Radioautographic Visualization and Biochemical Identification of *O*-Phosphoserine- and *O*-Phosphothreonine-containing Phosphoproteins in Mineralizing Embryonic Chick Bone

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ABSTRACT We injected $NaH_2{}^{33}PO_4$ into normal 14-d-old embryonic chicks and examined the long bones by both radioautography and biochemical analyses from 10 to 240 min after the injection was completed. At 30 min, determination of the radiographic grain density revealed that ^{33}P was concentrated principally in fibroblasts, preosteoblasts, and osteoblasts. With time, there was a progressive increase in the density of silver grains located over both the osteogenic cells and the regions of uncalcified (osteoid) and calcified extracellular organic matrices. Biochemical analyses identified ^{33}P-*O*-phosphoserine as the major ^{33}P component in glutaraldehyde-treated whole demineralized bone tissue and in EDTA-soluble, nondiffusible proteins extracted from the bones, both at the same time periods that ^{33}P-induced silver grains were visualized by radioautography. ^{33}P-*O*-phosphothreonine was also identified in experiments using a dosage of 10 mCi per embryo. The results provide the first combined direct biochemical and radioautographic identification that phosphoproteins are synthesized in bone and are located morphologically at the sites of mineralization. The data provide further evidence that phosphoproteins play a critical role in the biological calcification of vertebrate tissues.

Phosphoproteins are present in all the normally and pathologically calcified vertebrate tissues thus far studied (1). Evidence of the ability of phosphoproteins to bind calcium ions (2, 3), together with other molecular, ultrastructural, and biochemical characteristics as well as theoretical considerations, has provided the basis for the hypothesis that these components play a significant role in the formation of a solid phase of calcium phosphate in the mineralized tissues of vertebrates (4–6).

Until recently, however, there was little direct proof that the phosphoproteins were even synthesized in the mineralized tissues and not transported to these tissues from other sites as are albumin and α_2HS-glycoproteins in bone matrix (7–9). The first indication that phosphoproteins were indeed synthesized in mineralized tissues came from the elegant radioautographic studies of Weinstock and Leblond (10). These investigators followed the localization of ^{33}P first in the odontoblasts and later at the mineralization front of the extracellular matrix of the dentin of rats injected with $^{33}P_i$ (inorganic orthophosphate).[1] Although the visualized radiolabel was not identified biochemically as being an integral component of a protein, the data were consistent with subsequent studies of Munksgaard et al. (11), who demonstrated active biosynthesis of phosphoprotein by dentinal tissue in organ culture, and the in vivo biosynthesis studies of Dimuzio and Veis (12).

In the case of bone tissue, phosphoproteins containing Ser(P) (*O*-phosphoserine) and Thr(P) (*O*-phosphothreonine), whose molecular sieve and ion exchange chromatographic properties parallel those of the EDTA-soluble and well char-

[1] *Abbreviations used in this paper:* P_i, inorganic orthophosphate; Ser(P), O-phosphoserine; Thr(P), O-phosphothreonine.

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acterized bone matrix phosphoproteins (13), have been shown to be synthesized by bone in organ culture (14). More recent work with isolated bone cells in culture strongly suggests that these phosphoproteins are synthesized principally by osteoblasts (15).

In this study, based on the earlier approach of Weinstock and Leblond (10), we report the radioautographic localization of ^{33}P in the periosteal cells and osteoblasts of embryonic chick tibiae within 30 min after the injection of $NaH_2{}^{33}PO_4$, later in osteoblasts and extracellular tissue osteoid, and finally at the mineralization front and more heavily calcified regions of the extracellular organic matrix. Biochemical analyses of aliquot samples of the same bony tissues used for the radioautography studies established that both Ser(^{33}P) and Thr(^{33}P) are present in the whole, glutaraldehyde-treated demineralized bone used for the radioautographic studies; in whole native bone; and in the EDTA-soluble, nondiffusible phosphoproteins of the tissue. Preliminary data have been reported (16).

MATERIALS AND METHODS

Normal, 14-d-old embryonic chicks (Spafas, Inc., Norwich, CT) were injected with 2, 5, or 10 mCi of $NaH_2{}^{33}PO_4$ (New England Nuclear, Boston, MA) through the eggshells onto the allantoic membrane. Chicks were killed after 30 min (2 mCi) and 10, 30, and 240 min (5 and 10 mCi). Afterwards, whole tibiae with periosteum and a thin cuff of adherent muscle tissue were dissected, fixed 2–7 d in 2.5% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4, at 4°C, and then decalcified in 10% EDTA in 2.5% glutaraldehyde at pH 7.4 for 2 wk with frequent changes of solution. The use of EDTA and glutaraldehyde in this manner has been shown to retain the bone phosphoproteins and osteocalcin components in the tissue (M. J. Glimcher and D. Kossiva, unpublished observations). After decalcification, tibiae were dehydrated in graded ethanols and embedded in Spurr medium (17). For light microscopic studies, tissue blocks were sectioned at 0.5 μm thickness on fresh glass knives with a Porter-Blum MT-2 ultramicrotome, mounted on glass slides, and stained with toluidine blue. For electron microscopy, silver sections (~80 nm) were cut on a diamond knife with an LKB Ultrotome III (LKB Instruments, Inc., Gaithersburg, MD) and collected on carbon-reinforced, nickel grids (18). Slides were prepared for radioautography with Kodak NTB-2 according to the methods of Kopriwa and Leblond (19). 2-wk exposure times were sufficient for all radioautography. Developed silver grains were counted at × 1,000 total magnification (× 100 primary magnification) in a Zeiss light microscope fitted with an objective reticule. Radioautographs were photographed at a primary magnification of × 80 on Kodak Pan-X film using a Zeiss 35-mm camera. Grains were counted over five regions: B1, empty portions of a slide (containing no tissue section); B2, the portions of a section containing muscle and nonosseous connective tissue; P, the outer periosteal regions of tibial diaphyses containing fibroblasts and osteoprogenitor cells; O, regions containing the osteoid matrix and the single layer of osteoblasts adjacent to it; and M, the mineralization front and the more heavily mineralized matrix. The relatively unmineralized osteoid portion of the extracellular matrix was defined by its lighter staining characteristics with toluidine blue, and as that portion of the extracellular matrix extending from the border of the osteoblasts to the edge of the mineralizing front as defined by the discernible change in metachromasia. Thin tissue sections on nickel grids were stained with 8% uranyl acetate in 50% ethanol-water and 0.2% aqueous lead citrate (20), examined in a JEOL 100C electron microscope operated at 60 kV, and photographed on Kodak 4489 film.

For the biochemical analyses, the following samples at each time period and at each dosage level of $NaH_2{}^{33}PO_4$ were used: whole, undemineralized bone; whole undemineralized bone first treated with glutaraldehyde and then demineralized in glutaraldehyde-EDTA solution exactly as described for the radioautography studies; nondialyzable components present in the EDTA extracts of whole native bone not treated with glutaraldehyde. Aliquot portions of these samples were hydrolyzed in 4 N HCl, 106°C, for 6 h and subjected to preparative ion exchange amino acid chromatography for the isolation of Ser(^{33}P) and Thr(^{33}P) (21, 22).

RESULTS

Fig. 1 defines the principal histological regions in a developing tibia from the 14-d-old embryonic chick. At this time, chick development shows formation of a primary center of ossification at the mid-diaphysis of the tibia. Table I presents the results of grain density analyses of radioautographs from whole tibiae of 14-d-old chicks, killed 30 min after injection with 2 mCi/embryo of $NaH_2{}^{33}PO_4$. The grains are present in the cells of both regions P and O, but are most heavily concentrated in the osteoblasts and the relatively uncalcified osteoid bone matrix (region O). Some grains are also present in the more heavily calcified extracellular bone matrix (region M). When a dosage of 10 mCi per embryo was used, the overall uptake and density of the radioactive label was too great to allow quantitative grain counting of sections even at 10 min. Biochemical analyses were, however, performed.

A complete time-series of experiments was carried out at a dosage of 5 mCi per chick embryo. Light microscopic radioautographs for this time-course uptake of label at 10, 30, and 240 min after injection are presented in Figs. 2–4, respectively, and qualitatively show a temporal shift in the location and distribution of developed silver grains from osteoprogenitor cells to osteoblasts and the heavily mineralized bone matrix. Since precise uptake of the label over the different regions of

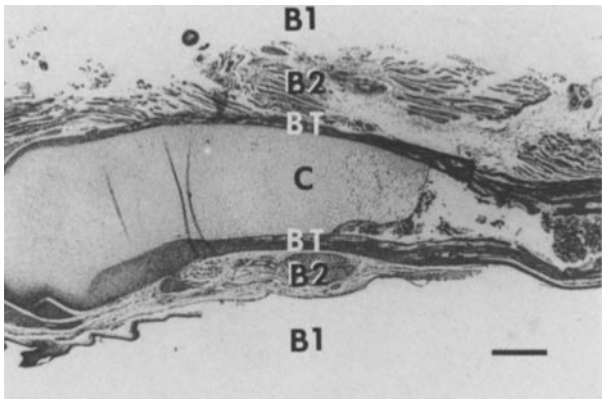


FIGURE 1 Light micrograph of the developing tibia from a 14-d-old embryonic chick showing bone tissue (*BT*) present at the primary center of ossification along the mid-diaphysis of the tibia. *C*, cartilage; *B1*, slide background; *B2*, muscle and nonosseous connective tissue. Bar, 200 μm. × 35.

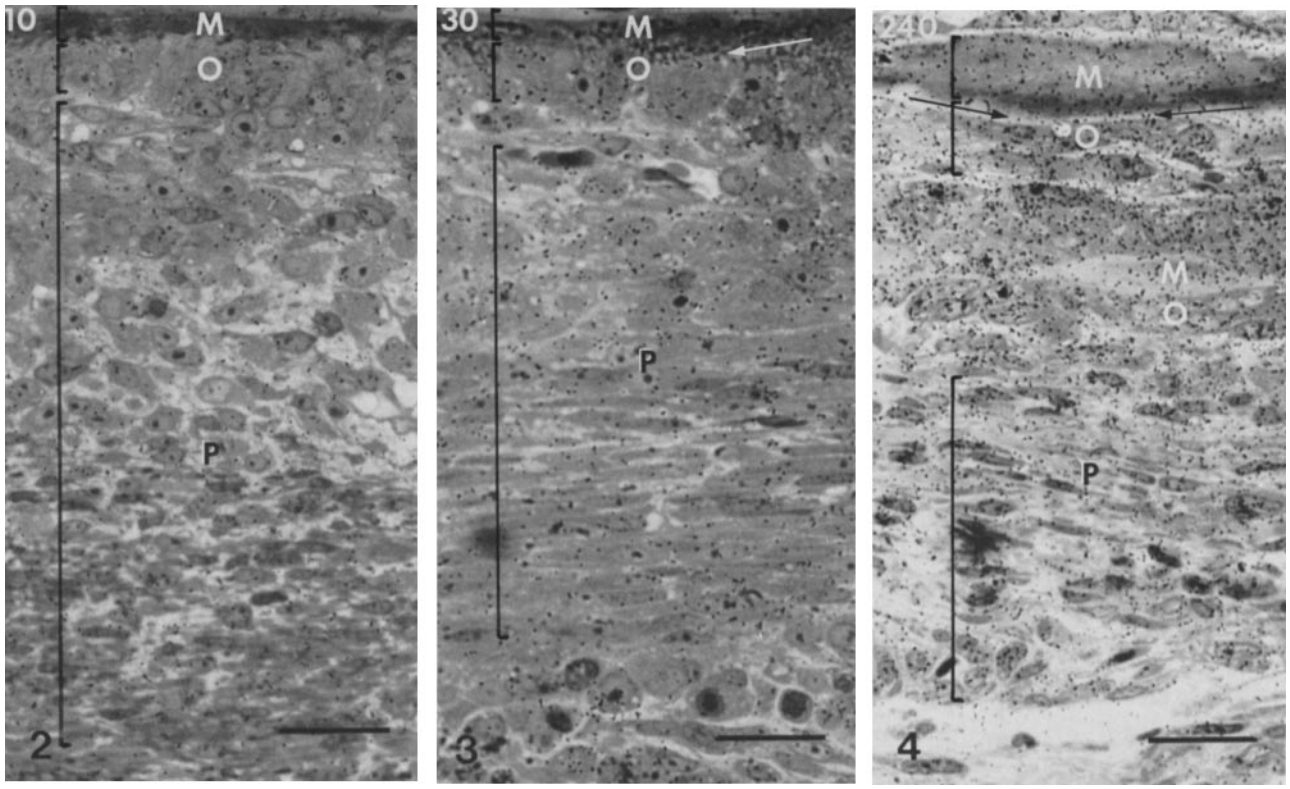
TABLE I

Localization and Quantitation of ^{33}P Grain Distribution in Embryonic Chick Tibiae 30 Min after Injection of 2 mCi/Embryo of $NaH_2{}^{33}PO_4$

Region analyzed	Grain density	Significance
B1: Slide background (no tissue)	0.0028 ± 0.0019	B2-B1 NS P-B1 $P < 0.001$
B2: Nonosseous tissue	0.0050 ± 0.0046	P-B2 $P < 0.02$ O-B1 $P < 0.001$
P: Outer periosteal cells	0.0117 ± 0.0062	O-B2 $P < 0.001$ O-P $P < 0.001$
O: Osteoblasts and osteoid	0.0450 ± 0.0083	O-M $P < 0.001$ M-B1 $P < 0.001$
M: Mineralized matrix	0.0129 ± 0.0066	M-B2 $P < 0.01$ M-P NS

Grains were counted on light microscope radioautographs of labeled embryonic chicks killed at 30 min after label injection; then fixed and decalcified as described in Materials and Methods. Emulsions were exposed for 2 wk, slides developed, and grains counted over five regions of interest (see Materials and Methods). Grain density is the mean (number of grains/square micron section or tissue area) ± standard deviation of triplicate determinations from each of three slides over the regions indicated ($n = 9$ for each region). *P* values show the statistical significance (Student's *t* test) of the differences between grain densities over the slide regions examined. *NS*, not significant.

interest was not directly apparent from the radioautographs, grain density measurements were made over a number of the same tissue sections initially photographed. Quantitative results are given in Table II. Within the first 30 min after injection of $NaH_2{}^{33}PO_4$, grains are concentrated throughout all regions of the tissue, including preosteogenic cells, osteoblasts, and osteoid matrix, and the heavily mineralized extracellular zones. The differences in grain density distribution over different zones at this time are statistically significant. At 30 min compared to 10 min, there is also an increase in grain density over all regions, especially those containing preosteogenic cells, osteoblasts, and osteoid. The highest number of grains is located over the osteoblasts and osteoid. After 240 min, additional increases in grain density have occurred. The



FIGURES 2–4 Light microscope radioautographs showing distribution of silver grains along the embryonic chick tibial mid-diaphysis 10, 30, and 240 min, respectively, after injection of 5 mCi $NaH_2{}^{33}PO_4$. Fig. 2: At 10 min, grains are present principally over outer periosteal regions of the tissue containing fibroblasts and osteoprogenitor cells (*P*). Fig. 3: At 30 min, extensive labeling has also occurred over regions (*O*) containing the single layer of osteoblasts adjacent to the narrow extracellular osteoid matrix (arrow). Grains are apparent in addition over the mineralization front and more heavily mineralized matrix (*M*). Fig. 4: By 240 min, grains are most numerous over regions containing osteoblasts and osteoid matrix (*O*) and the mineralization front and more heavily mineralized extracellular matrix (*M*). Regions of fibroblasts and osteoprogenitor cells (*P*) contain relatively fewer counts at this time. Bar, 10 μm. × 1,400.

TABLE II

Embryonic Chick Tibiae Uptake of ^{33}P at 5 mCi/Embryo Dosage

Region analyzed	Grain density		
	10 min after injection	30 min after injection	240 min after injection
B1: Slide background (no tissue)	0.0127 ± 0.0067 *n* = 12	0.0058 ± 0.0007 *n* = 9	0.0136 ± 0.0031 *n* = 12
B2: Nonosseous tissue	0.0119 ± 0.0062 *n* = 12	0.0081 ± 0.0015 *n* = 9	0.1171 ± 0.0676 *n* = 12
P: Outer periosteal cells	0.0143 ± 0.0086 *n* = 14	0.0249 ± 0.0061 *n* = 16	0.4045 ± 0.1389 *n* = 12
O: Osteoblasts and osteoid	0.0166 ± 0.0093 *n* = 14	0.0334 ± 0.0095 *n* = 10	0.7018 ± 0.0895 *n* = 12
M: Mineralized matrix	0.0131 ± 0.0089 *n* = 14	0.0183 ± 0.0142 *n* = 10	0.4264 ± 0.1484 *n* = 12

Grains were counted as detailed in Table I with chicks sacrificed at 10, 30, and 240 min after injection with 5 mCi $NaH_2{}^{33}PO_4$. Emulsion exposures of 2 wk were used. Total grain density determinations over the regions of interest are denoted by *n*. The qualitative changes in location and distribution of silver grains in the radioautographs of Figs. 2–4, indicating a shift with time of label from osteoprogenitor cells ultimately to the mineralized extracellular bone matrix, are supported by the grain density data. The statistical significance (Student's *t* test) of the respective differences between grain densities over the regions of these slides (see Table I) is summarized as follows: (*a*) 10 min after injection—*NS* for all regions compared except $P < 0.2$ for O-B2; (*b*) 30 min after injection—$P < 0.001$ for all regions compared except $P < 0.02$ for O-P, O-M, and M-B1; $P < 0.05$ for M-B2; and $P < 0.1$ for M-P; (*c*) 240 min after injection—$P < 0.001$ for all regions compared except NS for M-P.

highest grain density persists in the osteoblast-osteoid region, but by this time the rate of grain accumulation is greatest over the more heavily calcified extracellular matrices (~650%) compared to the other zones of interest (Table III).

Electron microscopic radioautography revealed silver grains over the rough endoplasmic reticulum and Golgi apparatus of the osteoblasts particularly at 10 and 30 min after injection (Fig. 5). Silver grains were most prominent over the extracellular bone matrix 4 h after injection.

TABLE III

Rate of ^{33}P Accumulation in Embryonic Chick Tibiae after Injection of 5 mCi/Embryo of $NaH_2{}^{33}PO_4$

Region analyzed	Rate of ^{33}P accumulation (No. grains/μm^2/min) × 10^{-4}		Rate Increase of ^{33}P Accumulation (%)
	10–30 min after Injection	30–240 min after Injection	
B1: Slide background (no tissue)	−3.45	0.371	-
B2: Nonosseous tissue	−1.90	5.91	-
P: Outer periosteal cells	5.30	18.1	242
O: Osteoblasts and osteoid	8.40	31.8	279
M: Mineralized matrix	2.60	19.4	646

For each of the regions of interest, the number of grains per square micrometer section or tissue area obtained from Table II at the times indicated were subtracted and divided by the duration of embryo incubation (20 or 210 min) with $NaH_2{}^{33}PO_4$. Rate increase is {[(30–240 min rate) − (10–30 min rate)]/(10–30 min rate)} × 100%.

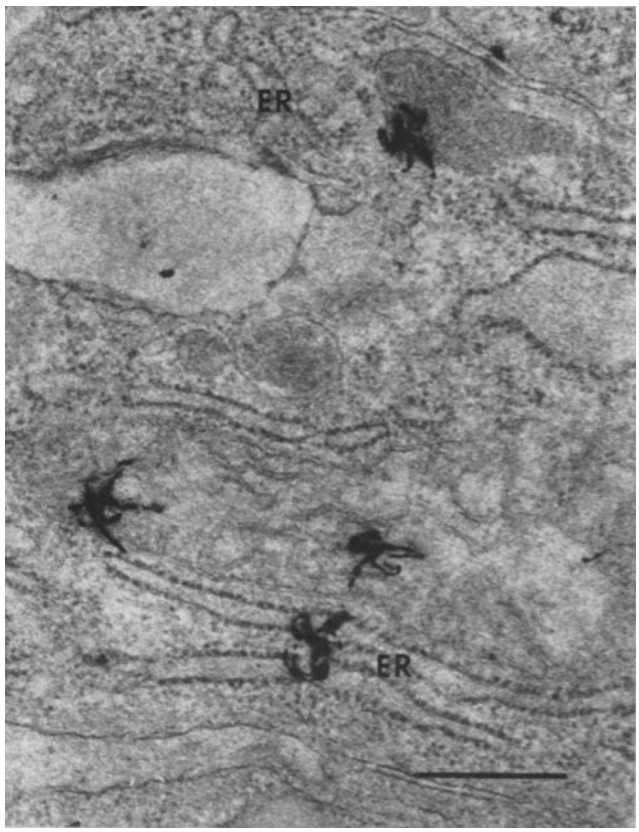


FIGURE 5 Electron microscope radioautograph of silver grains over endoplasmic reticulum (*ER*) of osteoblasts 30 min after injection of $NaH_2{}^{33}PO_4$. Some silver grains were also observed over Golgi apparatus of osteoblasts in other thin tissue sections. Bar, 1 μm. × 20,000.

TABLE IV

Recovery of ^{33}P as Ser(^{33}P) in Whole Bone Tissues after Injection of 5 mCi $NaH_2{}^{33}PO_4$ into 14-d-old chick embryos

Time after injection	CPM recovered as Ser(^{33}P)/mg tissue
min	
10	96
30	487
240	17,941

After dissection and removal of samples for microscopic processing, the remaining whole appendicular and axial skeletons of labeled embryos were fixed 7 d with 2.5% glutaraldehyde and then thoroughly decalcified in a solution of 10% EDTA in 2.5% glutaraldehyde. Aliquot portions of embryonic bone were next hydrolyzed in 4 N HCl, 106°C, for 6 h and subjected to ion exchange amino acid chromatography for isolation of Ser(^{33}P) and Thr(^{33}P).

Ser(^{33}P) was isolated in whole undemineralized bone samples; in the EDTA-soluble, nondiffusible tissue components; and, most importantly, in the glutaraldehyde-fixed and glutaraldehyde-EDTA demineralized bone tissue at the same time periods corresponding to the radioautographic study at dosages of 5 and 10 mCi per embryo. A typical experiment utilizing glutaraldehyde-fixed, demineralized bone tissue is shown in Table IV.

Thr(^{33}P) was identified in the whole bone sample; in the EDTA-soluble components; and in the glutaraldehyde-treated, glutaraldehyde-EDTA demineralized bone tissue at 240 min after the injections of 10 mCi of $NaH_2{}^{33}PO_4$ per embryo. The number of counts in Thr(^{33}P) varied from 10 to 20% of that found in Ser(^{33}P).

Glutaraldehyde-EDTA demineralization of whole, fixed tissue removed <5% of the total Ser(^{33}P) content of the tissue, demonstrating that the vast majority of the Ser(P)-containing phosphoproteins remained in the tissue. Furthermore, 60% or more of the total ^{33}P in the glutaraldehyde-fixed, EDTA-extracted tissue was accounted for by Ser(^{33}P) [and in those instances where it was detected, Thr(^{33}P)]. Considering the recovery of Ser(P) from chicken bone phosphoproteins after partial acid hydrolysis (22), it is clear that the majority (85–95%) of the ^{33}P grains visualized radioautographically correspond to Ser(^{33}P)- and Thr(^{33}P)-containing phosphoproteins. Over 97% of the total Ser(^{33}P) content of whole bone was extracted in EDTA, ~80–90% of which was present in nondiffusible components (3,500-mol-wt cutoff dialysis membranes).

DISCUSSION

The present data provide additional direct radioautographic and biochemical evidence that the matrix phosphoproteins of bone are synthesized in bone matrix and are not simply transported to the tissue from other sites as are albumin and α_2HS-glycoproteins (7–9). The results are consistent with previous studies that rigorously established biochemically that chick bone and isolated osteoblastic cells of bone synthesize the characteristic phosphoproteins of bone matrix in organ and isolated cell culture, respectively (14, 15).

The ^{33}P-labeled phosphoprotein(s) identified in the present experiments are, like those isolated and characterized from whole postnatal chick bone (21) and those synthesized in organ and cell culture (14, 15), almost completely extractable

in EDTA, are nondiffusible and contain both Ser(P) and Thr(P). We believe it may be concluded that the ^{33}P-labeled components visualized in this study by radioautography represent principally the phosphoproteins isolated and characterized previously (14, 21–23). The localization of silver grains over endoplasmic reticulum and Golgi apparatus of osteoblasts provides further strong evidence that the phosphoproteins are synthesized principally by these cells, a conclusion consistent with the results recently obtained in cell culture by Gotoh et al. (15).

The temporal progression of ^{33}P from the osteoblasts to the mineralizing front and calcified extracellular matrices of bone corresponds well with similar observations made in dentin by Weinstock and Leblond (10). Together with other biochemical and ultrastructural data and with theoretical physical-chemical considerations (4–6), this report offers further support for the concept that phosphoproteins of bone, dentin, and other mineralized tissues play a significant role in the calcification of these tissues.

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