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Role of Organic Phosphate in Mineralization of Bone *in vitro*

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Calvarial periosteae were dissected from 17-day-old embryonic chicks and folded with the osteogenic cells in apposition. The folded explants were cultured for up to six d on serum and plasma clots or in serum-free hormone-supplemented completely-defined medium. Osteoid consistently formed in such cultures in both types of media, and this osteoid mineralized when appropriate levels of β -glycerophosphate were added to each type of medium. The data presented suggest that the levels of organic phosphate might be more important than inorganic phosphates as a limiting factor in the initiation of mineralization of bone *in vitro*.

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Introduction.

Disturbances of phosphate metabolism have been associated with mineralization defects of bones and teeth. Hypophosphatemia, for example, can result in the production of interglobular dentin and rachitic bone lesions,¹ and hypophosphatasia is also associated with dental and osseous abnormalities. Vitamin D-resistant rickets with familial hypophosphatemia has also been shown to result in dental and osseous mineralization defects.² Supplementation with inorganic phosphates will partially reverse the rachitic lesion by stimulating bone formation.³ Studies *in vitro* have demonstrated that appropriate levels of calcium and inorganic phosphate are required for mineralization of a calcifiable matrix.⁴ However, the levels of inorganic phosphate that are required for *in vitro* mineralization are above physiological levels for no known reason. The possible role of organic phosphates in the mineralization process has never been investigated.

We have previously developed an *in vitro* bone formation system involving the culture

of explanted periosteae on plasma clots.⁵ In the present study we describe the use of a serum- and plasma-free, completely-defined medium in which osteogenesis and mineralization will occur. In both systems, osteoid was shown to mineralize when β -glycerophosphate (BGP) was added in appropriate concentrations. The requirement for and role of organic phosphates in the mineralization process are discussed.

Materials and methods.

The culture methods have been previously described in detail.⁵ Briefly, periosteae were removed from 17-day-old chick embryo calvariae in cold Hanks balanced salt solution (HBSS) under a dissecting microscope.* These periosteae were then folded with the osteogenic layer of cells in apposition within the fold. The folded explants were then placed on millipore filters (AA.80 μ), and cultured either on a clot, or on a grid positioned at the gas-liquid interface on a liquid, serum-free medium.

Preparation of culture media. — Two types of culture media were used: (1) a clot medium comprised of 50% HBSS supplemented to 2.5 mM calcium,⁶ 30% rooster plasma,⁷ 10% rooster serum,⁷ and 10% extract from nine-day embryonic chicks;⁸ and (2) a serum-free medium consisting of BGJ_B supplemented with Vit C 50 μ g/ml, transferrin 5 μ g/ml, insulin 100 ng/ml, PTH 8x10⁻¹¹ M, calcitonin 6x10⁻¹¹ M, EGF 5 ng/ml, FGF 10 ng/ml and thyroxin 1x10⁻⁸ M.

To study the role of organic phosphate in the mineralization process, both types of media were supplemented with either 10 mM, 5 mM, or 2.5 mM β -glycerophosphate (BGP). The choice of BGP as the organic phosphate addition was based on previous experiments⁵ using this compound.

This paper was the First Prize winner, post-doctoral category, in the Hatton Awards Competition, during the IADR's 59th General Session, Chicago, Illinois, March, 1981.

*American Optical

Incubation conditions. — All cultures were incubated at 37°C in 5% CO₂ in humidified air for up to six d. The media were changed every 48 h.

Analysis of cultures. — At the end of the culture, tissues were fixed in acetate buffered formalin for one d. Five-micron paraffin sections were prepared using standard histological techniques. The sections were stained with either hematoxylin and eosin or the Van Gieson and the Von Kossa stain. Some specimens were subjected to X-ray diffraction analysis to analyze for the presence of hydroxyapatite.

Results.

Clot system. — Tissues grown on clots containing 5 or 10 mM BGP for six d all formed mineralized bone within the fold (Table). The mineralized part of the matrix contained osteocytes and was surrounded by a seam of non-mineralized osteoid, which was, in turn, surrounded by osteoblasts (Fig. A). The osteoid was bi-refringent when observed with polarized light. X-ray diffraction analysis of mineralized cultures demonstrated the presence of hydroxyapatite.⁹ Osteoid formed in the presence of 2.5 mM BGP only mineralized in about 50% of the cultures. Explants cultured without BGP contained only unmineralized osteoid. The osteoblasts in these cultures appeared somewhat flattened in comparison with osteoblasts in mineralized cultures.

Serum-free system. — In explants cultured on serum-free medium, mineralization occurred in all cultures at 10 mM BGP (Table), and the bone formed was morphologically similar to that formed in the clot system (Fig. B). At 5 mM and 2.5 mM BGP,

mineralization was observed in approximately 80% of cultures, although those cultured in the presence of 2.5 mM BGP appeared more lightly mineralized. Cultures grown in the absence of BGP did not mineralize, and explants cultured in BGJ medium containing no additional hormones showed little or no evidence of osteoid formation.

Discussion.

In examining the results obtained on both the clot system and the completely-defined medium system, it is apparent that osteogenesis will occur readily in both media. If the media are not supplemented with BGP, there is no mineralization, despite the fact that both calcium and inorganic phosphate levels in the clot and defined medium systems are approximately in the physiological range.

Mineralization occurs when appropriate amounts of BGP are added to the culture medium. It appears, however, that different levels of BGP are required to initiate and support mineral formation in the two media. The normal level of organic phosphate is approximately 10 mM.¹⁰ Since the organic phosphate levels in the clot system are about half the physiologic levels, because of the dilution factor acquired in the preparation of a plasma clot in HBSS, while there are no organic phosphates in the completely-defined, serum-free medium, both media are clearly deficient in organic phosphate. Therefore, if the dilution factors are taken into account, it can be calculated that the clot system requires supplementation with 5 mM organic phosphate in order to re-establish a 10 mM concentration, while the serum-free medium requires a 10 mM supple-

TABLE
THE EFFECT OF β -GLYCEROPHOSPHATE (BGP) ON THE MINERALIZATION OF CULTURES ON PLASMA CLOTS AND ON SERUM-FREE DEFINED MEDIUM.

[BGP]	Percent Mineralized Cultures*	
	Defined Medium	Plasma Clot
10 mM	100 (6)	100 (17)
5 mM	78 (9)	100 (29)
2.5 mM	82 (6)	50 (4)
0 mM	0 (6)	0 (22)

*All cultures, whether mineralized or not, contained osteoid. The number of cultures at each concentration is given in brackets.

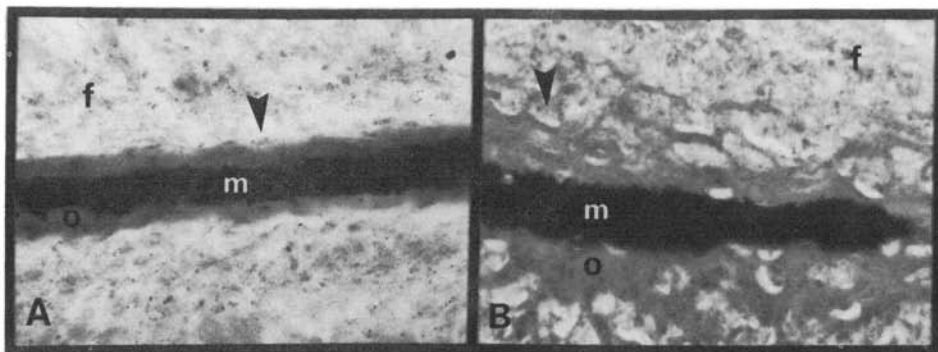


Fig. — (A) Von Kossa and Van Gieson Stain of a 5 μ section of culture grown on a clot containing 10 mM BGP. Note the presence of mineral (m) surrounded by a seam of unmineralized osteoid (O). Osteoblasts (arrow) and fibrous periosteum (f) are present. (B) Von Kossa and Van Gieson Stain of a 5 μ section of a culture grown on completely-defined medium. Note the similarity between this culture and the one grown on the plasma clot. Note also the presence of mineralized matrix (m) surrounded by a seam of unmineralized osteoid (O). Osteoblasts (arrow) and fibrous periosteum (f) are present.

mentation. It would appear, therefore, that osteoid mineralizes reproducibly when organic phosphate levels are restored to near-physiological levels. The data obtained with the clot system support this hypothesis, but the data obtained with the defined medium suggest that mineralization can still occur in medium containing lower-than-physiologic levels of organic phosphates. One possible explanation of this might be the absence in serum-free medium of inhibitors of mineralization or crystallization that might be present in the clot system. In addition, BGP might be more readily hydrolyzed by bone cell phosphatases than the broad spectrum of organic phosphates known to exist in serum,¹⁰ thus implying that some serum organic phosphates might even act as competitive inhibitors of phosphatase activity. In view of this, lower levels of BGP might initiate mineralization in serum-free medium at lower concentrations than in serum-containing medium.

An additional point of interest is the observation that osteoblasts appeared more active with BGP supplementation. A similar phenomenon was observed by Raisz *et al.*,¹¹ who found increased bone collagen synthesis in the presence of increased inorganic phosphate levels. Further experiments to quantitate osteoblast activity in response to organic phosphate supplementation and subsequent mineralization are in progress.

Conclusion.

These data demonstrate that osteogenesis

can take place in a completely-defined, serum-free medium. The initiation of mineralization appears to be more sensitive to the presence of physiologic levels of organic phosphates and possibly to inhibitors of mineralization, than simply to levels of inorganic phosphate.

REFERENCES

1. NIKIFORUK, G. and FRASER, G.: Etiology of Enamel Hypoplasia and Interglobular Dentine, *Metab Bone Dis and Rel Res* 2:17-23, 1979.
2. SHAFER, W.G.; HINE, M.K.; and LEVY, B.M.: *A Textbook of Oral Pathology*, 3rd ed., Toronto: W.B. Saunders Co., 1974.
3. HARRIS, W.H.; HEANEY, R.P.; DAVIS, L.A.; WEINBERG, E.H.; COUTTS, R.D.; and SCHILLER, A.L.: Stimulation of Bone Formation *in vivo* by Phosphate Supplementation, *Calc Tiss Res* 22:85-98, 1978.
4. BINDERMAN, I.; GREENE, R.M.; and PENNYPACKER, J.P.: Calcification of Differentiating Skeletal Mesenchyme *in vitro*, *Science* 206:222-224, 1979.
5. TENENBAUM, H.C. and HEERSCHKE, J.N.M.: Differentiation of Osteoblasts and Formation of Mineralized Bone *in vitro*, *Calc Tissue Int*, In press, 1981.
6. NIJWEIDE, P.J. and van der PLAS, A.: Calcium and Strontium Metabolism of Bone, *Proc Kon Ned Akad Wet C78*:410-417, 1975.
7. PAUL, J.: *Cell and Tissue Culture*, Churchill and Livingston, 1975.
8. GAILLARD, P.J.: Developmental Changes in the Composition of Body Fluids in Relation to the Growth and Differentiation of Tissue Cultures, *Protoplasma XXIII*:145-174, 1934.

9. TENENBAUM, H.; CHENG, P.; PRITZKER, K.; and HEERSCHE, J.N.M.: Manuscript in preparation.
10. ALBRITTON, E.C.: **Standard Values in Blood**, Philadelphia, PA: W.B. Saunders Co., 1952.
11. RAISZ, L.G.; DIETRICH, J.W.; and CANALIS, E.M.: Factors Influencing Bone Formation in Organ Cultures, *Isr J Med Sci* 12:108-114, 1976.