of MV associated with the onset of mineralization (20, 21), we report here for the first time the isolation of native MV from cultured chondrocytes which possess the ability to accumulate large amounts of Ca$^{2+}$ and P in vitro and induce mineralization.

In this paper we describe the isolation of MV produced by primary cultures of hypertrophic chondrocytes, the characterization of their constituent proteins and phospholipids, some conditions which affect their elaboration by cells in culture, and initial results regarding their ability to accumulate mineral ions in vitro. Our studies indicate that release of alkaline phosphatase-rich MV by chondrocytes in culture is closely related to the attainment of cell confluency and/or the state of differentiation which leads to re-expression of alkaline phosphatase synthesis. Furthermore, they show that the vesicles so produced differ significantly in protein, but not phospholipid composition, from those released by crude collagenase digestion. Our findings indicate that there may be differences between vesicles produced early and those produced later in the post-confluent period of culture with respect to their ability to accumulate mineral ions. A preliminary report briefly describing some of these findings has been published (22).

**EXPERIMENTAL PROCEDURES**

*Materials*—Dulbecco’s Modified Eagle’s Medium (DMEM), with L-glutamine, sodium pyruvate, and glucose (1 g/liter), fetal bovine serum, and antibiotic-antimycotic were obtained from Gibco (Grand

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1 The abbreviations used are: MV, matrix vesicles; DMEM, Dulbecco’s modified Eagle’s medium; TBS, Tris [hydroxymethyl] methyl-2-amino-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CDMV, culture-derived matrix vesicles; SCL, synthetic cartilage lymph.
Island, NY). Trypsin (type III, 2 x crystallized from bovine pancreas), hyaluronidase (type VI-S), alkaline phosphatase substrate (p-nitrophenylphosphate), TES, and SDS-PAGE protein M, standards were obtained from Sigma. Collagenase (CLS II) was purchased from Worthington, and [32P]orthophosphoric acid (carrier-free), [35Ca]calcium chloride (NEZ-013, 1.6 Ci/mol), [methyl-1-2H]thymidine (67 Ci/mmol), and omnifluor were from New England Nuclear. All culture flasks (75 and 150 cm2) and 24-well dishes were obtained from Corning Glass Works (Corning, NY). All other chemicals used were of reagent grade and were supplied by Fisher.

Cell Culture—Chondrocytes were isolated from the hypertrophic region of epiphyseal growth plate cartilage of the tibia of 8–10-week-old hybrid broiler-strain chickens (Columbia Farma, West Columbia, SC) as previously reported (22–24). Cell viability was determined by trypan blue exclusion and counting with a hemocytometer. Chondrocytes were generally seeded at a density of 2.7 x 10^6 cells/cm² of culture surface area. The cultures were supplied with DMEM, fetal bovine serum (10%, v/v), penicillin/streptomycin (10,000 units and 10 μg/ml, respectively; 2%, v/v) and antimycotic (Fungisone [25 μg/ml]); 1%, v/v), the total volume being 0.15–0.20 ml/cm² of culture surface area. Cultures were maintained in an incubator at 37°C with a 5% air, 5% CO2 atmosphere. The culture medium was changed every 3 days for the duration of the experiments.

[3H]Thymidine Incorporation—To measure the rate of cell division, [3H]thymidine was added to the culture media at an activity of 70% ethanolic. 5 min. The fixed cells were washed with 1% sucrose in phosphate-buffered (pH 7.4) glutaraldehyde before being embedded in Spurr standard medium (27). Sections were stained with uranyl acetate and lead nitrate and examined under a Phillips EM-300 electron microscope.

Phospholipids—Chondrocyte cell cultures were initiated as outlined previously. From the 3rd day of culture forward, 10 μCi of [32P]orthophosphate/5 ml of DMEM was added at each successive feeding. Phospholipids were extracted from the isolated chondrocytes and MV as previously described (28). Briefly, samples (with added carrier phospholipids) were extracted with chloroform/methanol (2:1, v/v) twice according to the procedure of Folch et al. (29). The phospholipid composition of the lipid fraction was analyzed by two-dimensional chromatography on Whatman SG-81 silica gel-loaded paper (30). The radioactivity of the lipid-bearing areas from the chromatograms was measured by liquid scintillation counting. For the cell and plasma membrane fractions (to which no carrier lipid was added) the amount of phospholipid in each class was determined by P₃ analysis of the lipid-bearing areas (31) after digestion with 70% perchloric acid.

Electrophoresis of Membrane Proteins—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of MV proteins was performed using 10% polyacrylamide slab gels (130 x 200 x 1.5 mm), essentially as described by O’Farrell (32). The gels were stained for protein either by the Coomassie Blue method of Weber and Osborn (33), or with the silver method of Wray et al. (35). SDS-PAGE protein molecular mass standards were: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa). In some cases, the Coomassie Blue stained gels were scanned using a Zenith soft laser scanning densitometer.

Ion Uptake by Matrix Vesicles—Uptake of 4Ca by the carrier–
derived MV was as described previously (13), except that the assay
Formation of Matrix Vesicles in Culture

Fig. 2. Effect of initial cell seeding density on the production of alkaline phosphatase-rich MV by hypertrophic chondrocytes in culture. MV were harvested by differential ultracentrifugation of the spent culture medium (see “Experimental Procedures”). The flask surface area was 75 cm²; the seeding densities tested were: 1.7 × 10⁴ (○), 3.3 × 10⁴ (△), 6.6 × 10⁴ (△), 1.3 × 10⁵ (○), and 2.7 × 10⁵ (●) cells/cm². Arrow heads mark the time at which the cells became confluent. Note the markedly higher and more rapid release of alkaline phosphatase-rich MV at the higher seeding densities. pNPP, p-nitrophenylphosphate.

Fig. 3. Relationship between cumulative production of alkaline phosphatase-rich MV and the length of time in culture by chondrocytes grown in DMEM + 10% fetal bovine serum in cell culture. Cells were seeded at 2.7 × 10⁴ cells/cm². Inset, rate of MV production after each successive cell feeding. Values presented are the mean ± S.E. of seven successive cultures. Note the reproducible cyclic pattern in the rate of MV production. The marked decline in rate of MV formation which occurred shortly after seeding (days 4–9) corresponds to the time of cell attachment, spreading, and early rapid cell division. pNPP, p-nitrophenylphosphate.

RESULTS

Hypertrophic chondrocytes released from chicken epiphyseal growth plate cartilage attached to the culture vessels within 24 h of plating, began to flatten out and undergo mitosis, and usually attained confluency between days 10 and 15 of culture (Fig. 1). Approaching confluence, the rate of alkaline phosphatase-rich MV production increased, rapid formation of MV normally occurring for about 10 days (Fig. 3, inset). Beyond this point, the rate of MV production often declined, although some lots of serum cultures maintained high rates of MV production for as long as 35–40 days.

Effects of Seeding Density—The initial cell-seeding density had a profound effect on MV production (Fig. 2), optimal seeding density being about 3 × 10⁴ cells/cm² of growing area. At lower seeding densities, the time to confluency was considerably lengthened, and the rate of MV production was measurably reduced. When cultures were seeded at much higher densities (6 × 10⁴–1.5 × 10⁵ cells/cm²) there was no significant increase in the release of alkaline phosphatase-rich MV into the culture medium (data not shown).

Time Course of Matrix Vesicle Production—Fig. 3 shows the mean production of MV in seven successive primary cell cultures using a single lot of fetal bovine serum. Individual lots of fetal bovine serum varied in their ability to support cell growth and MV production. After a brief burst in output of alkaline phosphatase-rich vesicles immediately after seeding the cells, MV production was minimal for several days. As the cultures approached confluency, the rate of alkaline phosphatase-rich MV production increased, rapid formation of MV normally occurring for about 10 days (Fig. 3, inset). Beyond this point, the rate of MV production often declined, although some lots of serum cultures maintained high rates of MV production for as long as 35–40 days.

Relationship between Cell Division, Cellular Alkaline Phosphatase, and Matrix Vesicle Production—Studies showed that there was a parallel relationship between the cumulative
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FIG. 5. Transmission electron micrographs of MV harvested at different stages of cell culture. Cells were grown as described in the Legend to Fig. 3 and samples of spent medium were subjected to differential centrifugation to sediment the released MV after treatment of the medium with highly purified hyaluronidase to disaggregate proteoglycans (see "Experimental Procedures"). The MV pellets were washed with phosphate buffer, fixed in isosmotic glutaraldehyde and OsO₄, and processed for transmission electron microscopy as described under "Experimental Procedures." MV released by collagenase digestion during chondrocyte isolation (a) and CDMV harvested at day 2 (b), day 12 (c), and day 37 (d) of the culture period are shown. Note the decrease in size and density of most MV isolated on day 12 (c) when rates of MV-alkaline phosphatase production were low (Fig. 4), and the reappearance of large numbers of electron-dense MV late in the culture (d) when alkaline phosphatase levels were high. The alkaline phosphatase-rich CDMV (b and d) are similar in morphology to those isolated from fresh tissue (a). (Bars equal 1.0 μm.)

incorporation of [³H]thymidine, cellular levels of alkaline phosphatase, and release of alkaline phosphatase-rich MV into the culture medium (Fig. 4). The levels of cellular and MV alkaline phosphatase were low for the first 12 days of culture, but during the subsequent 12-day period in which an almost constant rate of [³H]thymidine incorporation occurred, cellular alkaline phosphatase activity increased progressively (Fig. 4, inset). Under these experimental conditions, release of alkaline phosphatase-rich MV was closely correlated with cellular alkaline phosphatase levels (r = 0.85, n = 9). The peak rate of MV production occurred just before the time when the rate of [³H]thymidine incorporation began to decline.

Transmission Electron Microscopy of Culture-derived Matrix Vesicles (CDMV)—Fig. 5 compares vesicles released by the collagenase method (a) with those produced by the chondrocytes at days 2 (b), 12 (c), and 37 (d) in culture. Vesicles released into the medium by the cultured chondrocytes at different stages of the culture were generally similar in appearance to those seen in vivo or after release by collagenase digestion. The CDMV contained electron-dense amorphous material, a feature seen most frequently early (day 2) and late (day 37) in the culture period at a time when alkaline phosphatase levels were high. Midway in culture, before major production of alkaline phosphatase-rich MV reappeared (see Figs. 3 and 4), isolated CDMV were smaller and less electron-dense (Fig. 5c) than those seen earlier or later in the culture period. Note that a number of the CDMV obtained on day 2 were distorted and contained mineral crystallites (Fig. 5b).

SDS-PAGE Protein Profiles of Culture-derived Matrix Vesicles—The protein composition of sucrose gradient-purified CDMV and collagenase-released MV, and tissue-derived and culture-derived chondrocyte plasma membrane fractions were analyzed by SDS-PAGE stained with Coomassie Blue and analyzed using a soft laser scanning densitometer (Fig. 6). (Two other methods of visualization of proteins from the various vesicle fractions were also used: silver staining (35) and autoradiography of ¹⁴C-amino acid-labeled proteins. These methods gave very similar protein profiles, data not shown.) Although the protein profiles of CDMV and collagenase-released MV had similarities (e.g. major bands at 37, 44, and 55 kDa, CDMV had larger amounts of high-molecular mass and smaller amounts of low-molecular mass proteins. The major protein bands of CDMV (Fig. 6a) were found at 67, 100, 73, 55, and 44 kDa (in that order) and lesser bands at 37, 34, 31, and 26 kDa; collagenase-released MV (Fig. 6d) had major bands at 48, 33, 35, 55, and 44 kDa (in that order) and lesser bands at 37, 50, 100, 57, and 73 kDa. Only minor differences existed between the protein patterns of cultured chondrocyte-derived plasma membrane fractions (Fig. 6c) and those obtained from plasma membrane fractions isolated from fresh tissue microsomes (Fig. 6e). Both plasma membrane
the reappearance of alkaline phosphatase activity in the Matrix Vesicles—By using $^{32}P$ orthophosphate supplied continuously to the culture medium at defined specific activity, the phospholipid composition of the CDMV (Table I) was similar to that previously observed for collagenase-released MV (28, 38) and that reported by Glaser and Conrad (18) for CDMV. There was, however, a noticeably lower level of phosphatidylcholine, higher sphingomyelin, and less evidence of phospholipase A-degraded lipids. The principal differences between the CDMV and the plasma membrane fraction were a decreased proportion of phosphatidylcholine (45% less), increased sphingomyelin (220% more), and increased phosphatidylserine (260% more), confirming that a major change in phospholipid composition of the plasma membrane accompanies the formation of MV (39).

$^{45}Ca$ and $^{32}P$, Metabolism by Culture-derived Matrix Vesicles—Initial studies revealed that MV produced by preconfluent cultured cells were capable of accumulating small amounts of $^{45}Ca$ from SCL (Fig. 8, inset). No organic phosphate substrate was needed for this process; however, rate of uptake was slower than that observed with homogenization-derived MV-enriched microsomal fractions (13). Further studies with SCL-washed MV derived from post-confluent cultures showed that these MV were also capable of accumulating large amounts of $^{45}Ca$ and $^{32}P$, from the SCL. However, here, addition of an alkaline phosphatase substrate (1 mM ATP, Fig. 7) or 2 mM AMP (data not shown) to the $^{45}Ca$ and $^{32}P$-labeled SCL was needed for mineral ion accumulation.

**DISCUSSION**

Chondrocytes isolated from the hypertrophic zone of the growth plate of 8–10-week-old broiler-strain chickens, grown in DMEM supplemented with 10% fetal bovine serum, synthesized type II, not type I collagen (data not shown), produced abundant matrix proteoglycans, and released phospholipid-, protein-, and alkaline phosphatase-rich MV into the culture medium. Our studies indicate that production of alkaline phosphatase-rich MV is a function of cellular differentiation. Chondrocytes isolated from the proliferating and hypertrophic regions of the growth plate differed significantly in their ability to produce alkaline phosphatase-rich MV; those from the proliferating region released fewer MV, even after extended culture, than those isolated from the hypertrophic region. These findings confirm and extend previous studies by Glaser and Conrad (18) who showed that embryonic chick chondrocytes released alkaline phosphatase-rich MV into the culture medium. These studies are fundamentally different, however, from those of Golub et al. (19), who manipulated the medium in which the freshly isolated chondrocytes were placed so as to induce release of vesicles.

Production of MV by hypertrophic chondrocytes varied markedly depending on the stage of culture: immediately after isolation from the tissue, the cells released large amounts of alkaline phosphatase-rich MV, but as they attached to the culture flask, flattened and began to divide, alkaline phosphatase production rapidly declined to minimal levels. Production of alkaline phosphatase and release of alkaline phosphatase-rich MV into the culture medium, which were closely correlated, began to increase only after the cells had attained confluency. However, production of alkaline phosphatase-rich MV was not simply due to cellular degeneration. Fig. 4 (inset) shows that the rate of MV formation increased steadily during an extended period when cell division occurred at a nearly constant rate. Further, it is important to note that the growth plate chondrocytes did not exhibit classic contact inhibition,
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**FIG. 7.** Incorporation of $^{32}$P-orthophosphate into MV phospholipids by cultured hypertrophic chondrocytes. For details, see "Experimental Procedures." Closed symbols, cumulative synthesis of MV phospholipids; inset, rate of MV phospholipid synthesis at successive stages of cell culture (open symbols). Sphingomyelin ($\Delta, \Lambda$), phosphatidylincholine ($\square, \bigcirc$), phosphatidylethanolamine ($\downarrow, \circlearrowleft$), and phosphatidylserine ($\uparrow, \bigcirc$) are shown. Note that $^{32}$P labeling of CDMV phospholipids closely paralleled the appearance of alkaline phosphatase activity ($\bigcirc$) in the CDMV fraction. $pNPP$, p-nitrophenylphosphate.

**TABLE I**

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* Mean value differs significantly from the matrix vesicle values reported earlier (28, 38); $p \leq 0.01$.

* Mean value differs significantly from chondrocyte plasma membrane value reported earlier (12); $p \leq 0.01$.

but in some areas began to form nodular multilayers (Fig. 1). Again note that cell numbers continue to increase after the attainment of confluency (Fig. 4). These findings, coupled with the finding that MV production was affected by initial cell-seeding density, suggest that maintenance of alkaline phosphatase phenotypic expression and release of MV is dependent on tissue-derived factors and/or the effects of cell-cell contact. Studies in progress indicate that both serum and tissue-derived growth factors are essential for this type of phenotypic expression.

A significant question here is whether the MV released by the cultured chondrocytes are analogous to those observed in situ in the growth plate. From a variety of criteria this appears to be so. First, the ultramicroscopic morphology of the CDMV is very similar to that seen in tissue MV, or in MV isolated by crude collagenase digestion (Fig. 5), (14, 28). Second, the CDMV show the same type of phospholipid compositional relationship to the plasma membrane of chondrocytes as that seen in MV isolated directly from the fresh tissue (28, 38). They possess the well-established enrichment in sphingomyelin and phosphatidylserine, and depletion of phosphatidylcholine (Table 1) reported in several earlier studies (18, 28, 38). Third, the CDMV are enriched in alkaline phosphatase, a classic MV marker enzyme (14, 23). Fourth, they possess the ability to accumulate large amounts of $^{45}$Ca and $^{32}$P in vitro using the metastable SCL. Finally, they possess a distinctive

*SPH, sphingomyelin; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol; DPG, diphasphatidylglycerol. Values are the mean ± S.E. percentages of the total lipid P; the number of samples analyzed is given at right in parentheses.*
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**FIG. 8.** Time course of \(^{45}\text{Ca}\) and \(^{32}\text{P}\) uptake from SCL by MV collected during the post-confluent period of chondrocyte culture. \(^{45}\text{Ca}\) uptake, \(\bullet\), \(^{32}\text{P}\) uptake, \(\square\) + ATP. \(\odot\), \(\odot\) – ATP. \(\bullet\), \(\odot\) – ATP. The biphasic uptake curve of mineral ions is typical of MV-induced mineralization (11, 13). Net ion accumulation by the CDMV at late stages of incubation had \(^{45}\text{Ca},^{32}\text{P}\) uptake ratios of 1.55, values indicative of formation of \(^{45}\text{Ca}-\text{deficient hydroxyapatite.}

Note that ATP markedly stimulated uptake of both mineral ions. Inset, time course of \(^{45}\text{Ca}\) uptake from SCL by MV harvested early in the post-confluent period of culture. \(\text{Ca}^{2+}\) uptake, which occurred in the absence of added organic phosphate substrates to the SCL, although limited and relatively slow, was nevertheless much more than seen with post-confluent CDMV in the absence of alkaline phosphate substrates.

and reproducible protein electrophoretic profile which, although related to that of the chondrocyte plasma membrane, appears to be unique to MV. (This finding is evidence against the idea that MV are simply products of cellular degeneration.)

These studies also help resolve a long-standing question regarding the intrinsic protein constituents of MV. Vesicles isolated by crude collagenase digestion are subject to degradation of the exposed surface proteins because of the nonspecific protease constituents present (36). On the other hand, MV fractions isolated by nonenzymatic methods which rely on homogenization and fractionation (12) are heterogeneous because of the presence of intracellular membrane components. The CDMV reported here overcome several important criticisms of these other studies. First, the CDMV are isolated by the mildest of procedures: differential centrifugation of the vesicle-containing culture medium. Second, because no proteolytic digestion or homogenization is involved, the CDMV should be in a virtually native state. The fact that the CDMV are capable of accumulating large amounts of \(^{45}\text{Ca}\) and \(^{32}\text{P}\) suggests that this is indeed true. Thus, it is significant that CDMV (Fig. 6b) differ significantly in protein composition from MV isolated by crude collagenase digestion (Fig. 6d) or from plasma membrane fractions obtained by homogenization and sucrose gradient fractionation methods (Fig. 6, c and e). The presence of several characteristic intense higher-molecular mass (67, 73, and 100 kDa) protein bands which are nearly absent from collagenase-released MV indicates that some proteolytic degradation of MV proteins must occur during crude collagenase digestion. Nevertheless, it should be pointed out that MV isolated from different species may differ in sensitivity to protease degradation. For example, MV derived from rat alveolar bone (39) and cartilage (39–40), and fetal bovine cartilage (40), by collagenase digestion contain significantly more high-molecular mass protein than the collagenase-released chicken MV reported here.

The absence, or significantly lower levels of certain high-molecular mass, and the enrichment of several lower-molecular mass (e.g., 33, 35, and 37 kDa) bands in plasma membrane fractions obtained from both cultured and native chondrocytes indicates that modification of the protein, as well as the phospholipid constituents occurs in the plasma membrane at sites of MV formation. Since the protein profiles of the native (Fig. 6e) and cultured chondrocyte (Fig. 6c) plasma membrane fractions were very similar, it is unlikely that the differences seen between collagenase-released MV and CDMV resulted from changes in gene expression by the cultured cells.

Comment needs to be made regarding the uptake of \(^{45}\text{Ca}\) and \(^{32}\text{P}\), by the CDMV. The levels of mineral ions accumulated by the CDMV (10.0 μmol of \(^{45}\text{Ca}\) and 6.3 μmol of \(^{32}\text{P}\) of protein) were far higher than those observed by Glaser and Conrad (18), amounts that exceed the ability of transport systems to accumulate in the absence of precipitate formation. These levels of uptake are similar to those previously observed with MV-enriched microsomes (13) in which it was established by x-ray diffraction that apatite formation had occurred. That apatite formation did occur during CDMV incubation is supported by the fact that these high levels of \(^{45}\text{Ca}\) and \(^{32}\text{P}\), uptake occurred at ratios consistent with apatite formation. Thus, the data indicate that the CDMV were capable of inducing mineral formation within a period of about 14 h, when provided with appropriate alkaline phosphate substrates.

Furthermore, the finding that the ability of CDMV to accumulate mineral ions varies depending upon the state of culture from which they were produced may provide insight...

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\(^{5}\) The hyaluronidase used is a highly purified endoglycosidase from bovine testis which was used to degrade the polysaccharide chains of the proteoglycans abundantly produced by the cultured chondrocytes and released into the culture medium.
into the nature of MV-induced calcification. For example, it may explain some apparent discrepancies between data obtained by various groups relative to the involvement of organic phosphate substrates in MV-mediated calcification. Initial studies by Ali and Evans (9), by Hsu and Anderson (10), and by Väähänéen (15) indicated that ATP was stimulatory, if not obligatory for MV calcification. On the other hand, our earlier studies with nonenzymatically released MV (12, 13), and studies by Felix et al. (11) with collagenase-released MV, suggest that preformed labile mineral within MV is a key component for the mineralizing activity of such preparations. Here we find evidence for the production of both types of vesicles by the cultured cells: those not requiring organic phosphate substrates, isolated early after release of cells from the native tissue; and those dependent upon phosphate substrates, produced by cells during the post-confluent period of culture. It is evident that MV isolated early in the culture period contained mineral ions carried over by the freshly isolated cells. In fact, the early CDMV (Fig. 5b) show the presence of numerous MV containing mineral crystallites. On the other hand, it is highly unlikely that the mineral seen in these early CDMV was present extracellularly at the time of cell isolation. This is based on the fact that, before seeding, the cell pellets were washed with pH 6 buffer to remove residual mineral. Thus, it is possible that different types of MV exist in cartilage tissue, and that they may be capable of inducing calcification by different mechanisms.

REFERENCES