Fetal Bovine Bone Cells Synthesize Bone-specific Matrix Proteins

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ABSTRACT We isolated cells from both calvaria and the outer cortices of long bones from 3- to 5-mo bovine fetuses. The cells were identified as functional osteoblasts by indirect immunofluorescence using antibodies against three bone-specific, noncollagenous matrix proteins (osteonectin, the bone proteoglycan, and the bone sialoprotein) and against type I collagen. In separate experiments, confluent cultures of the cells were radiolabeled and shown to synthesize and secrete osteonectin, the bone proteoglycan and the bone sialoprotein by immunoprecipitation and fluorography of SDS polyacrylamide gels. Analysis of the radiolabeled collagens synthesized by the cultures showed that they produced predominantly (~94%) type I collagen, with small amounts of types III and V collagens. In agreement with previous investigators who have employed the rodent bone cell system, we confirmed in bovine bone cells that (a) there was a typical cyclic AMP response to parathyroid hormone, (b) freshly isolated cells possessed high levels of alkaline phosphatase, which diminished during culture but returned to normal levels in mineralizing cultures, and (c) cells grown in the presence of ascorbic acid and β-glycerophosphate rapidly produced and mineralized an extracellular matrix containing largely type I collagen. These results show that antibodies directed against bone-specific, noncollagenous proteins can be used to clearly identify bone cells in vitro.

Several criteria have been used to characterize cells isolated and cultured from bone as osteoblasts, although none have proved specific. These are that (a) freshly isolated bone cells possess high alkaline phosphatase activity (1), (b) bone cells show a strong cyclic AMP response to parathyroid hormone (2), (c) bone cells secrete predominantly type I collagen when grown in the presence of ascorbic acid (3), and (d) bone cells produce and partially mineralize a matrix, given extended time in culture and defined culture conditions (4–9).

Recently, the isolation and purification of several noncollagenous matrix proteins from fetal calf bone (10) have provided new tools to identify bone cells with increased certainty. Antibodies against these proteins have been used to establish their tissue specificity in fetal calf bone (11–13). In this study we confirm the criteria listed above using bone cells obtained from the calvaria and long bones of fetal calves, the first nonrodent system to be explored. We show by indirect immunofluorescence and in biosynthetic experiments, that these cells produced three bone-specific, noncollagenous proteins; osteonectin (11), the bone proteoglycan (12), and the bone sialoprotein (13), thus proving conclusively that this methodology can clearly identify bone cells in vitro.

MATERIALS AND METHODS

Culture Conditions: Fetal calves (3–5 mo in utero [8] and still in the fetal sac) were obtained from Schneider Packing Co., St. Louis, MO within 15 min of being killed. The fetus was removed with sterile gloves and placed in a plastic bag on ice for transport to the laboratory (45 min).

Dissection of the bones was performed under a laminar flow hood. Both halves of the cranial vault, tibias, and femurs were removed under aseptic conditions. The periosteum of all bones, the cartilaginous ends of the long bones and the dense connective tissue of the sutures of the calvaria were removed. Radial slices of the cranial vault, 1–2 mm wide and 10–25 mm in length, were removed from the thinnest areas (1–3 mm thick) with a sterilized single-edged razor blade. Longitudinal slices of the tibias and femurs were shaved off the
out metaphysical and diaphysial cortex, rinsed in culture medium without serum, and cut into pieces with approximate dimensions of 1 x 1 x 2 mm. The bone pieces were transferred to 100 mm petri dishes and incubated with intermittent agitation for 40 min at 37°C in 8-10 ml of culture medium without serum and containing 1 mg/ml type IV collagenase (Sigma Chemical Co., St. Louis, Mo. or crude collagenase (Worthington Biochemical Corp., Freehold, NJ). The pericellular matrix was digested using 20% trypsin and sodium azide. After digestion, the medium containing the released cells placed in 20-ml sterile centrifuge tubes. An equal volume of complete culture medium (see below) was added to the centrifuge tubes to stop the collagenase activity and the tubes centrifuged at slow speed for 3-5 min. The medium was removed and the cell pellet resuspended in 2-3 ml of complete medium. 1/2 ml of the cell suspension (1 x 10^6 cells) was plated out into 35-mm petri dishes, onto 20-mm² glass coverslips or into 75-ml culture flasks and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO₂. Each 100 ml of complete media contained 400 µl Dulbecco’s modified Eagle’s nutrient media, with 4.5 µg/mL of glucose, 40 µg/mL of F-2, 20% heat-inactivated fetal calf serum, human origin. The cells were incubated for 4 h at 37°C. The coverslips were washed three times with phosphate-buffered saline without calcium and magnesium, and containing 0.2% EDTA. After 5-10 min of incubation at 37°C, the cell suspension was removed, centrifuged, resuspended in 3-5 ml of culture medium and plated out onto six to ten 35-mm petri dishes (1 x 10⁵ cells each) using 1% trypan blue and a hemocytometer. To induce mineralization, we stimulated the cells with 2-3 µM of 1,25-dihydroxyvitamin D₃. In confluent cultures where most cells overgrowth and cell multilayering occurred mineralized first. Visual observation of the onset of mineralization was possible within 7-10 d and most cultures appeared to add mineralized matrix progressively up to 14 d from the onset. The calcium and phosphorus concentrations of the medium were ~1.73 mM and 1.1 mM, respectively. Mineralized areas were removed manually from the culture dishes and fixed for x-ray diffraction and infrared spectroscopic examination. Alkaline Phosphatase: Samples analyzed were (a) freshly-isolated cultures and (b) cultures at confluence treated either with or without β-glycerophosphate and ascorbic acid for 2 to 6 d. The cells were rinsed in Hank’s balanced salt solution, scraped off the dish and transferred to test tubes in 500 µl of 0.2 M sucrose and sonicated (Bisonick II [Bronnwell Scientific, Rochester, NY]) on ice for 30 s at the high setting. Alkaline phosphatase activity of the sonicated solution was determined using the Sigma procedure and reagents (Sigma Chemical Co.) #246-A. P-Nitrophenyl phosphatase (66 µM) was added to 6.5 ml of a 0.1 M solution of the buffer (2% aminophenylisobutylphosphate and 1% Tris-HCl buffer as described by Laemmli containing 0-mercaptoethanol, 19), boiled for 5 min and cooled to room temperature. After incubation, the coverslips were washed in four changes of phosphate-buffered saline with gentle agitation for a total of 20 min. The coverslips were then immersed in deionized-distilled water for 10 min and washed thoroughly. The washing procedures were repeated with a 1:20 dilution of fluorescein-conjugated IgG, and anti-rabbit made in sheep for type I collagen and anti-β-actin made in goat for osteonectin, bone proteoglycan, and bone sialoprotein (Cappel Laboratories, Cochranville, PA). Coverslips were mounted on glass slides in 85% glycerol and examined with a Leitz-Ortholux II photomicroscope with a fluorescent vertical illuminator. Photomicrographs of antibody treated and controls were exposed and processed under identical conditions. Noncollagenous Protein Biosynthesis: Primary bone cells were cultured for 1-2 weeks in 35-mm petri dishes and grown to confluency. The medium was removed and the cultures washed with Dulbecco’s-Vogt medium with 9.5 µg glucose/ml, without methionine or fetal calf serum. For osteonectin, 10 µCi/ml of L-[^35]S-methionine, (800 Ci/mmol, translation grade [New England Nuclear, Boston, MA]) was added to one ml of the Dulbecco-Vogt medium and incubated for 4 h at 37°C. Bone sialoprotein, to 1 ml of Dulbecco-Vogt plus 20% fetal calf serum, 10 µCi/ml of L-[^35]S-methionine was added and incubated at 37°C for 24 h. For bone proteoglycan, 100 µCi/ml of ^35S-SO₄ (carrier free [ICN Pharmaceuticals, Inc., Cleveland, OH]) was added to 1 ml of the Dulbecco-Vogt and incubated for 4 h at 37°C. Cyclic AMP production was terminated by pouring off the medium, and the supernatant, containing the cyclic AMP, was washed five times with equal volumes of diethylether to remove trichloroacetic acid and lyophilized. The supernatant fraction, and the resulting precipitate was washed with 5% trichloroacetic acid and 0.05 M Tris-HCl, pH 7.4, 0.3 mM phenylmethylsulfonyl fluoride, 20 mM EDTA acid, and 10 mM N-ethylmaleimide. The medium and cell layer fractions were combined, homogenized and centrifuged at 10,000 g to remove insoluble material. The homogenate, containing cyclic AMP, was added to a final concentration of 15% to the supernatant fraction, and the resulting precipitate was washed with 5% trichloroacetic acid and subsequently dissolved in 0.5 M acetic acid. Lathyritic rat skin collagen was added (50 µg/ml) as a carrier before treatment with pepsin (Calbiochem-Behring Corp., 0.1 mg/ml) to digest noncollagenous proteins. After stirring at 4°C for 24 h, the collagen was precipitated from the 0.3 M acetic acid solution by the addition of solid NaCl to 1.7 M and were collected by centrifugation at 10,000 g for 20 min, washed with 4.5 M NaCl, 0.5 M Tris-HCl, pH 7.4, and finally with 18% ethanol. After drying, the precipitates were electrophoresed on SDS 5% polyacrylamide slab gels (19) with and without reduction by 1 mM dithiothreitol and with delayed reduction with diithiothreitol (21) to separate αI(1) from αI(III). The gels were prepared for fluorography by using dimethylsulfoxide and diphenylloxazole (22) and were exposed to Kodak XAR film at ~70°C for 48 h. Quantitation of the different ω-chains was determined by scanning the film with a laser densitometer, or by using a densitometer and scanning the film with a laser densitometer, or by using a densitometer and scanning the film with a laser densitometer. Electron Microscopy: Bone cells were grown on coverslips or
Confluent cultures of fetal bovine bone cells elicit a typical cyclic 3',5'-AMP response to 100 ng/ml of parathyroid hormone (1–34 fragment). Peak response is demonstrated at 5 min. Each point represents the mean from four cultures, each analyzed in duplicate.

Confluent cultures of fetal bovine bone cells demonstrate minimal alkaline phosphatase activity either basally or after stimulation with ascorbic acid (50 μg/ml) and/or β-glycerophosphate (10 mM), both for 2 d. However, after 6 d of treatment cultures each treatment a significant rise in alkaline phosphatase activity, comparable to that measured in freshly isolated cells. Each bar value represents the mean from three cultures each analyzed in duplicate. con, control. ac, ascorbic acid. bgp, β-glycerophosphate.

Indirect fluorescein immunofluorescent micrographs of 5-d cultures of bone cells exposed to antibodies to bovine osteonectin (a), bone proteoglycan (b), bone sialoprotein (c), and a representative preimmune serum control (d). The osteonectin response is very strong throughout most of the cytoplasm, while both the bone proteoglycan and bone sialoprotein appear to be limited to the Golgi region. × 300.
in petri dishes with flexible plastic inserts (Falcon Labware, Oxnard, CA). Fixation took place in 1.0% glutaraldehyde in 0.1 M phosphate-sucrose buffer, pH 7.3, for a ½ h at room temperature. Postfixation was for 1 h in 1% phosphate-sucrose-buffered osmium tetroxide at 4°C. The cells were dehydrated in an alcohol series and embedded in an epoxy resin. 1-2 μm thick sections were prepared for light microscopy and stained with toluidine blue and basic fuschin. Thin sections (90-100 nm) were cut with a diamond knife (Dupont Instruments, Inc., Wilmington, DE) on an LKB ultramicrotome. The sections were collected on copper grids and stained with uranyl acetate and lead citrate and viewed at initial magnifications of 3,000-10,000 on a Philips 300 electron microscopy at 60 kV.

Von Kossa Staining: Mineralization in ascorbic acid and β-glycerophosphate-treated cultures was detected by Von Kossa's silver method (23). The cultures were treated with 5% silver nitrate for 30 min, rinsed and counterstained for 60 s in 0.3% toluidine blue.

RESULTS
Under the isolation and culture conditions employed, confluent cells obtained from either calvaria or outer cortices of long bones all showed a typical increase in cyclic 3',5'-AMP in response to parathyroid hormone (Fig. 1). Alkaline phosphatase levels were high at isolation (Fig. 2) but fell after 2 d in culture and remained depressed during subsequent culture. When medium containing either 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, or both was added daily to confluent cultures, alkaline phosphatase levels again became elevated after 6 d of treatment (Fig. 2). The combination of ascorbic acid and β-glycerophosphate induced the best stimulation; however, stimulation by the two substances simultaneously was not additive (Fig. 2).

5-d primary or secondary cultures of the bovine bone cells were exposed to antibodies specific for the bovine bone proteins: osteonectin; the bone proteoglycan; and the bone sialoglycoprotein. In all cases, the cells were positive for each protein (Fig. 3, a–c) when compared to the preimmune serum control (3d). Osteonectin immunofluorescence was most intense (Fig. 3a), while that of both the bone proteoglycan and the bone sialoglycoprotein was distinct but less intense (Fig. 3, b and c). These cells were also strongly positive to antibodies against type I collagen (Fig. 4, a and b). In the absence of ascorbic acid, fluorescence was visible intracellularly (Fig. 4a). With the addition of ascorbic acid to the cultures, fluorescence was observed both intracellularly and extracellularly along matrix fibrils (Fig. 4b). In contrast, little, if any, extracellular fluorescence was seen when ascorbic acid–treated cultures were exposed to the osteonectin antibody (Fig. 4c), presumably because it was released into the media. However, the cytoplasmic distribution of osteonectin staining appeared more perinuclear in the ascorbic acid–treated cells (compare Figs. 4c and 3a).

Confluent cultures, placed on ascorbic acid and β-glycerophosphate produced and mineralized an extracellular matrix, commencing 7 d after the start of treatment. The matrix

![Figure 4](image_url) Indirect fluorescein immunofluorescence of antibodies directed against type I collagen and osteonectin after the addition of 50 μg/ml ascorbic acid in nonconfluent cultures. In the absence of added antibodies to type I collagen stain the cell cytoplasm, primarily (a), while both the cells and the extracellular matrix stain when ascorbic acid is added to the culture medium (b). Osteonectin (c) remains localized primarily to the perinuclear region with ascorbic acid treatment (compare to Fig. 3a).

![Figure 5](image_url) Indirect fluorescein immunofluorescent micrographs of demineralized, in vitro bone matrix, treated with antibodies to type I collagen (a), osteonectin (b) or with preimmune serum (c). Because of the extended period of tissue treatment required to demineralize the matrix, the remaining cell remnants present dark (fluorescein negative) profiles. × 300.
production and mineralization were supported for 3–4 wk in culture. The mineral present was shown to be hydroxyapatite by x-ray diffraction and infrared spectroscopy (data not shown). When this in vitro-produced matrix was demineralized and stained immunocytochemically for osteonectin and type I collagen, it was positive for both (Fig. 5). We surmise that anti-osteonectin fluorescence is less intense than collagen because of its loss during demineralization. In addition, during processing cells were lost from the matrix surface and the cells which remained retained little or no fluorescence.

To confirm that the cultured cells produce both noncollagenous and collagenous bone matrix proteins, we labeled cultures with either $^{[35]S}$methionine, Na$^{35}$SO$_4$, or $[^3H]$proline. Fluorograms ($^{[35]S}$methionine) of SDS gels from labeled media immunoprecipitated with anti-osteonectin (Fig. 6a) or anti-bone sialoprotein (Fig. 6b) indicate synthesis and secretion of these two proteins. Similarly, anti-bone proteoglycan immunoprecipitate ($^{35}$SO$_4$) (Fig. 7) showed the production of this macromolecule by the bovine osteoblast cultures.

Fluorograms of SDS gels of the collagens produced by passaged confluent bovine osteoblasts (Fig. 8) showed that similar amounts and types of collagen were produced by cultures from both calvaria and long bones. Quantitation of types I and III collagens was accomplished by the use of delayed reduction with dithiothreitol to separate $\alpha 1(I)$ from $\alpha 1(III)$ chains. All cultures produced approximately 94% type I and approximately 6% type III collagen. Long-term exposure of the gels to x-ray films revealed that a small amount (0.5%) of type V collagen was also produced as indicated by the presence of $\alpha 1(V)$ chains (data not shown).

The ultrastructure of bone cells stimulated with ascorbic acid and $\beta$-glycerophosphate exhibited cytoplasmic elements consistent with an active connective tissue secretory cell (Fig. 9). Collagen fibrils were abundant adjacent to the cells.

Mineralization in confluent cultures of cells treated for up to 14 d with $\beta$-glycerophosphate and ascorbic acid was confirmed by the Von Kossa method (Fig. 10). Small focal points of mineralization which were visible on day 7, enlarged and fused with one another until about two-thirds of the culture dish was covered with mineralized matrix.

**DISCUSSION**

This study showed that bone cells, isolated from fetal calf calvarial and long bones by conventional means, synthesized substantial quantities of three tissue-specific noncollagenous proteins of fetal calf bone: osteonectin, bone proteoglycan and bone sialoprotein. These cells can, therefore, be identified as functional osteoblasts. The bovine bone cells also were positive for alkaline phosphatase upon isolation from the tissue, produced cyclic AMP upon stimulation with parathyroid hormone, synthesized type I collagen, and mineralized their matrix when treated with $\beta$-glycerophosphate and ascorbic acid. Thus, all criteria established earlier for presumptive bone cell identity (1–9) were also expressed by the bovine osteoblasts described in this study.

Although the precise function of the three noncollagenous...
FIGURE 9  Osteoblast from a confluent culture exposed for 6 d to 50 μg/ml ascorbic acid and 10 mM β glycerol phosphate. The cytoplasm of the cell is rich in rough endoplasmic reticulum (ER) and contains a prominent Golgi apparatus (G). Large numbers of collagen fibrils (CO) are found extracellularly. × 33,000.

FIGURE 10  Von Kossa/toluidine staining of a confluent culture treated with ascorbic acid and β-glycerophosphate for 10 d. Focal points (arrow) of heavy mineralization are found within areas of more diffuse mineralization. × 128.
bone proteins is unknown, they appear to be necessary for the formation of normal, structurally-sound bone tissue. In one form of bovine osteogenesis imperfecta, now being employed as a model for human osteogenesis imperfecta, osteonectin and the bone proteoglycan are reduced by >90% and the bone sialoprotein by 40–50% (unpublished data). Because of the relatively small size of the bone proteoglycan molecule as compared to the cartilage proteoglycan aggregate (12), it is likely that it does not prevent mineralization but, provides another as yet undiscovered function.

The alkaline phosphatase data shows that either ascorbic acid or β-glycerophosphate can induce alkaline phosphatase activity in confluent primary cultures of bovine bone cells. The mechanism of how either induction occurs must remain speculative. The ascorbic acid mechanism might be related to an increase in membrane turnover (presumably also including alkaline phosphatase) brought about by an increase in matrix synthesis and release. β-Glycerophosphate was originally used to induce mineralization in vitro (9, 13) because it has been used successfully both chemically and histochemically as an alkaline phosphate substrate. Perhaps β-glycerophosphate operates through a substrate induction mechanism. Addition of ascorbic acid to the nutrient medium appeared to be essential for detection of type I collagen and osteocalcin (Fig. 4b, 4c) in the extracellular matrix of confluent cultures.

Our experience supports the findings that significant mineralization in tissue culture occurs only after confluency has been reached and substantial cell overgrowth and multilayering has occurred (7–9). In addition, mineralization appears to require a certain volume of matrix. It has been our consistent observation that before mineralization onset, β-glycolcer phosphate and ascorbic acid together produce a much greater matrix volume and in a shorter time period than do either alone. This results in appearance of mineral within 14 d in culture in agreement with Ecarot-Charier et al. (9), 1–2 wk earlier than others have reported (4–8).

As others have shown in rodent cell culture (8), mineralization of bovine bone matrix occurs in matrix sandwiched between two bone cell layers, one a single cell layer which adheres to the dish, the other a continuous multilayer cell that sits atop the matrix. At the initiation of mineralization focal points of mineral appear and expand in size in areas where the cell layer atop the matrix remains as a multilayer cell (unpublished observations).

The small amounts of type III collagen found are as expected, considering that reticular connective tissue associated with numerous blood capillaries is positive for type III collagen in fresh frozen sections of undermineralized fetal calf bone (unpublished findings). In addition, interstitial (progenitor) mesenchyme lying between the forming bone spicules in intramembranous bone formation areas (calvarial, subperiosteal bone) was also positive for type III collagen (12). Bone cell cultures were previously shown (24) to contain small amounts of type V collagen. The absence of type II collagen from the cultures using indirect immunofluorescent microscopy suggests that they were free of cartilage cell contamination. Experiments designed to show that the bovine bone cells produced bone gla protein (osteocalcin) gave poor results by both indirect immunofluorescent antibody staining and bio- synthetic labeling. This failure could, however, be related to the young age of the fetuses from which the cells were isolated. It is known that in the fetal bones of at least three species, osteocalcin synthesis does not reach substantial levels until birth and early neonatal life (25–27). It is noted that the only cells unequivocally shown to produce osteocalcin are rat osteosarcoma cell lines of Rodan (28), the precise origin of which remains unknown.

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REFERENCES

1. Peck, W. A., S. J. Birge, and S. A. Fedak. 1964. Bone cells: biochemical and biological studies following enzymatic isolation. Science (Wash. D.C.) 146:146–147.
2. Peck, W. A., J. Carpenter, K. Messinger, and D. Delta. 1973. Cyclic, 3’,5’ adenosine monophosphate in isolated bone cells: response to low concentrations of parathyroid hormone. Endocrinology. 92:662–667.
3. Scott, D. M., G. N. Kent, and D. V. Cohn. 1980. Collagen synthesis in cultured osteoclast-like cells. Arch. Biochem. Biophys. 201:384–391.
4. Binderman, I. D., D. Dukkin, A. Haaral, E. Katsi, and L. Sachs. 1974. Formation of bone tissue in culture isolated bone cells. J. Cell Biol. 61:427–439.
5. Harrell, M. I., Binderman, and M. Guetz. 1976. Culture tissue of bone cells: mineral transport, calcification and hormonal affeets. J. Med. Sci. 12:115–133.
6. Marvao, V., and G. W. Bernard. 1976. Isolated fetal mesenchymal cells form lamellar bone in vitro. J. Dent. Res. 57(Supplement A):99.
7. Williams, D. C., G. B. Boder, E. R. Toomey, D. C. Paal, C. C. Hillman, Jr., K. L. King, R. von Faak, C. C. Johnson, Jr. 1980. Mineralization and metabolic response in serially passaged adult rat bone cells. Calcif. Tissue Int. 30:235–246.
8. Sode, H., H. A. Kodana, Y. Amagai, S. Yamamoto, and S. Kasi. 1983. In vitro differentiation and calcification in a new clonal osteosarcoma cell line derived from newborn mouse calvaria. J. Cell Biol. 96:193–199.
9. Ecarot-Charier, B., F. H. Florieux, M. van der Rest, and G. Pereira. 1983. Osteoblast isolated from mouse calvaria initiate mineralization in culture. J. Cell Biol. 96:639–643.
10. Termine, J. D., A. B. Belcourt, M. Conn, and H. K. Kleinman. 1981. Mineral and collagen-binding proteins of fetal calf bone. J. Bone. 256:1040–1040.
11. Termine, J. D., H. K. Kleinman, S. W. Whitson, K. M. Conn, M. L. Garvey, and G. R. Martin. 1981. Osteocin, a bone-specific protein linking mineral to collagen. Cell. 26:99–105.
12. Fisher, L. W., J. D. Termine, S. W. Whitson, M. Yamasanbishi, H. H. Kimura, V. C. Hasell, H. K. Kleinman, J. R. Hassell, and B. Nicholas. 1983. Proteoglycans of developing bone. J. Bone. 256:654–659.
13. Fisher, L. W., S. W. Whitson, L. V. Aviso, and J. D. Termine. 1983. Matrix sialoprotein of developing bone. J. Bone. 256:1273–1277.
14. Deutsch, D. J., J. E. Attar, C. Robinson, and J. A. Weatherall. 1979. Rate and timing of enamel development in the deucedous bovine incisor. Arch. Oral Biol. 24:407–413.
15. Tenenbaums, H. C., and J. N. M. Hoessen. 1982. Differentiation of osteoblasts and formation of mineralized bone in vitro. Calcif. Tissue Int. 34:76–79.
16. Termine, J. D., E. D. Eanes, D. J. Greenfield, and M. U. Nylen. 1973. Hyaluronic acid-adsorbed bone mineral. Calcif. Tissue Res. 12:47–60.
17. Gelman, A. G. 1970. A protein binding assay for adenosine 3’,5’ cyclic monophosphate. Proc. Natl. Acad. Sci. USA. 67:303–312.
18. Dahl, H. M. K. (1980). Cyclic, 3’,5’ adenosine monophosphate in migrating and nonmigrating new epidermal cells. J. Cell. Biol. 104:367–373.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 227:680–685.
20. Rowe, D. W., and J. R. Shapiro. 1982. In Symposium on Inheritable Disorders of Connective Tissue. Acheson, W., P. Bornstein, and M. Glimcher, editors. C. V. Mosby, St. Louis. 269–282.
21. Sykes, B. C., B. Pudlic, M. Francis, and R. Smith. 1976. The estimation of two collagens from human dermis by interrupted gel electrophoresis. Biochem. Biophys. Res. Commun. 72:1472–1480.
22. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83–88.
23. Mallory, F. B. 1938. Pathological Technique. Saunders, Philadelphia. PA. 144.
24. Wistow, M., S. Fischer, W. Desau, and P. K. Muller. 1981. Collagen types synthesized by isolated bone cells. Exp. Cell Res. 133:115–125.
25. Price, P. A., J. W. Lothring, and S. K. Nishimoto. 1981. Absence of the vitamin K dependent bone protein in fetal rat mineral. J. Bone. 255:1482–1492.
26. Price, P. A., J. W. Lothring, S. A. Bax, and A. H. Reddi. 1981. Development appearance of the vitamin K-dependent protein of bone during calcification. J. Bone. 256:3781–3784.
27. Termine, J. D. 1981. Chemical characterization of fetal bone matrix constituents. In The Chemistry and Biology of Mineralized Connective Tissues. A. Veis, editor. Elsevier-North Holland, Amsterdam. 349–353.
28. Nishimoto, S. K., and P. A. Price. 1980. Secretion of the vitamin K-dependent protein of bone by rat osteosarcoma cells. Evidence from intracellular precursor. J. Bone. 255:6579–6883.