

Alkaline Phosphatase Induces the Mineralization of Sheets of Collagen Implanted Subcutaneously in the Rat

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Abstract

To determine whether alkaline phosphatase (ALP) can cause the mineralization of collagenous matrices *in vivo*, bovine intestinal ALP was covalently bound to slices of guanidine-extracted demineralized bovine dentin (DDS). The preparations were implanted subcutaneously over the right half of the rat skull. Control slices not treated with the enzyme were implanted over the left half of the skull of the same animals. Specimens were harvested after periods varying from 1 to 4 wk. It was shown that ALP-coupled DDS rapidly accumulated hydroxyapatite crystals. 4 wk after implantation, the content of calcium and phosphate per microgram of hydroxyproline amounted up to 80 and 60%, respectively, of that found in normal bovine dentin. Our observations present direct evidence that ALP may play a crucial role in the induction of hydroxyapatite deposition in collagenous matrices *in vivo*. (*J. Clin. Invest.* 1992. 89:1974-1980.)
Key words: biomaterials • dentin • implants • organic phosphates • remineralization

Introduction

Alkaline phosphatases (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1.) are cell surface glycoproteins that hydrolyze a variety of monophosphate esters. Usually three isoenzymes are distinguished: liver/bone/kidney (L/B/K),¹ placental, and intestinal. Although the bone isoenzyme has long been thought to play a role in the mineralization of bone and cartilage (1) and is widely used as a marker of biomineralization, its physiological significance is still a matter of debate. The fact that the enzyme is widely distributed in the body, in calcifying as well as noncalcifying tissues, raises questions as to the specificity of the relationship between alkaline phosphatase (ALP) and mineralization (2).

Evidence for its role has come up from studies on hypophosphatasia, an inherited disorder of osteogenesis characterized by a deficient L/B/K ALP (3). Weiss and co-workers (4) have demonstrated that a mutation in the L/B/K ALP gene

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1. Abbreviations used in this paper: ALP, alkaline phosphatase; beta-GP, beta-glycerophosphate; DDS, demineralized dentin slices; L/B/K, liver/bone/kidney; pNPP, *p*-nitrophenylphosphate.

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that abolishes enzymatic activity causes profound skeletal hypomineralization. Also, the presence of the enzyme in matrix vesicles, the sites of early formation of mineral crystallites in cartilage and bone, suggests a role of ALP in mineralization (5-8).

Several possible actions of the enzyme in mineralization processes have been proposed (2, 3): increasing the local concentration of inorganic phosphate, destructing locally mineral crystal growth inhibitors, acting as P_i-transporter or acting as Ca-binding protein. Robison (1) was first in proposing that ALP hydrolyzes organic phosphate esters, thus producing an excess of free inorganic phosphate that would cause local supersaturation and initiate the biomineralization process.

Recently, Tenenbaum and Heersche (9) have shown in explant cultures of embryonic periosteum that *in situ* mineralization of osteoid occurs upon the addition of external phosphates, such as β-glycerophosphate. In line with this, we have adduced evidence that ALP can cause the remineralization of decalcified dentin slices when incubated in media containing β-glycerophosphate (10, 11).

A criticism that applies to many *in vitro* mineralization studies is that exogenous phosphate esters are added to the culture systems in relatively high concentrations (up to 10 mM). Although it has been argued that about 10 mM organic phosphate occurs in circulation *in vivo* (9), other authors doubt whether these organic phosphates are hydrolyzable under physiological conditions (12), and whether there is sufficient substrate in extracellular fluids to exert a significant effect on the concentration of inorganic phosphate (13, 14). In addition, according to some investigators, the rate of hydrolysis of phosphate esters by ALP at physiological pH would be too low to be relevant to the process of mineralization (15).

It was the aim of the present study to determine whether ALP can cause the mineralization of collagenous substrates under *in vivo* conditions. For this purpose ALP of intestinal origin was covalently bound to sheets of collagen, and thus created biomaterial implanted subcutaneously in the rat.

Methods

Preparation of dentinal collagen sheets. Bovine permanent incisors were collected at the local slaughterhouse immediately after killing of the animals (age 1-3 yr) and frozen at -80°C until use. After thawing, the gingiva and periodontal ligament were removed and the roots cut with a diamond disk parallel to their longitudinal axis from the apex to the cervical area under constant irrigation with tap water and split with a chisel. The roots were then cleaned and freed from pulp. They were washed with ice-cold PBS in the presence of proteinase inhibitors (16), and the outer dentin (containing the mantle dentin layer and the cementum) was removed with a diamond disk under cooling with tap water.

Demineralized dentin slices (DDS) were prepared after demineralization of the roots with 0.6 M HCl or 0.5 M acetic acid at 4°C (10).

After demineralization, sections were cut on a cryotome set at 30 μm . The DDS were further extracted with 4 M guanidine. HCl and 0.4 M EDTA (pH 7.5) for 3 d at 4°C. Before use, the DDS were washed in double-distilled water for 1 h and placed in double-distilled water supplemented with antibiotics (10) at 4°C overnight. They were then transferred to IMDM. The DDS were free of calcium but still contained some collagen-bound phosphate residues ($0.039 \pm 0.015 \mu\text{g}/\mu\text{g}$ hypro after demineralization with HCl).

Preparation of dura mater sheets. Pieces of human dura mater were used as supplied by the manufacturer (see last paragraph of Methods). They contained $0.011 \pm 0.002 \mu\text{g}$ collagen-bound phosphate/ μg hypro.

Binding of ALP. Bovine intestinal ALP was covalently bound to the dentinal collagen sheets by using the coupling agents glutaraldehyde or carbodiimide (17).

Glutaraldehyde coupling. The DDS were incubated in PBS containing 0.1% glutaraldehyde for 2 h at 20°C in the presence of ALP (700 U/ml). The material was then extensively washed with PBS and stored at 4°C in 0.1 M glycine buffer (pH 10.5) containing 1 mM Mg^{2+} and 0.1 mM Zn^{2+} .

Carbodiimide coupling. The DDS were incubated for 2 d at 4°C in 0.13 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. HCl (pH 4.5) in the presence of ALP (700 U/ml). They were then exhaustively and successively washed in distilled water, 1 M NaCl in 0.1 M Na-acetate (pH 4.0), distilled water, 0.1 M NaHCO_3 (pH 8.3), and finally distilled water. The material was stored in glycine buffer at 4°C (see preceding paragraph). The enzyme retained its activity under these storage conditions for at least 1 yr. Lyophilized human dura mater was treated in a similar fashion.

Control specimens were treated identically to the experimental ones, except that incubations were carried out in the absence of ALP.

In vitro experiments. To study the deposition of mineral in the collagen sheets, ALP-treated DDS and pieces of dura mater, as well as their respective controls, were incubated in IMDM supplemented with 10% heat-inactivated NRS and antibiotics (10) for varying time periods at 37°C. Each specimen was incubated in 0.3 ml medium containing $1.1 \pm 0.05 \text{ mM}$ phosphate (of which 0.9 mM was inorganic) and $1.6 \pm 0.1 \text{ mM}$ total calcium (10). Radiolabeled calcium ($1 \mu\text{Ci}$ [^{45}Ca]Cl₂/well) was added and the collagen sheets monitored for uptake of the label. As phosphate source β -glycerophosphate (β -GP) was added in a concentration of 10 mM. Media were changed every 2 or 3 d. At the end of the experiment, the specimens were decalcified in 0.5 ml 1 M HCl for 1 h at 37°C. Samples of 300 μl were added to Optifluor® scintillation cocktail (Packard Instrument Co., Inc., Downers Grove, IL) and counted in a Packard Tricarb 4530 scintillation counter.

In vivo experiments. ALP-treated DDS and dura mater were tested for their ability to mineralize under in vivo conditions as follows: female Wistar rats (about 200 g each) were anesthetized with Hypnorm® and an incision was made through the skin covering the skull following the sagittal suture. Pockets were prepared by blunt dissection on both sides of the incision. The samples consisting of DDS (measuring $\sim 0.8 \times 1.5 \text{ cm}^2$ each) or portions of dura mater (measuring about $0.3 \times 1 \text{ cm}^2$ each) were implanted deep into the pockets: ALP-treated ones on the right side of the skull and controls on the left side. The incision was closed with nylon sutures and allowed to heal for time periods varying from 1 to 4 wk. The animals were then anesthetized, the wounds reopened, and the implants excised. A small portion of each implant was removed, together with its surrounding connective tissues, and prepared for histological examination (see next paragraph). The remaining part was freed from soft connective tissues and used for chemical analyses.

Light and electron microscopy. Implants were fixed in a solution of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4, for 24 h at room temperature. They were postfixated in 1% OsO_4 in cacodylate buffer pH 7.4 for 1 h at 4°C, dehydrated through a graded series of ethanols and embedded in LX-112 epoxy resin (Ladd Research Industries, Inc., Burlington, VT). Sections of 2- μm thickness were made with glass knives and stained with methylene

blue, or according to the Von Kossa method. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined in an electron microscope (EM 10C; Carl Zeiss, Inc., Thornwood, NY).

Enzyme histochemistry. To study the distribution of ALP bound to the DDS, cryostat sections were treated according to the indoxyl-tetrazolium salt method (18, 19). 5-Bromo-4-chloro-3-indolylphosphate was used as a substrate. Control sections were incubated without substrate.

Biochemical determinations. ALP activity was determined as follows: specimens were extracted in 0.2 ml glycine buffer (0.1 M glycine, 1 mM MgCl_2 , 0.1 mM ZnCl_2 , pH 10.5) containing 0.1% Triton X-100 for 1 h at 4°C. Then, samples were supplemented with 2 ml glycine buffer (37°C). After 10 min, *p*-nitrophenylphosphate (pNPP) was added as a substrate (final concentration 6 mM) and the optical density monitored at 405 nm by using a Beckman 25 spectrophotometer (model 25; Beckman Instruments, Inc., Fullerton, CA). Enzyme activity (U) was expressed as micromole pNP released per minute (at 37°C and pH 10.5).

Hypro was determined colorimetrically (20, 21). Phosphate was determined according to the method of Kirkpatrick and Bishop (22), and calcium by atomic absorption spectrometry.

X-ray diffraction. DDS were harvested and powdered with a pestle and mortar in liquid nitrogen. The powdered material was washed with a small volume of ethanol, lyophilized, and examined by means of a Philips PW 1327 powder camera with nickel-filtered Cu K_α radiation generated at 50 kV, and 30 mA anode current for 4 h. Pure hydroxyapatite (specific surface area 8 m^2/g) served as a control.

Chemicals and reagents. IMDM, NRS, Fungizone® (amphotericin), streptomycin, and penicillin were purchased from Gibco Laboratories (Grand Island, NY). Lyophilized human dura mater (Lyodura®) was obtained from Braun (Melsungen, Germany). β -GP disodium salt was from Merck (Darmstadt, Germany), multiwell culture dishes from Costar, Data Packaging (Cambridge, MA), pNPP from Sigma Chem. Co., St. Louis, MO. Bovine intestinal ALP (sp. act. 2,500 U/mg) was purchased from Boehringer Mannheim GmbH, Mannheim, Germany, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. HCl from Pierce (EDC) (Oud Beijerland, NL). [^{45}Ca]Cl₂ (1.12 GBq/mg) was from New England Nuclear, (NEN Chemicals, Germany).

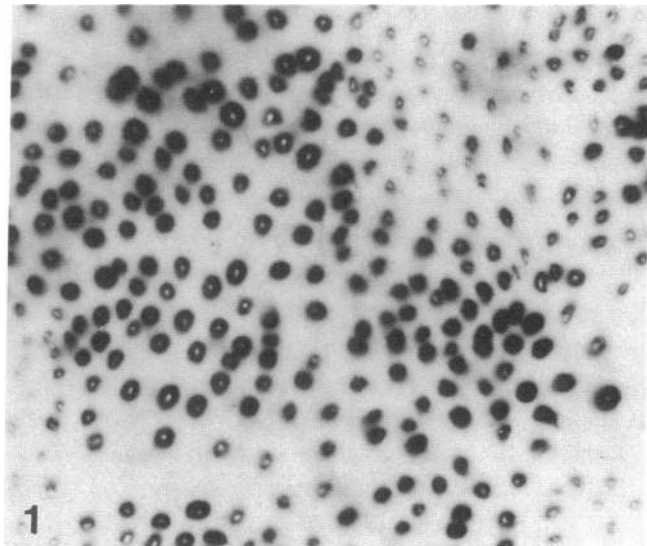


Figure 1. DDS sectioned perpendicular to the dentinal tubules and stained for alkaline phosphatase activity. The dark rings around the tubules represent alkaline phosphatase activity bound to the dentin with carbodiimide. $\times 875$.

Table I. ⁴⁵Ca-uptake in ALP-treated DDS

	+β-GP	-β-GP
DDS-glutar	460±58 (4)	11±8 (4)
Controls	8±5 (4)	
DDS-carbo	427±22 (4)	4±2 (4)
Controls	2±1 (4)	

DDS were incubated for 3 d in IMDM supplemented with 10% NRS. ALP was covalently bound to the dentin by means of glutaraldehyde (DDS-glutar, sp enzyme act 0.92±0.03 mU/μg hypro) or carbodiimide (DDS-carbo, sp enzyme act 1.30±0.29 mU/μg hypro). Controls were treated with the crosslinking agent only. Measurements are given as dpm ×10⁻³±SD (n).

Results

Dentinal collagen slices

In vitro experiments. Histochemistry revealed that in both carbodiimide-treated and glutaraldehyde-treated DDS, the enzyme was not distributed uniformly throughout the dentin. It was particularly bound to the outer surfaces of the sheets and the wall of the dentinal tubules (Fig. 1). Control sections were negative.

As shown in Table I, DDS treated with ALP accumulated radiolabeled calcium when incubated in the presence of β-GP. In the absence of the monophosphate ester very little [⁴⁵Ca] was found in the dentin. When incorporation of the label in carbodiimide-treated specimens was followed as a function of time, a rapid influx of [⁴⁵Ca] was observed during the first day. Thereafter, a more gradual increase was noted (Table II). Control sheets treated with carbodiimide only remained almost free of radioactivity (Tables I, II). ALP-containing slices hardened as time progressed and stained intensely with Von Kossa stain. At the electron microscope level aggregates of mineral were found in close association with the collagen fibrils of the DDS (Fig. 2).

X-ray diffraction revealed spectra that corresponded with those of hydroxyapatite. Next to a prominent peak corresponding to *d* spacings of 2.18, 2.78, and 2.72 Å (planes 211, 112, and 300), distinct peaks at *d* spacings of 3.17 and 3.44 Å (planes 102 and 002), and minor peaks at *d* spacings of 2.26, 1.94, 1.84, and

Table II. ⁴⁵Ca-uptake in ALP-treated DDS and Dura Mater as a Function of Time

	Time in days				
	1	2	3	4	6
DDS	278±105	472±166	419±70	526±216	537±50
Controls	8±5	12±4	7±3	5±1	6±5
Dura mater	8±2	30±14	74±25	108±117	279±234
Controls	10±3	3±0.6	2±0.4	2±0.7	2±0.2

DDS and dura mater sheets were incubated in medium supplemented with 10% NRS and 10 mM β-GP. ALP was bound to the collagen by means of carbodiimide (sp enzyme act in dentin, 0.95±0.51 mU/μg hypro; dura mater, 0.54±0.10 mU/μg hypro). Controls were treated with carbodiimide only. Measurements are given as dpm ×10⁻² per μg hypro±SD (n = 4 per time).

1.72 Å (planes 310, 222, 213, and 004) were observed in the diffractograms. Peak broadening (e.g., the planes 102, 002, and 310) indicated that the mineral was less crystalline than pure hydroxyapatite.

In one experiment, whole rat serum (pH 7.4, 3.2±0.3 mM total phosphate, 2.7±0.1 mM total calcium) was used as incubation medium, without extra phosphate added. It was observed that ALP-treated DDS (crosslinked with carbodiimide) accumulated radiolabeled calcium, whereas control DDS (no enzyme) remained free of mineral (Table III). The rate of ⁴⁵Ca-uptake was less than in IMDM supplemented with 10% NRS and 10 mM β-GP. X-ray diffraction showed spectra typical of hydroxyapatite.

In vivo experiments. Healing of the skin wounds was uneventful. Inspection of the sites of implantation during reentry revealed that, particularly with respect to the carbodiimide-treated implants, the surrounding tissues were not inflamed. Glutaraldehyde-treated implants, however, were often surrounded by a somewhat oedematous connective tissue. In both

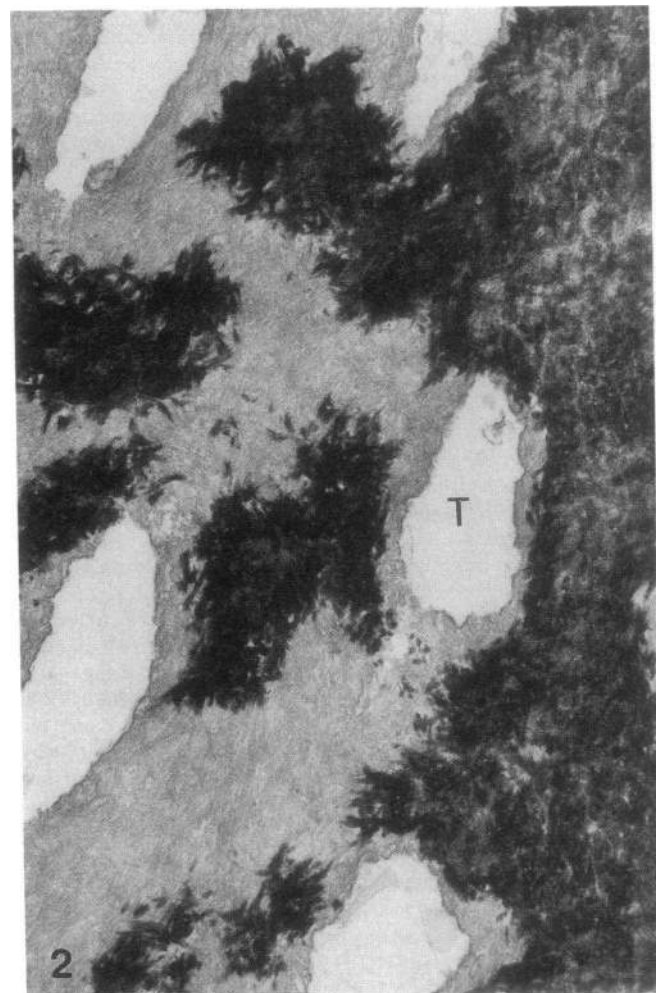


Figure 2. Electron micrograph of ALP-treated DDS (crosslinked with carbodiimide) incubated for 3 wk in IMDM supplemented with 10% NRS and 10 mM β-GP. Electron dense regions represent mineral. Note that mineral aggregates are deposited in close spatial relation to collagen. T, dentinal tubule. ×9,500.

Table III. ⁴⁵Ca-uptake in ALP-treated DDS Incubated in Whole Rat Serum

	Time in days		
	1	4	7
Rat serum			
DDS	4±1 (5)	81±39 (5)	114±46 (6)
Controls	3±1 (5)	5±2 (5)	4±3 (6)
IMDM			
DDS	142±83 (6)	363±53 (6)	397±108 (6)
Controls	5±2 (6)	5±1 (6)	5±2 (6)

For comparison with rat serum, DDS were incubated in IMDM supplemented with 10% NRS and 10 mM β-GP. ALP was bound to the slices with carbodiimide (sp enzyme act 0.91±0.38 mU/μg hypro). Control slices were treated with carbodiimide. Results are given as dpm × 10⁻² per μg hypro±SD (n).

implant types the enzyme-coupled DDS were hard and had acquired an opaque appearance. Control specimens were soft and translucent upon macroscopic inspection.

Chemical analyses of the implants of experiment I (coupled with carbodiimide) demonstrated that mineral uptake by ALP-treated DDS increased as time progressed (Table IV). Both phosphate/hypro and calcium/hypro showed a positive correlation with time (PO₄, *r* = 0.77, *df* = 27, *P* < 0.005; Ca, *r* = 0.73, *df* = 27, *P* < 0.005). In experiment II (Table V) the influx of phosphate and calcium in the carbodiimide-coupled specimens occurred at a more rapid rate. Also, the concentration of mineral ions per microgram hypro after 4 wk was higher than in experiment I. The calcium content per microgram hypro was about 80% of that found in normal bovine dentin, while the phosphate content was about 60%. It must be emphasized, however, that the specific activity of the enzyme in experiment II was higher than that in experiment I (1.30 vs. 0.92 mU/μg hypro).

Table IV. Phosphate and Calcium Content of ALP-treated DDS Implanted in the Rat (Experiment I)

Time	PO ₄ /hypro	Ca/hypro
4 d		
DDS	1.01±0.80 (6)	0.97±0.63 (6)
Controls	0.12±0.04 (8)	0.06±0.05 (8)
7 d		
DDS	2.18±2.00 (8)	1.92±1.81 (8)
Controls	0.12±0.02 (7)	0.06±0.02 (7)
2 wk		
DDS	4.33±1.56 (7)	3.65±1.20 (7)
Controls	0.10±0.02 (9)	0.08±0.07 (9)
4 wk		
DDS	6.30±1.96 (8)	4.97±1.63 (8)
Controls	0.08±0.05 (12)	0.13±0.07 (12)

Enzyme was bound to the DDS with carbodiimide (sp enzyme act 0.91±0.38 mU/μg hypro). Control slices were treated with carbodiimide only. PO₄ and Ca are given as microgram per microgram hypro±SD (n).

Table V. Phosphate and Calcium Content of ALP-treated DDS Implanted in the Rat (Experiment II)

Time	PO ₄ /hypro	Ca/hypro
1 wk		
DDS-carbo	6.95±1.31 (2)	6.07±1.50 (2)
Controls	0.11±0.09 (2)	0.28±0.10 (2)
2 wk		
DDS-carbo	6.98±0.01 (2)	5.81±0.12 (2)
Controls	0.04±0.01 (2)	0.05±0.02 (2)
DDS-glutar	8.05±0.86 (2)	6.62±1.81 (2)
Controls	0.89 (1)	1.36 (1)
3 wk		
DDS-carbo	8.09±0.94 (4)	6.06±0.62 (4)
Controls	0.04±0.02 (4)	0.04±0.03 (4)
DDS-glutar	6.60±3.40 (2)	4.56±2.15 (2)
Controls	0.10±0.00 (2)	0.15±0.01 (2)
4 wk		
DDS-carbo	8.73±1.39 (4)	7.79±1.31 (4)
Controls	0.03±0.01 (4)	0.04±0.01 (4)
DDS-glutar	3.87±3.56 (4)	2.95±2.38 (4)
Controls	0.04±0.01 (4)	0.04±0.02 (4)
Normal dentin	14.02±2.55 (5)	9.47±1.92 (5)

Enzyme was bound to the DDS with carbodiimide (carbo, sp enzyme act 1.30±0.29 mU/μg hypro) or glutaraldehyde (glutar, sp enzyme act 0.92±0.03 mU/μg hypro). Control slices were treated with cross-linking agents only. PO₄ and Ca are given as microgram per microgram hypro±SD (n). For comparison, phosphate and calcium of normal bovine dentin (determined after hydrolysis) are included.

As with the carbodiimide-coupled implants also the glutaraldehyde-coupled specimens accumulated considerable amounts of mineral (Table V). In the latter implant type, however, the amount of mineral seemed to decrease again at the later time intervals.

When expressed in terms of molar concentrations, it appeared that the Ca/P ratio (±SD) in the remineralized dentin was 1.99±0.31 (*n* = 29) in experiment I, and 2.00±0.23 (*n* = 12) in experiment II for the carbodiimide-treated specimens. According to Student's *t* test, this ratio was significantly higher (*P* < 0.005) than that in normal bovine dentin (1.60±0.12; *n* = 5). For glutaraldehyde-treated specimens the Ca/P ratio amounted to 1.80±0.20 (*n* = 8), which was not statistically significant from that in normal dentin (*P* > 0.05).

Light and electron microscopy confirmed the chemical analyses and proved that all experimental (but not control) DDS contained mineral at the time intervals observed (Fig. 3). In most specimens, however, the DDS were not remineralized throughout their entire width. Where the DDS were in direct contact with fibroblast-like cells, a rim of nonmineralized matrix had often persisted (Fig. 4).

The connective tissue surrounding the implants was not mineralized. It contained collagen and fibroblasts which were aligned predominantly parallel to the outline of the remineralized DDS, thus constituting a capsule-like configuration (Fig. 4). No evidence was obtained that the connective tissue was firmly attached to the dentin. No bone, cartilage, or cementum were formed in relation to the remineralized DDS. The connective tissue surrounding the implants contained few inflammatory cells except in the glutaraldehyde-treated specimens

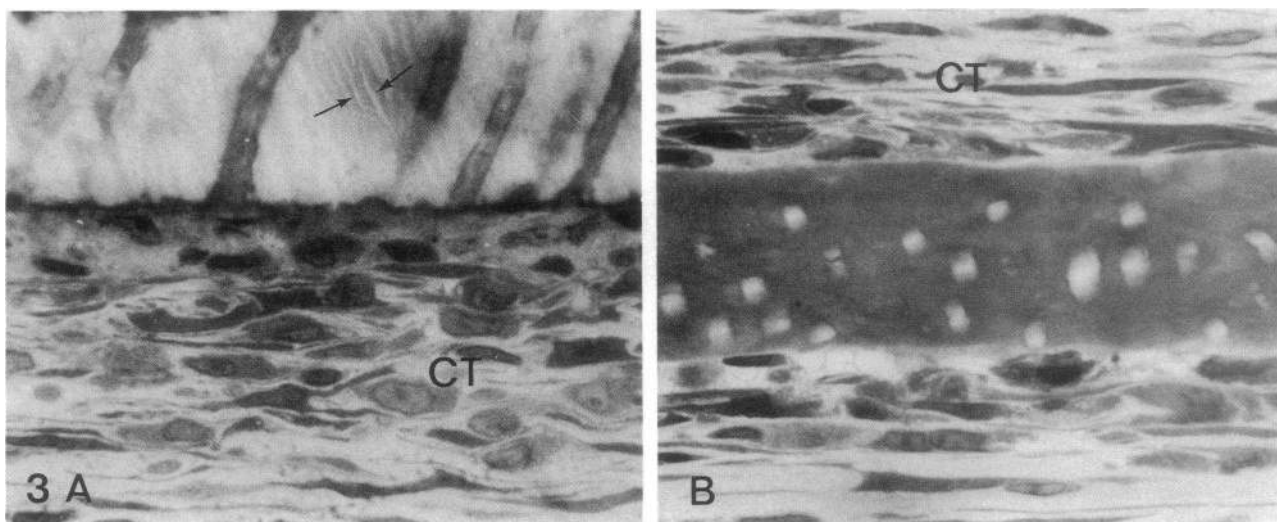


Figure 3. ALP-treated (A) and control (B) DDS implanted subcutaneously in the rat for 4 wk. The enzyme was bound to the dentin with carbodiimide. Note that the DDS in A (sectioned perpendicular to its upper surface) stains faintly with methylene blue and shows many cracks (arrows) caused by sectioning. The control implant in B was not remineralized; it stains intensely with the basic dye and exhibits no cracks. CT, connective tissue. $\times 875$.

where polymorphonuclear leukocytes, but especially macrophages, were frequently found. In the vicinity of the implants, multinuclear giant cells were occasionally observed, particularly in the glutaraldehyde-treated DDS (Fig. 5).

In an additional experiment comprising five animals (experiment III) ALP-treated DDS (carbodiimide coupled) were harvested 3 wk after implantation and examined for hydroxyapatite deposition. X-ray diffraction analysis revealed a profile typical of hydroxyapatite crystals (see above).

Dura mater sheets

In vitro experiments. As with the ALP-treated DDS, the enzyme-treated dura mater sheets accumulated radiolabeled cal-

cium during a 6-d incubation period in the presence of 10 mM β -GP (Table II). The rate at which this occurred, however, was far less than with the dentin. The specific enzyme activity in dura mater was almost half of that in DDS.

In vivo experiments. In contrast with the DDS, the dura mater (which had a lower specific enzyme activity) did not show any tendency to calcify. Chemical analyses revealed that in none of the enzyme-treated implants calcium and phosphate levels were higher than in the control specimens. This was the case at all time intervals observed (1, 2, 3, and 4 wk after installation). It must be noted that healing of the tissues after implantation was uneventful and not accompanied by severe inflammatory reactions.

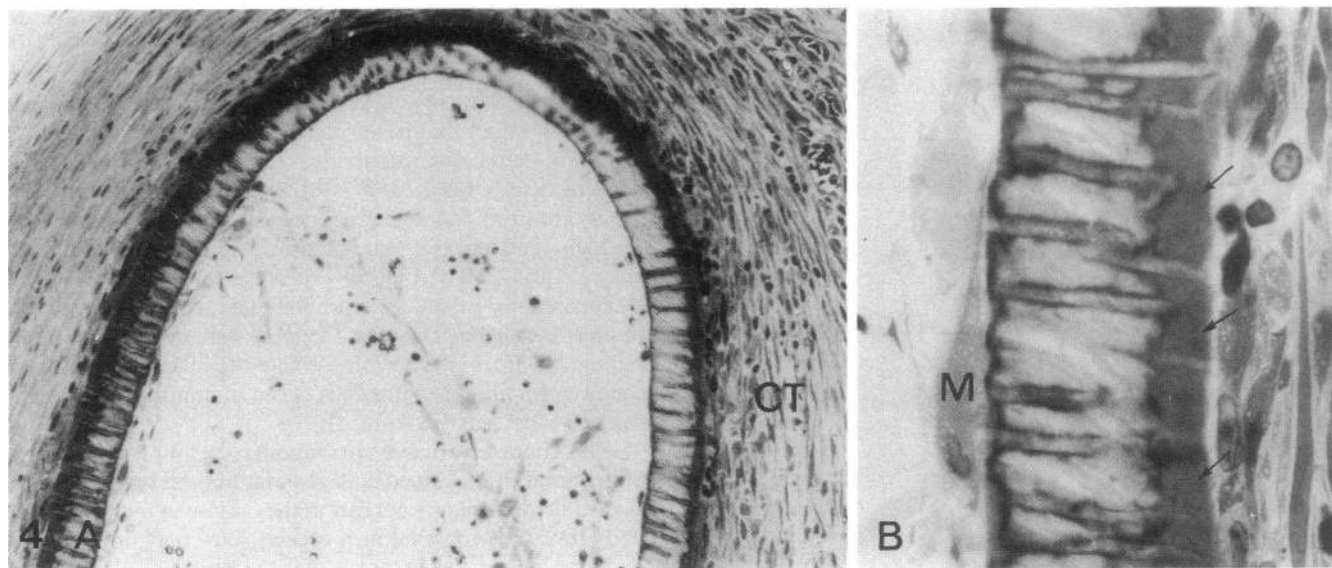


Figure 4. ALP-treated DDS (crosslinked with carbodiimide) implanted subcutaneously for 3 wk. During implantation, the specimen was folded. This resulted in a narrow space which was not populated by fibroblasts. The dentin was remineralized except for its outer layer adjacent to the connective tissue (CT) encapsulating the implant. Nonmineralized parts of the DDS (shown at a higher magnification in B) stain intensely with methylene blue (arrows). M, macrophage-like cell. (A) $\times 220$; (B) $\times 875$.

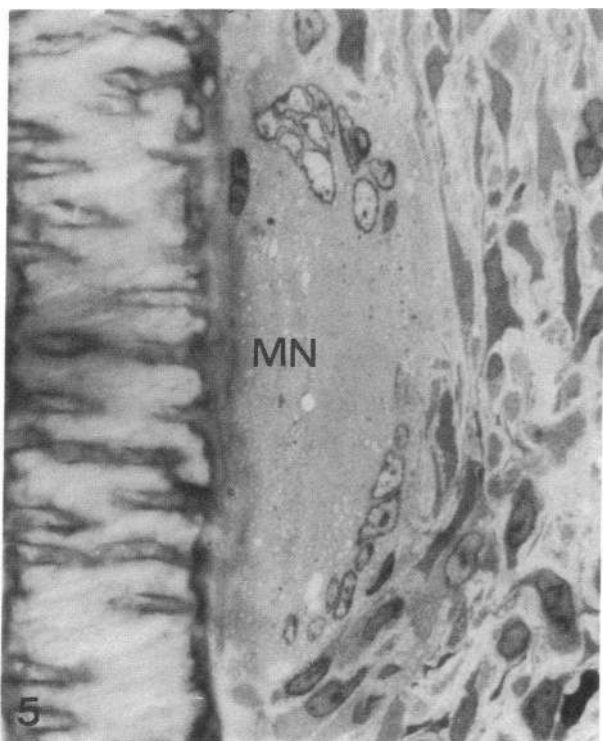


Figure 5. ALP-treated DDS (crosslinked with glutaraldehyde) implanted subcutaneously for 3 wk. Note the presence of a multinuclear cell (MN) adjacent to the remineralized dentin. $\times 875$.

Discussion

The present study has shown that demineralized and extracted dentin slices of $30\ \mu\text{m}$ thickness can be remineralized to a large extent in a short period of time under *in vitro* as well as *in vivo* conditions. The mineral exhibited an x-ray diffraction profile typical of hydroxyapatite. Its deposition was induced by ALP covalently bound to the dentin by the crosslinking agents glutaraldehyde and carbodiimide. Without the enzyme, mineral was not laid down. As far as we are aware, this is the first direct evidence that an ALP can induce the mineralization of a collagenous substrate in the animal body. Since the enzyme was of intestinal origin, the mineral-inducing properties of ALP are not strictly bound to the L/B/K isoenzyme (see Introduction).

According to Nimni and co-workers (17), glutaraldehyde-treated collagen implanted in the animal body is, at the long term, subject to calcification. Levy and co-workers (23) reached a similar conclusion on the basis of their work on subcutaneously implanted type I collagen sponges treated with the crosslinking agents glutaraldehyde or formaldehyde. The degree of calcification of these collagen implants, however, was very low, as compared with ALP-treated dentinal collagen slices. According to the work of the latter authors, glutaraldehyde-treated collagen, implanted for 21 d in weanling rats, contains $0.29\ \mu\text{g}$ calcium/ μg hypro. Our results indicate that ALP-treated collagen can accumulate over 20 times as much calcium per microgram hypro within the same period of time.

As to the acceptance by the body of the implanted collagen sheets, it was noted that at the time intervals observed little signs of inflammation were present with respect to the carbodiimide-coupled sheets. The glutaraldehyde-treated specimens, however, exhibited more signs of inflammation. Besides

fibroblasts encapsulating the implants, macrophages were present and multinuclear giant cells. This suggests that a mild foreign body reaction had occurred (see also reference 17). Perhaps, local cytotoxicity was due to unstable glutaraldehyde polymers that persisted in the interstices of the crosslinked collagenous substrate (24). Further, it was noted that in the specimens treated with glutaraldehyde, a decrease occurred in the mineral content at the later time intervals after implantation. The reason for this is unclear.

From the present results it is evident that there is enough organic phosphate esters present in healing skin for collagen-bound ALP to produce phosphate ions in concentrations high enough to cause precipitation of hydroxyapatite crystals in the DDS. Taking into account that dentinal collagen can mineralize in whole rat serum not supplemented with β -GP, we believe that phosphorylated serum components (leaking into the implantation site) could represent a principal phosphate source in the *in vivo* situation.

Since it was often observed that the dentinal matrix implants did not fully mineralize in regions that were in direct apposition to the fibrous capsule, we consider it unlikely that the bulk of the phosphate esters hydrolyzed by the enzyme originated from fibroblast-like cells. On the contrary, our observations might be interpreted as providing evidence that the soft connective tissues around the implants exerted inhibitory influences on dentin remineralization, thus preventing a rim of dentin from being remineralized.

By using a collagenous tissue that is quite different from dentin in terms of origin, architecture, and composition, we were able to demonstrate that, under *in vitro* conditions, mineral deposition in collagen as induced by ALP is not unique to dentin. Mineral did also accumulate in enzyme-treated human dura mater (thoroughly extracted and freed from noncollagenous components by the manufacturer) when incubated in media supplemented with β -GP (10 mM). Under *in vivo* conditions, however, ALP-treated dura mater did not mineralize within an experimental period of 4 wk.

The reason for this difference with respect to dentin is not yet understood. It is conceivable that the dura mater collagen contained some inhibitory molecules. Alternatively, dentin remineralization was perhaps promoted by traces of phosphoproteins covalently bound to the extracted collagenous matrix (25–30). Another explanation for the difference between DDS and dura mater might be sought in the relatively low specific enzyme activity of the latter. Considerably more ALP activity was bound to the dentinal slices than to the dura mater pieces (Table II). Consequently, the concentration of phosphate ions released by the enzyme could have been too low to initiate mineral deposition *in vivo*. Although several attempts were undertaken to increase the specific enzyme activity of the dura, we did not succeed.

In summary, we have presented direct evidence in support of the view (1) that ALP can play a principal role in the mineralization of collagenous matrices in the animal body. Our findings also indicate that in skin wounds sufficient organic phosphate esters and calcium ions are present to fuel the mineralization process.

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References

1. Robison, R. 1923. The possible significance of hexosephosphoric esters in ossification. *Biochem. J.* 17:286-293.
2. Wuthier, R. E., and T. C. Register. 1985. Role of alkaline phosphatase, a polyfunctional enzyme, in mineralizing tissues. In *The Chemistry and Biology of Mineralized Tissues*, W. T. Butler, editor. EBSCO Media, Birmingham, AL. 113-124.
3. Whyte, M. P. 1989. Hypophosphatasia. In *The Metabolic Basis of Inherited Disease*. Ch. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 2843-2856.
4. Weiss, M. J., D. E. C. Cole, K. Ray, M. P. Whyte, M. A. Lafferty, R. A. Mulivor, and H. Harris. 1988. A missense mutation in the human liver/bone/kidney alkaline phosphatase gene causing a lethal form of hypophosphatasia. *Proc. Natl. Acad. Sci. USA.* 85:7666-7669.
5. Wuthier, R. E. 1982. A review of the primary mechanism of endochondral calcification with special emphasis on the role of cells, mitochondria and matrix vesicles. *Clin. Orthop. Relat. Res.* 169:219-242.
6. De Bernard, B., M. Cherardini, G. C. Lunazzi, C. Modricky, L. Moro, E. Panfili, P. Pollesello, N. Stagni, and F. Vittur. 1985. Alkaline phosphatase of matrix vesicles from preosseous cartilage is a Ca^{2+} binding glycoprotein. In *The Chemistry and Biology of Mineralized Tissues*. W. T. Butler, editor. EBSCO Media, Birmingham, AL. 142-145.
7. McLean, F. M., P. J. Keller, B. R. Genge, S. A. Walters, and R. E. Wuthier. 1987. Disposition of preformed mineral in matrix vesicles. Internal localization and association with alkaline phosphatase. *J. Biol. Chem.* 262:10481-10488.
8. Anderson, H. C. 1989. Biology of disease. Mechanism of mineral formation in bone. *Lab. Invest.* 60:320-330.
9. Tenenbaum, H. C., and J. N. M. Heersche. 1982. Differentiation of osteoblasts and formation of mineralized bone in vitro. *Calcif. Tissue Int.* 34:76-79.
10. Beertsen, W., and T. Van den Bos. 1989. Calcification of dentinal collagen by cultured rabbit periosteum: the role of alkaline phosphatase. *Matrix.* 8:159-171.
11. Beertsen, W., and T. Van den Bos. 1991. Alkaline phosphatase induces the deposition of calcified layers in relation to dentin. An in vitro study to mimick the formation of afibrillar acellular cementum. *J. Dent. Res.* 70:176-181.
12. Khouja, H. I., A. Bevington, G. J. Kemp, and R. G. G. Russell. 1990. Calcium and orthophosphate deposits in vitro do not imply osteoblast-mediated mineralization: mineralization by betaglycerophosphate in the absence of osteoblasts. *Bone (Elmsford).* 11:385-391.
13. Neuman, W. F., V. DiStefano, and B. J. Mulryan. 1951. The surface chemistry of bone. III. Observations on the role of phosphatase. *J. Biol. Chem.* 193:227-235.
14. Fisher, L. W., and J. D. Termine. 1985. Noncollagenous proteins influencing the local mechanisms of calcification. *Clin. Orthop. Relat. Res.* 200:362-385.
15. De Bernard, B., P. Bianco, E. Bonucci, M. Constantini, G. C. Lunazzi, P. Martinuzzi, C. Modricky, L. Moro, E. Panfili, P. Pollesello, et al. 1986. Biochemical and immunohistochemical evidence that in cartilage an alkaline phosphatase is a Ca^{2+} -binding glycoprotein. *J. Cell Biol.* 103:1615-1623.
16. Van den Bos, T., and W. Beertsen. 1987. Effects of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) on the synthesis of dentin matrix proteins in the mouse. *Collagen Relat. Res.* 7:135-147.
17. Nimni, M. E., D. Cheung, B. Strates, M. Kodama, and K. Sheikh. 1987. Chemically modified collagen: a natural biomaterial for tissue replacement. *J. Biomed. Mater. Res.* 21:741-771.
18. McGadey, J. 1970. A tetrazolium method for non-specific alkaline phosphatase. *Histochemie.* 23:180-184.
19. Van Noorden, C. J. F., and G. N. Jonges. 1987. Quantification of the histochemical reaction for alkaline phosphatase activity using the indoxyl-tetra-nitro BT method. *Histochem. J.* 19:94-102.
20. Stegemann, H., and K. H. Stalder. 1967. Determination of hydroxyproline. *Clin. Chim. Acta.* 18:267-273.
21. Guis, M. B., R. N. Sloodweg, and G. J. M. Tonino. 1973. A biochemical study of collagen in the periodontal ligament from erupting and non-erupting bovine incisors. *Arch. Oral Biol.* 18:253-263.
22. Kirkpatrick, D. S., and G. H. Bishop. 1971. Simplified wet ash procedure for total phosphorus analysis of organophosphates in biological samples. *Anal. Chem.* 43:1707-1709.
23. Levy, R. J., F. J. Schoen, F. S. Sherman, J. Nichols, M. A. Hawley, and S. A. Lund. 1986. Calcification of subcutaneously implanted type I collagen sponges. Effects of formaldehyde and glutaraldehyde pretreatments. *Am. J. Pathol.* 122:71-82.
24. Huang-Lee, L. L. H., D. T. Cheung, and M. E. Nimni. 1990. Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived crosslinks. *J. Biomed. Mater. Res.* 24:1185-1201.
25. Veis, A., and A. Perry. 1967. The phosphoprotein of the dentin matrix. *Biochemistry.* 6:2409-2416.
26. Cookson, D. J., B. A. Levine, R. J. P. Williams, M. Jontell, A. Linde, and B. De Bernard. 1980. Cation binding by the rat-incisor-dentine phosphoprotein. A spectroscopic investigation. *Eur. J. Biochem.* 110:273-278.
27. Glimcher, M. J. 1981. On the form and function of bone: from molecules to organs. Wolff's law revisited, 1981. In *The Chemistry and Biology of Mineralized Connective Tissues*. A. Veis, editor. Elsevier/North Holland, New York. 617-673.
28. Lussi, A., M. A. Crenshaw, and A. Linde. 1988. Induction and inhibition of hydroxyapatite formation by rat dentine phosphoprotein in vitro. *Arch. Oral Biol.* 33:685-691.
29. Glimcher, M. J. 1990. The possible role of collagen fibrils and collagen-phosphoprotein complexes in the calcification of bone in vitro and in vivo. *Bio-materials.* 11:7-10.
30. Beertsen, W., and T. Van den Bos. On the role of alkaline phosphatase in the mineralization of collagenous substrates. In *Biological Mechanisms of Tooth Movement and Craniofacial Adaptation*. Z. Davidovitch, editor. EBSCO Media, Birmingham, AL. In press.