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# Calcification of chick vertebral chondrocytes grown in agarose gels: a biochemical and ultrastructural study

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## SUMMARY

**Chick embryo vertebral chondrocytes (CHECOV cells) grown in agarose gels form spherical colonies containing cells of hypertrophic morphology and a metachromatically staining matrix. Biochemical analysis of these cultures resulted in the following findings. (i) Calcification of CHECOV cultures can be induced by addition of $P_i$ (at least 1.9 mM) or β-glycerol phosphate (BGP). (ii) Alkaline phosphatase activity reaches a maximal value at the time when mineral deposition is initiated. (iii) Added BGP is converted to $P_i$; maximal production of $P_i$ occurs at the time of maximal alkaline phosphatase activity. (iv) BGP-supplemented cultures produce a degree of calcification that corresponds to the amount of BGP conversion to $P_i$.**

**It can be concluded that $P_i$ is rate-limiting for the calcification of chondrocyte cultures. BGP promotes calcification of these cultures by acting as a substrate for the alkaline phosphatase-mediated production of inorganic phosphate.**

Key words: agarose, alkaline phosphatase, calcification, chondrocytes

## INTRODUCTION

The chondrocytes of the epiphyseal growth plate and other sites of endochondral ossification undergo a sequence of differentiation events characterized by proliferation, matrix synthesis and hypertrophy. The final stage of this sequence is calcification, which occurs by deposition of hydroxyapatite (HA) in the extracellular matrix of the longitudinal septa. The principal components of this matrix are proteoglycan aggregates, types II and X collagen, and the C-propeptide of type II collagen (chondrocalcin).

The means by which formation of HA is initiated in calcifying cartilage remains unknown. Indeed, there is no consensus on even the general principle involved. Some workers have suggested that calcification requires the presence of a nucleating agent (Bonucci, 1987; Irving and Wuthier, 1968; Poole et al., 1984; Schmid and Linsenmayer, 1985). Others have suggested that removal or modification of an inhibitor would be sufficient; the main candidate for this role is proteoglycan (Buckwalter, 1983). A third school of thought holds that calcification is initiated by a local increase in calcium phosphate supersaturation, perhaps mediated by matrix vesicles (Wuthier, 1982) or by displacement of calcium bound to proteoglycan (Hunter, 1987). Also to be accounted for is a consistent correlation between alkaline phosphatase activity and the calcification of cartilage and bone (Robison, 1923).

Testing hypotheses of cartilage calcification has proven difficult. The proximity of bone to calcifying cartilage complicates ultrastructural studies, and conventional tissue-processing techniques result in demineralization and loss of proteoglycan (Hunziker and Schenk, 1984). Biochemical analyses of calcifying cartilage are hampered by contamination with metaphyseal bone and the presence of multiple "zones" within small volumes of tissue.

Cell culture systems offer a potential solution to these problems. Contamination of cartilage samples with bone is avoided, and, as chondrocyte cultures undergo a temporal rather than spatial differentiation sequence, the presence of cells at different stages of differentiation is minimized. Although it is difficult to maintain the differentiated phenotype even of articular chondrocytes, several systems in which chondrocyte calcification reproducibly occurs have recently been described. From these studies, it appears that calcification of chondrocyte cultures is stimulated by serum (Kato et al., 1988), substratum-independent growth (Tacchetti et al., 1989), ascorbate (Leboy et al., 1989; Wu et al., 1989) and 24,25-dihydroxy vitamin D (Hinek et al., 1987). Calcification is inhibited by transforming growth factor β and bromodeoxyuridine (Kato et al., 1988). β-Glycerol phosphate (BGP) is used as a medium supplement in some calcifying chondrocyte culture systems (Hinek et al., 1987; Tacchetti et al., 1989), but not in others (Bruckner et al., 1989; Kato et al., 1988; Wu et al., 1989). In a number of systems where its effect has been studied, BGP has been shown to stimulate calcification (Gerstenfeld and Landis, 1991; Leboy et al., 1989; Thomas et al., 1990).

None of these studies, however, has addressed the ques-

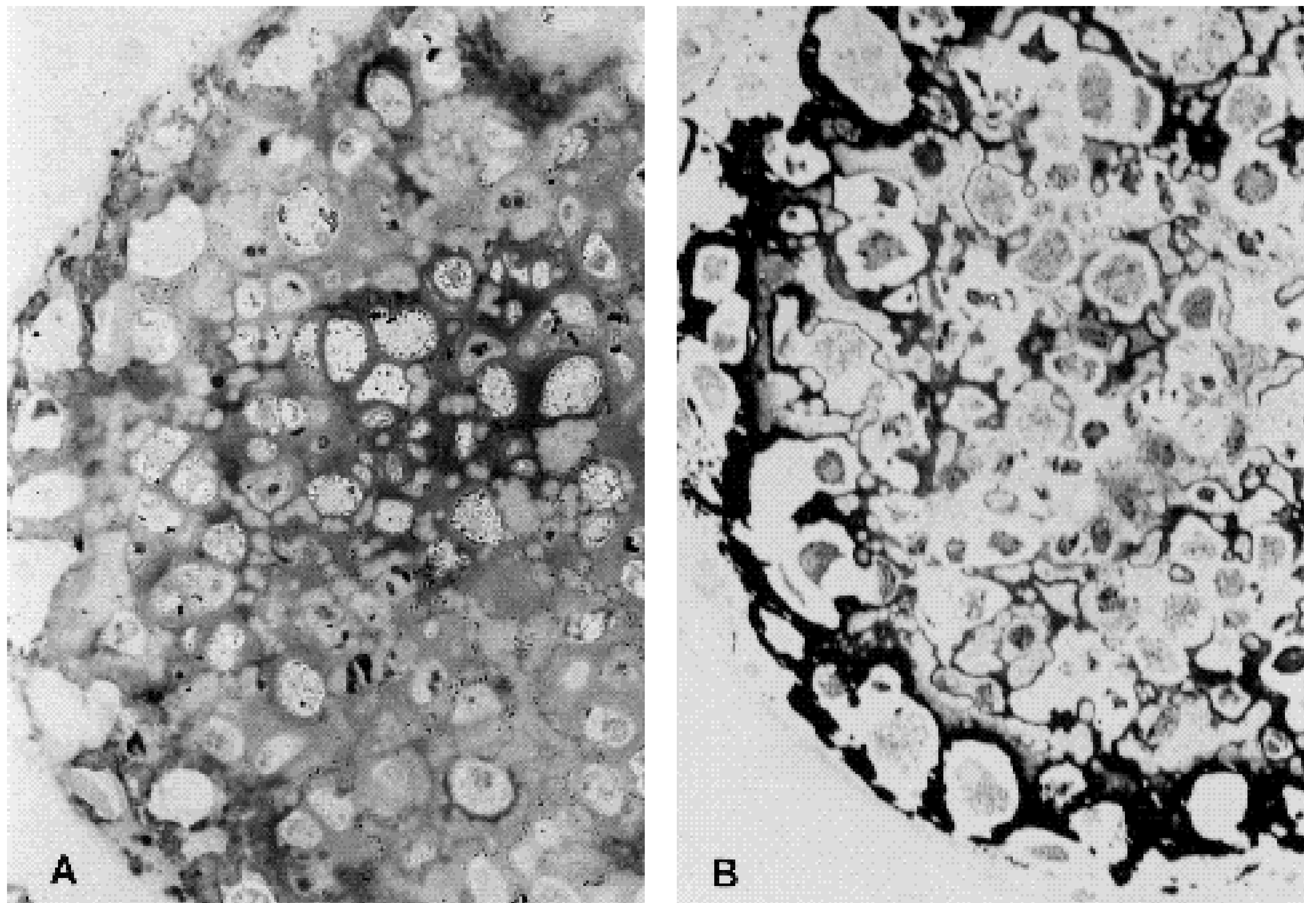


**Fig. 1.** Light microscopic observations of 16-day CHECOV culture grown with BGP. (A) Toluidine blue staining. ×400. (B) Von Kossa staining. ×400.

tion of the mechanism by which calcification is initiated in chondrocyte cultures. In the present study, a system is described in which calcification occurs in CHECOV (chick embryo chondrocyte of vertebra) cells grown in agarose gels. Biochemical and ultrastructural analyses of these cultures have been performed, and the roles of organic and inorganic phosphates in their calcification investigated.

## MATERIALS AND METHODS

### Materials

Collagenase (CLS-II) was obtained from Miles Laboratories. Trypsin (type II, from porcine pancreas), DNA (type I, from calf thymus) and *p*-nitrophenylphosphate were obtained from the Sigma Chemical Company. Dulbecco's modified Eagle's medium (DMEM; lyophilized powder), minimal essential medium (MEM) without phosphate and antibiotic-antimycotic solution (100× concentrate: 10,000 i.u./ml penicillin G, 10,000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B) were obtained from Gibco. Seaplaque agarose was obtained from Mandel Scientific. Bisbenzimide (Hoechst H33258) was obtained from Terochem Laboratories.

### Establishment of cultures

The following cell culture media were used. "2 × DMEM" was twice concentrated Dulbecco's modified Eagle's medium containing 25 mM HEPES. "Basic DMEM" was Dulbecco's minimal essential medium containing 25 mM HEPES plus 10% fetal calf serum and antibiotics. "DMEM with BGP" and "DMEM without BGP" were basic DMEM containing 50 μg/ml ascorbic acid with and without 5 mM β-glycerol phosphate, respectively.

The culture system used is a modification of the method described by Gerstenfeld and Landis (1991). Chick embryos (12-day-old) were decapitated, and the lower half of the vertebral column dissected free of soft tissue. The notochord was then removed by squeezing with flat-ended forceps. The cartilage was incubated in 10 mg/ml collagenase and 0. 3 mg/ml trypsin in basic DMEM for 30 min at 37°C to remove adherent fibroblasts. The tissue was then minced with fine scissors, and incubated in another aliquot of enzyme solution for 2 h. Following the second incubation, cells were pelleted by centrifugation, resuspended in basic DMEM, and counted using a hemocytometer.

Agarose was dissolved at 2% in water by heating at 100°C, and allowed to cool to 37°C. An equal volume of 2 × DMEM was then added to make a solution of 1% agarose in DMEM. The latter solution was then added to an equal volume of basic DMEM, and 1 ml aliquots used to coat 60 mm diameter plastic Petri dishes. The cell suspension was diluted to $10^6$ per ml in basic DMEM, and added to an equal volume of 1% agarose in DMEM. A 2 ml aliquot of this final suspension was then added to coated agarose dishes, and gels allowed to set at 4°C for 30 min. The final cell concentration, therefore, was $10^6$ cells per dish.

A 3 ml aliquot of DMEM with or without BGP was added, and

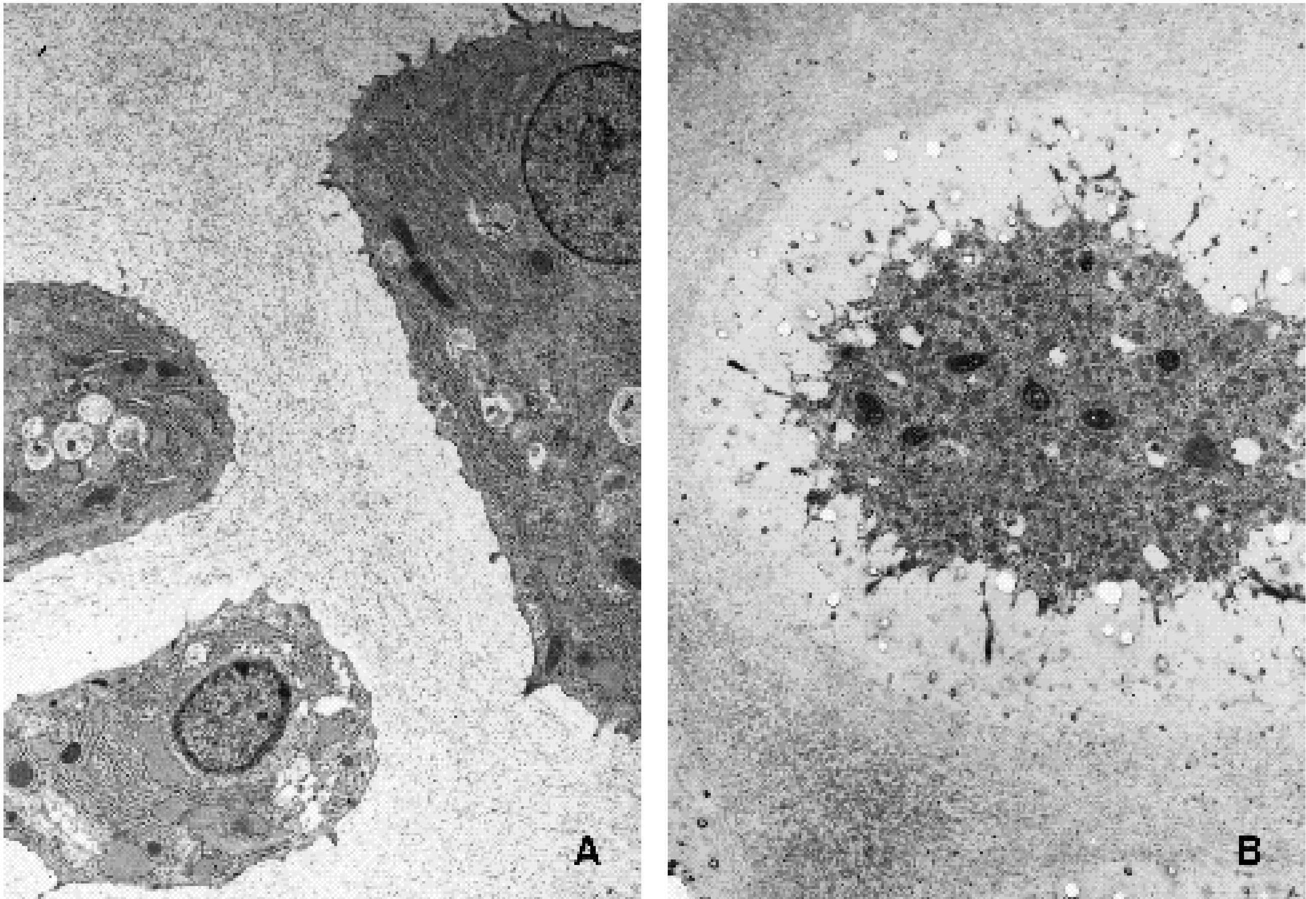


**Fig. 2.** Transmission electron microscopy of CHECOV cultures grown without BGP. (A) Day 7 culture. Note the abundant endoplasmic reticulum and intracellular granules. ×5,700. (B) Day 16 culture. Note the numerous mitochondria and cell processes, and the presence of a well-defined boundary between the territorial and inter-territorial matrices. ×6500.

cultures incubated at 37°C in a 5% $CO_2$ atmosphere. Medium was changed three times weekly, with freshly prepared ascorbic acid used for each medium change.

## Preparation of samples for light and electron microscopy

Pieces of agarose containing individual colonies were fixed in 2% glutaraldehyde/0.1 M sodium cacodylate, pH 7.3, and postfixed in 1% osmium tetroxide/0.1 M sodium cacodylate, pH 7.3. Following dehydration with a graded ethanol series and propylene oxide, samples were embedded in Spurr epoxy resin. For light microscopy, 1 μm sections were stained with either toluidine blue or Von Kossa. For electron microscopy, 70 nm sections were stained with 2% uranyl acetate and 1% lead citrate and viewed with a Philips 430 transmission electron microscope. Energy-dispersive X-ray spectroscopy was performed using a Link AN 10000 microanalysis system.

## Biochemical analyses

All procedures were performed at 4°C unless otherwise specified. At various times after establishment, cultures grown with or without BGP were extracted with detergent as follows. The medium was discarded, and gels washed three times with 5 ml of 150 mM NaCl/50 mM Tris-HCl, pH 7.4, by gentle shaking for 15 min at room temperature to remove residual medium. Washed gels were added to 3 ml of 0.2% Triton X-100/150 mM NaCl/50 mM Tris-HCl, pH 7.4, and sonicated for 30 s. Following centrifugation at 30,000 ***g*** for 10 min, supernatants were retained, and pellets washed with 3 ml of 0.1% Triton X-100 containing NaCl and Tris as above. First and second supernatants were pooled (Triton extract), and frozen for later analysis. Pellets were added to 2 ml of 0.5 M HC1, and mixed at room temperature for 6 h or overnight. Samples were then centrifuged as above, pellets washed with 2 ml of distilled water ($dH_2O$), and the supernatants pooled (acid extract).

Triton extracts were used for analysis of alkaline phosphatase activity as follows. 1 ml of sample was added to 1 ml of a solution of 5 mM *p*-nitrophenylphosphate/1 mM $MgCl_2$/0.84 M 2-amino-2-methyl-1-propanol, pH 10.2, and incubated at 37°C for 15 min. A 5 ml sample of 0.05 M NaOH was added to stop the reaction, and the absorbance was read at 405 nm. Acid extracts were used for analysis of phosphate by the method of Chen et al. (1956) and calcium by atomic absorption spectroscopy.

Separate cultures were used for DNA measurement by the method of Labarca and Paigen (1980). Gels were suspended in 3 ml of 4 M NaCl/0.1 M sodium phosphate, pH 7.4, and sonicated for 30 s. Following centrifugation at 30,000 ***g*** for 10 min, supernatants were retained, and pellets washed with 3 ml of 2 M NaCl/0.05 M sodium phosphate, pH 7.4. First and second supernatants were pooled, and frozen until further analysis. On thawing, 1 ml aliquots of extracts were added to 0.1 ml of reagent solution (11 μg/ml bisbenzimide in 2 M NaCl/0.05 M sodium phosphate, pH 7.4), and mixed. Fluorescence was measured with a Perkin-Elmer LS-5 spectrophotometer using an excitation wavelength of 356 nm and an emission wavelength of 458 nm.

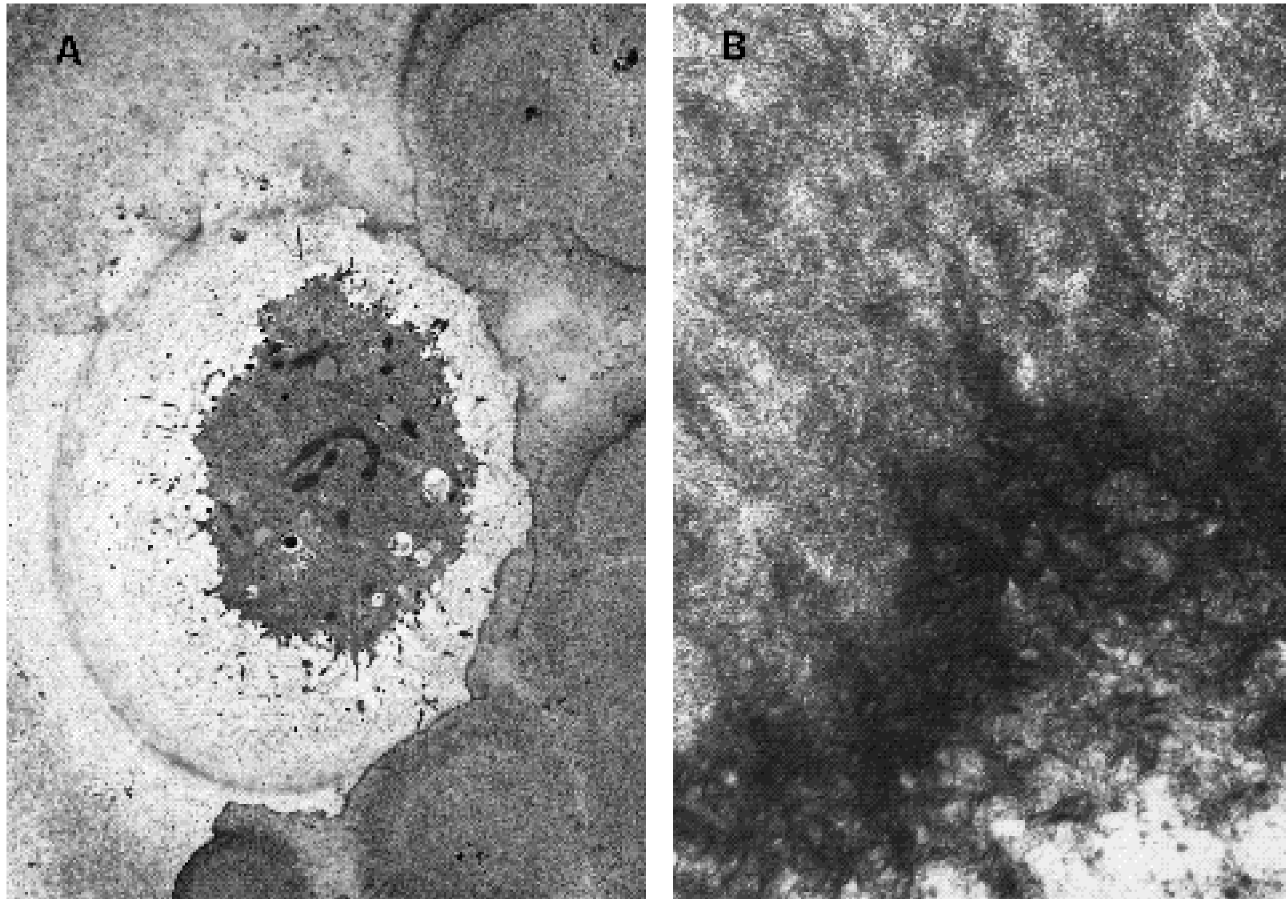


**Fig. 3.** Transmission electron microscopy of 16-day CHECOV culture grown with BGP. (A) Chondrocyte partially surrounded by mineralized matrix; compare with cell shown in Fig. 2B. ×5,700. (B) Mineral deposits at the boundary between the territorial and inter-territorial matrices. ×62,000.

## RESULTS

### Ultrastructural studies

Colonies from CHECOV cultures were processed for light and transmission electron microscopy using conventional (aqueous) techniques. Light microscopy images of 16-day cultures grown with BGP are shown in Fig. 1. The CHECOV colonies are spherical in shape. The cells resemble a section through the hypertrophic zone of epiphyseal growth plate, with no apparent stratification within the colonies (Fig. 1A). Using von Kossa staining, it was shown that calcification occurs only at the periphery of the colonies, and is restricted to the inter-territorial matrix (Fig. 1B). Sections stained with toluidine blue exhibit metachromatic staining, indicative of the presence of a high concentration of proteoglycan. Cultures grown without BGP exhibited similar cytology and metachromasia, but failed to stain with von Kossa (not shown).

An electron micrograph of a 7-day CHECOV culture grown without BGP is shown in Fig. 2A. The cells contain abundant endoplasmic reticulum. The extracellular matrix contains collagen fibrils and punctate deposits of proteoglycan, with little apparent differentiation between territorial and inter-territorial matrices. By day 16, cultures grown without BGP contain cells which lack endoplasmic reticulum but have numerous cell processes (Fig. 2B). Distinct territorial and inter-territorial matrices are seen at this stage, with no mineral in either. A 16-day culture grown with BGP is shown in Fig. 3A. The chondrocyte shown exhibits similar features to those of the same incubation time grown without BGP. Mineral deposits occupy much of the inter-territorial matrix, but are separated from the cell by a zone of unmineralized territorial matrix. Regions of dense mineral and “tidemarks” of intermediate mineral density are seen within the mineralized matrix. The latter may be artefacts caused by mineral dissolution during fixation and recrystallization during dehydration. A higher magnification view of the mineralization front is shown in Fig. 3B. Electron diffraction of this material indicates that it is a poorly crystalline hydroxyapatite (Fig. 4A). The elemental spectrum reveals calcium and phosphorus peaks in a ratio of 1.67:1 (Fig. 4B).

### Biochemical studies

Alkaline phosphatase activity was measured at various times in Triton extracts of CHECOV cultures grown in the presence or absence of BGP. Calcium and phosphate were measured in the subsequent acid extracts. DNA was measured on separate cultures at the same times.

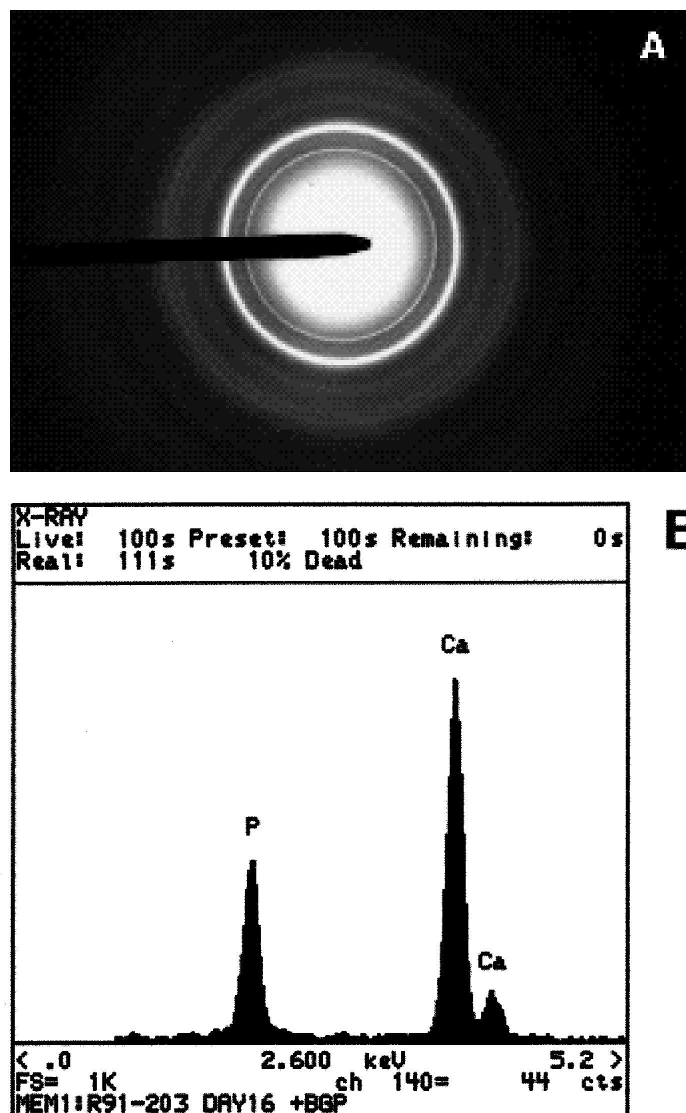


**Fig. 4.** Electron diffraction analysis of mineral deposits in 16-day CHECOV culture grown with BGP. (A) Selected area electron diffraction pattern obtained from area shown in Fig. 3B. (B) X-ray spectrum, showing characteristic peaks for P and Ca.

As shown in Fig. 5A,B, DNA contents reached a maximal value after approximately 8 d of culture, indicating that significant cellular proliferation occurred only in the early part of the culture period. The DNA content of cultures grown without BGP is slightly higher than that of cultures grown with BGP (see also Table 1). Outgrowth of cells from colonies of CHECOV cultures without BGP was often observed at later time-points, and may account for the elevated DNA content of these cultures.

Alkaline phosphatase activity was similar in both the presence and absence of BGP (Fig. 5G,H). Enzyme activity was initially almost undetectable, but increased to a maximal value at approximately the mid-point of the culture period, and decreased thereafter. Late in the culture period, therefore, alkaline phosphatase activity was relatively low, although the cultures were still actively calcifying.

As determined by the calcium and phosphate contents of acid extracts, only the cultures grown in BGP underwent calcification (Fig. 5C,D,E,F). Calcium and phosphate both became detectable in the acid extracts of cultures grown in BGP at day 10, and increased linearly throughout the remainder of the culture period.

### Effect of organic and inorganic phosphates on the calcification of CHECOV cultures

To determine whether inorganic phosphate ($P_i$) is required for calcification of chondrocyte cultures, CHECOV cells were grown for 21 d in $P_i$-free $\alpha$-MEM with BGP and ascorbic acid as above. For comparison, cultures grown in DMEM with or without BGP were harvested at the same time. As shown in Table 1, cultures grown in $P_i$-free medium contained less DNA than cultures grown in $P_i$-containing media, indicating that cellular proliferation is slower in the absence of $P_i$. The calcium and phosphate levels of acid extracts indicate that cultures grown in DMEM plus BGP, but not those grown in $P_i$-free $\alpha$-MEM plus BGP or those grown in DMEM minus BGP, underwent calcification. Therefore, BGP can supply phosphate for the growth of CHECOV cells, albeit at a lower level than in $P_i$-containing medium, but BGP cannot support the calcification of CHECOV cultures in the absence of $P_i$.

To determine whether added organic phosphates are hydrolyzed to produce $P_i$ by calcifying chondrocytes, aliquots of medium were removed, 48 h after each medium change, from CHECOV cultures grown in BGP-containing medium. Phosphate analysis of these aliquots showed that the $P_i$ concentration rose from an initial value of approximately 1 mM (corresponding to the $P_i$ content of DMEM) to a maximal level of approximately 3.2 mM by day 9, and remained at this level throughout the remainder of the incubation period (Fig. 6A). During the period of active calcification, therefore, almost half of the BGP added was being hydrolyzed within 48 h. The observed rise in $P_i$ concentration correlates with the increase in alkaline phosphatase activity (Fig. 5G,H).

To determine whether calcification of CHECOV cultures can be induced by an elevated $P_i$ concentration, as shown in other chondrocyte culture systems (Boskey et al., 1992; Ishikawa and Wuthier, 1992), cultures were grown in DMEM supplemented with various concentrations of $P_i$. After 21 d of incubation, acid-soluble phosphate was determined as previously described. As shown in Fig. 6B, essentially no calcification occurred in CHECOV cultures grown in basic DMEM ($P_i$ concentration approximately 0. 9 mM). Progressively higher levels of calcification occurred in DMEM supplemented with 1, 2, 3 and 4 mM $P_i$. The amount of calcification produced in DMEM + 5 mM BGP was intermediate between that of DMEM + 2 mM $P_i$ (total $P_i$ 2.9 mM) and DMEM + 3 mM $P_i$ (total $P_i$ 3.9 mM). As demonstrated above (Fig. 6A), the $P_i$ concentration in the medium of calcifying CHECOV cultures grown in the presence of 5 mM BGP was approximately 3.2 mM.

## DISCUSSION

Several chondrocyte culture systems in which calcification can be induced have recently been described. Generally,

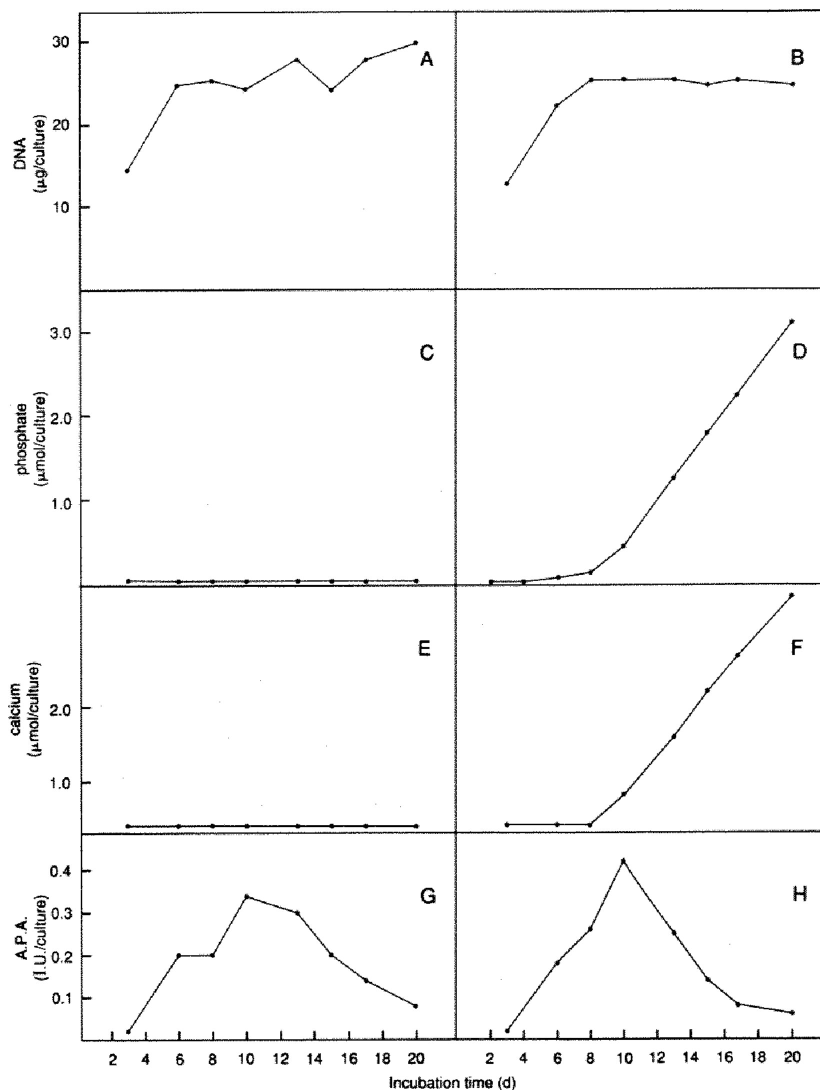


**Fig. 5.** Biochemical analyses of CHECOV cells grown in the presence and absence of β-glycerol phosphate. Phosphate and calcium analyses were performed on acid extracts; alkaline phosphatase analyses were performed on Triton extracts of the same cultures. Values given are means of determinations performed on three replicate cultures. DNA analyses were performed on different cultures. Values given are single determinations. (A), (C), (E), (G) without BGP; (B), (D), (F), (H) with BGP.

these systems have involved the use of growth plate chondrocytes or cells of the pre-osseous embryonic cartilage grown in the presence of BGP and/or ascorbic acid. Calcification has been reported using dispersed cell cultures of chondrocytes from adult chicken growth plate (Wu et al., 1989), chick embryo sternum (Leboy et al., 1989) and vertebra (Gerstenfeld and Landis, 1991), and bovine embryo growth plate (Hinek et al., 1987). Other systems involve culture in collagen gels (Thomas et al., 1990), agarose gels (Tacchetti et al., 1989) and concentrated cell pellets (Kato et al., 1988).

In initial studies, vertebral chondrocytes from 12-day chick embryos were grown in primary and secondary monolayer cultures. Calcification of monolayer cultures was occasionally observed in the presence of BGP and ascorbic acid, but this was not consistently reproducible under any conditions used. One possible reason for the inconsistent calcification of monolayer cultures is that contaminating fibroblasts overgrow the chondrocytes. Alternatively, differentiation of CHECOV cells may be promoted by substratum-independent growth. In this context, it has been shown that calcification (Tacchetti et al., 1989) and type X collagen gene expression (Adams et al., 1991) are induced by transferring chick embryo chondrocytes from monolayer to suspension culture.

In subsequent studies, CHECOV cells were grown in

**Table 1. Effect of organic and inorganic phosphate on the growth and calcification of CHECOV cultures**

| | Ca (μmole/dish) | $PO_4$ (μmole/dish) | DNA (μg/dish) |
|---|---|---|---|
| A. DMEM−BGP | 0.287±0.012[1] | 0.715±0.020[1] | 30.2±0.680[2] |
| B. DMEM+BGP | 1.97±0.042[3] | 3.57±0.091[3] | 27.6±1.72[3] |
| C. MEM−$P_i$+BGP | 0.227±0.046 | 0.854±0.262 | 22.1±0.361 |

Cultures were grown for 21 days prior to analysis. Ca and $PO_4$ were determined on acid extracts following Triton extraction. DNA was determined on separate cultures. All results are expressed as mean ± standard deviation of three determinations. Statistical significance was determined using ANOVA and the Tukey multiple comparisons test ($q$ = 0.005).

[1]Significantly different from B but not C.
[2]Significantly different from C but not B.
[3]Significantly different from C.

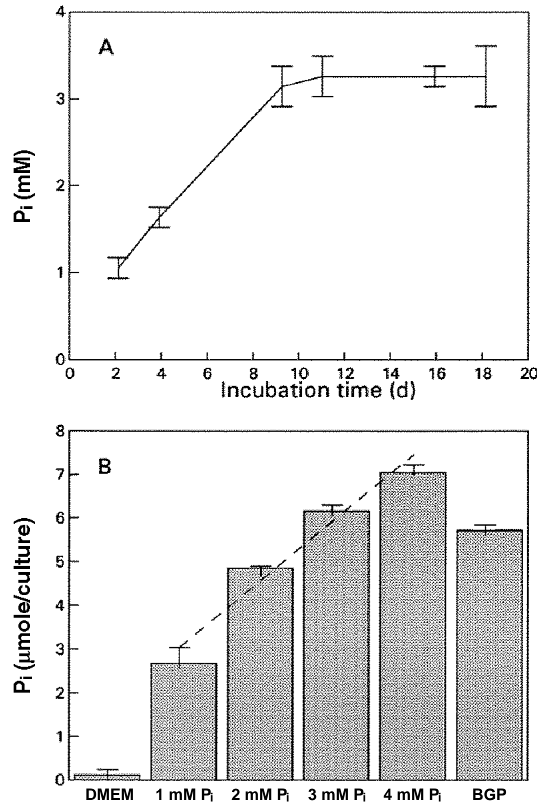


**Fig. 6.** Calcification of CHECOV cultures by phosphate added exogenously and phosphate from BGP. (A) CHECOV cultures were grown in DMEM with BGP, and $P_i$ was measured in the medium 48 h after addition of fresh medium. Results shown are the mean ± standard deviation of three determinations. (B) CHECOV cultures were grown in medium containing BGP or various concentrations of sodium dibasic phosphate for 21 d prior to analysis. Phosphate was measured in acid extracts following Triton extraction. Results are expressed as mean ± standard deviation of three determinations. The broken line is the regression line for acid-soluble phosphate versus added phosphate in the medium (1-4 mM), and has the formula: $y = 1.44x + 1.58$ ($r = 0.972$). The value for BGP is significantly different from that of 2 mM $P_i$ ($q = 0.01$) but not from that of 3 mM $P_i$ (Tukey multiple comparison test).

agarose gel suspension culture. Under these conditions, as shown by light and electron microscopic observations, CHECOV cultures undergo a process of differentiation and calcification generally similar to those seen in endochondral ossification, but with a rather different geometry. The cells form spherical colonies, similar to those seen in the agarose gel cultures of Tacchetti et al. (1989). Different cell morphologies are observed at different times of incubation: a typical secretory morphology, with abundant endoplasmic reticulum, early in the culture period; and a quite different morphology, characterized by abundant mitochondria and numerous cell processes, later in the culture period. Similar cell types in calcifying chondrocyte cultures have previously been observed by others (Gerstenfeld and Landis, 1991; Hinek et al., 1987; Tacchetti et al., 1989). At any given time, however, all cells within a colony appear similar, indicating that CHECOV cultures undergo a temporal, rather than a spatial, differentiation sequence. In common with calcifying cartilage, however, CHECOV cultures exhibit chondrocyte hypertrophy, metachromatic staining, and distinct territorial and inter-territorial matrices, with a poorly crystalline hydroxyapatite mineral phase restricted to the latter area. With respect to the latter observation, however, it should be noted that 'tidemarks" of increased mineral density were observed, indicating that dissolution and recrystallization of mineral may have occurred, as observed by others in conventionally fixed cartilage preparations (Arsenault and Hunziker, 1988).

The biochemical parameters used to measure growth, differentiation and calcification of CHECOV cultures were DNA, alkaline phosphatase activity, calcium and phosphate. The DNA content of CHECOV cells increased only during the early part of the culture period, reaching a maximal value at approximately eight days. Cultures grown without BGP exhibited a slightly elevated DNA content, perhaps due to an observed outgrowth of cells from colonies late in culture. Other workers have reported no effect of BGP on chick embryo chondrocyte proliferation (Thomas et al., 1990).

Calcification of CHECOV cultures was quantified as acid-soluble calcium and phosphate following extraction with Triton. By this criterion, calcification did not occur in cultures grown without BGP. In cultures grown with BGP, significant levels of acid-soluble calcium and phosphate were first seen at the mid-point of the incubation period, at a time when cell proliferation had ceased and when alkaline phosphatase activity was maximal. Thereafter, calcification increased in an approximately linear fashion until the end of the incubation period.

Alkaline phosphatase activity exhibited a similar sequence in cultures grown with or without BGP, suggesting that CHECOV cells undergo the same differentiation sequence whether or not calcification, the end result of that differentiation, is possible. In bone organ cultures, BGP decreases the amount of alkaline phosphatase activity (Tenenbaum, 1987). However, no effect of BGP on alkaline phosphatase activity was observed in chick embryo sternal chondrocytes (Leboy et al., 1989). Enzyme activity, which is almost undetectable in early cultures, increases steadily to a maximal value at approximately the mid-point of the culture period (day 10) and declines thereafter. The peak of alkaline phosphatase activity corresponds to the onset of calcification in cultures grown with BGP. A similar time course of alkaline phosphatase activity in calcifying chondrocyte cultures has been reported by others (Kato et al., 1988; Tacchetti et al., 1989).

CHECOV cultures grown in DMEM plus 5 mM BGP undergo calcification, those grown without BGP do not. During calcification, almost half the added BGP is converted to $P_i$ in 48 h. In osteoblast cultures induced to calcify by addition of BGP, almost all the BGP is hydrolyzed in 8 h (Bellows et al., 1992). In the present study, the rise in medium $P_i$ of CHECOV cultures grown with BGP was shown to parallel the increase in alkaline phosphatase activity. However, $P_i$ levels remained high after alkaline phosphatase levels decreased. This may indicate that alkaline phosphatase in the culture medium, rather than in the cell layer, is required for BGP hydrolysis, or that BGP, rather than alkaline phosphatase, is rate-limiting for calcification.

The effect of $P_i$ on CHECOV culture calcification was also examined. No mineral deposition was observed in CHECOV cultures grown in $P_i$-free medium supplemented with BGP, or in cultures grown in unsupplemented DMEM. Calcification could be induced, however, by increasing the $P_i$ concentration, as previously reported by others (Boskey et al., 1992; Ishikawa and Wuthier, 1992). Of particular interest, cultures grown with a total $P_i$ concentration of 3.9 mM resulted in a slightly higher level of mineral formation than cultures grown with 5 mM BGP; as noted above, these latter cultures contain approximately 3.2 mM $P_i$ at the onset of calcification. These findings suggest that BGP promotes calcification of chondrocyte cultures by serving as a source of $P_i$, and, more generally, that $P_i$ availability, at least in vitro, is rate-limiting for calcification.

In summary, these findings are consistent with the following mechanism of calcification in chondrocyte cultures. Mineral deposition requires a critical extracellular $P_i$ concentration (in the culture system used here, between 0.9 and 1.9 mM). This critical concentration can be achieved by supplementation of the culture medium with $P_i$ or with organic phosphate compounds such as BGP. In the latter case, the added BGP is hydrolyzed to $P_i$ by alkaline phosphatase, which reaches a maximal value at the onset of calcification.

Finally, the use of non-physiological organic phosphate compounds such as BGP in calcifying culture systems has led some authors to caution that the calcification observed may be artefactual (Khouja et al., 1990). Although the source of phosphate for cartilage calcification has not been identified, it is relevant to note that a decrease in organic phosphate and a corresponding increase in $P_i$ have been found in hypertrophic growth plate cartilage (Kakuta et al., 1985). Also, the activity of alkaline phosphatase is maximal in this zone (Arsenis, 1972). These findings suggest that the calcification of cartilage in vivo, like that in vitro, may involve the alkaline phosphatase-mediated hydrolysis of organic phosphate compounds.

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