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Studies on Dentinogenesis in the Rat

Ethane-1-Hydroxy-1,1-Diphosphonate (EHDP) Inhibits Crystal Growth in Predentin Calcification

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Summary. The effects of ethane-1-hydroxy-1,1-diphosphonate (EHDP) *in vivo* on crystal growth in the early stages of predentin calcification were studied in newborn Sprague-Dawley rats after intraperitoneal injection of 0.1 ml physiological saline solution containing EHDP at a concentration of 100 or 500 μg/ml. The animals were given one injection per day for four consecutive days, thus receiving a total amount of 8 or 40 mg EHDP respectively. Control rats were injected with the same volume of physiologic saline alone. The following electron microscope observations were made: In dentin, the EHDP-treated rats were found to exhibit crystallites of smaller size than the normal controls. In the mineralization zone, most of the needle-like crystallites did not reach 20 Å in width. Their further growth was inhibited, only a few reaching 25 Å in width in the intertubular dentin. Within extracellular membrane-bound bodies or "dentinal globules" normally found in early formed predentin matrix, needle-like crystallites appeared before mineral deposits were visible in the predentin matrix. The crystallites had a paired appearance with a central electron-lucid core. The crystallite pattern was disturbed in EHDP-treated rats. Although less pronounced, similar changes were found also in rats given the lower dose of EHDP.

Introduction

Pyrophosphate in physiological concentrations has been shown to impede the formation as well as the dissolution of apatite crystals *in vitro* (Fleisch *et al.*, 1966). Consequently, hydrolysis of the pyrophosphate layer on the crystals by a pyrophosphatase has been proposed as a possible mechanism regulating calcification (Russell and Fleisch, 1970). Synthetic disphosphonates such as disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) that contain the P-C-P bond are related to pyrophosphate in structure but cannot by hydrolysed biologically. These substances inhibit the growth (Fleisch *et al.*, 1968; Francis, 1969; Francis *et al.*, 1969; Fleisch *et al.*, 1970) and dissolution (Fleisch *et al.*, 1969a; Russell *et al.*, 1970) of hydroxyapatite crystals *in vitro*. *In vivo*, they prevent soft tissue calcification (Fleisch *et al.*, 1968; Francis *et al.*, 1969; Fleisch *et al.*, 1970; Francis and Flora, 1971). They inhibit bone resorption (Fleisch *et al.*, 1969a; Russell *et al.*, 1970) and prevent local osteoporosis due to immobilization in rats (Fleisch *et al.*, 1969b).

EHDP has also been tested as a possible agent for treatment of generalized osteoporosis. In osteoporotic patients, Jowsey *et al.* (1971) found an increase in the osteoid tissue after treatment with EHDP. These changes were considered to be caused by "decreased crystallization". However, no detailed description of the changes was given.

The existence of a specific pyrophosphatase in calcifying tissues has been speculated on but it has not yet been convincingly demonstrated. It seems likely that diphosphonates, acting more or less as a substitute for pyrophosphate, would interfere with a possible pyrophosphate-pyrophosphatase system *in vivo*. As of now, there are no data available on the effects of diphosphonates *in vivo* on the formation, growth and dissolution of hydroxyapatite crystals. In order to elucidate these matters we have studied the effects of EHDP on crystal growth in early stages of predentin calcification. The present communication is part of a series of ultrastructural investigations on dentin formation in the rat.

Material and Methods

Newborn (Spraque-Dawley) rats were injected intraperitoneally with 0.1 cc of a physiological saline solution containing sodium ethane-1-hydroxy-1,1-diphosphonate (EHDP)[1] at a concentration of 100 or 500 μg/cc. These doses of EHDP correspond to 0.5 and 2.5 mg P/kg body weight, respectively. The rats were given one injection per day for four consecutive days, thus receiving a total amount of 8 and 40 mg of EHDP respectively. Control animals were injected with the same volume of physiological saline solution alone.

The lower jaw first molar germs of decapitated rats were quickly dissected out and immersed in ice-cold 4% glutaraldehyde in 0.1 M cacodylate buffer, at pH 7.4. Fixation was continued for 2 hrs at 4°C. Some rats were anesthetized by Mebumal and perfused through their left cardiac ventricle with a) $1^0/_{00}$ Prokain in 0.05 M cacodylate buffer (pH 7.2) containing 0.2 M sucrose for a short time, immediately followed by b) 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) containing 2% Dextran (mwt 20000) for several minutes. Fixation of extirpated molar germs was continued at 4°C in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for two hrs.

Fixation was terminated by rinsing the specimens for a few hrs at 4°C in several changes of the cacodylate-sucrose buffer (pH 7.4). Thin slices of the specimens were cut by free-hand with a sharp razor blade under a stereomicroscope.

The slices were postfixed in 1% OsO_4 in the cacodylate-sucrose buffer (pH 7.4) for 1 hr at 4°C, quickly dehydrated in alcohol and embedded in Epon 812 according to Luft (1961).

Ultrathin sections were cut in an LKB Ultrotome, using glass knives. The sections were floated on 20% alcohol and collected within one minute on naked copper grids. Uncontrasted or uranyl acetate-lead citrate contrasted sections were examined in a Philips EM 300 electron microscope at 60 kV.

Approximately 50 crystallites were measured in each case on electron microscopic prints with a total magnification of 300000 ×. The original magnification in the electron microscope was 30000 ×.

Results

In normal rats, a mineralization zone approximately 3–4 μ wide is observed (Fig. 1). Within this zone, newly-formed needle-like crystallites measure 20–25 Å in width (Fig. 3a). They grow continually to a final width of about 30 Å in the intertubular dentin. The noncalcified prestage of dentin, or predentin, is approximately 15–20 μ wide.

Rats given EHDP were found to exhibit crystallites of smaller size than those observed in normal dentin. These changes were most pronounced in rats receiving the highest dose of EHDP (2.5 mg P/kg body weight). In the mineralization zone, most of the needle-like crystallites did not reach 20 Å in width (Fig. 3b). Their further growth was inhibited with only a few reaching 25 Å in

1 Kindly supplied by the Procter and Gamble Company.

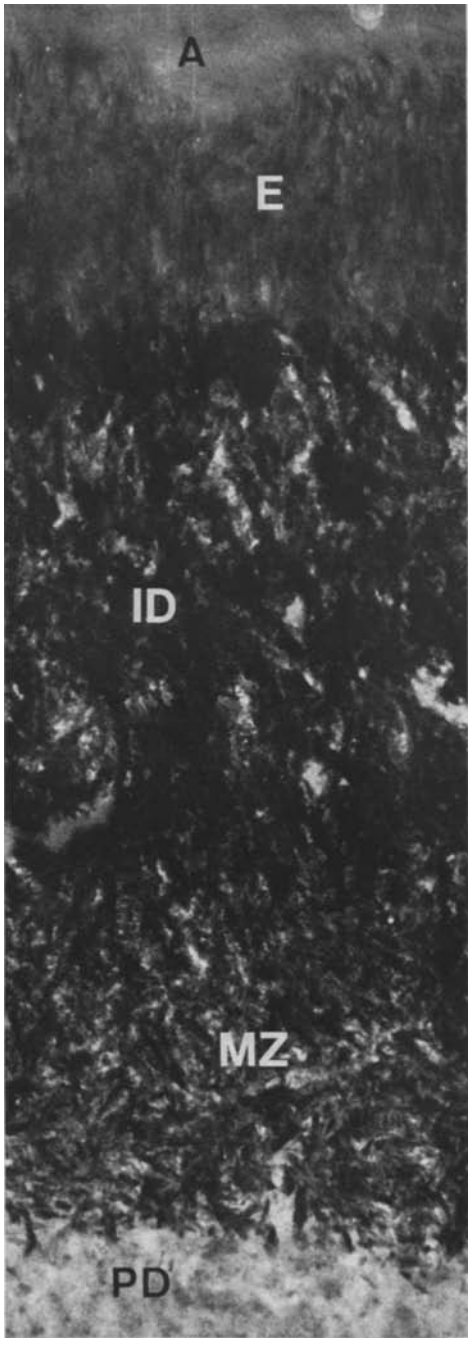


Fig. 1. Survey electron micrograph of calcifying predentin *PD*. A 3–4 μ wide mineralization zone *MZ* is observed in the dentin. A diffuse transformation into intertubular dentin *ID* is caused by continuous crystal growth. Initial enamel *E* formation by ameloblasts *A* is seen at top of figure. Uncontrasted, $\times$ 12000

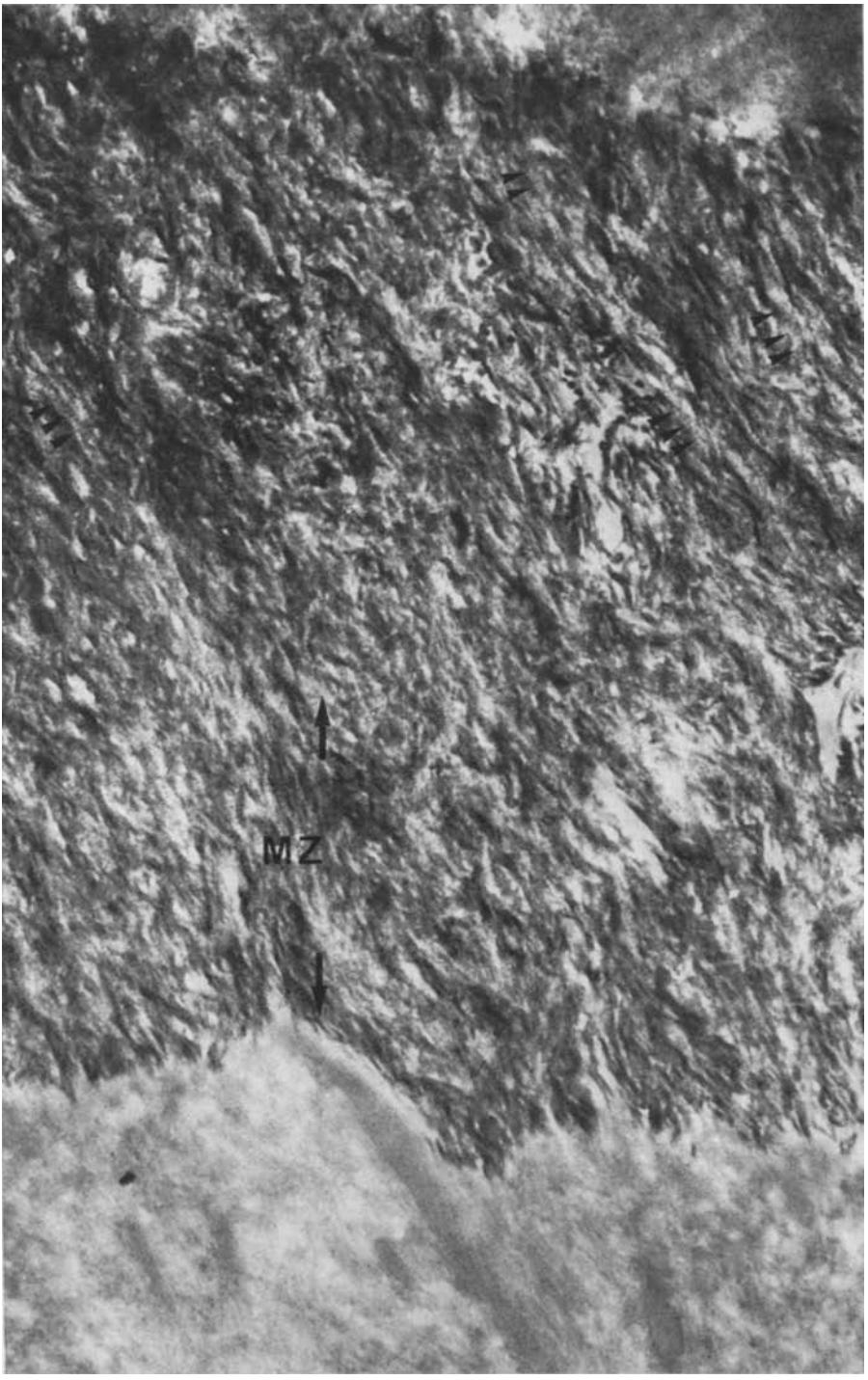
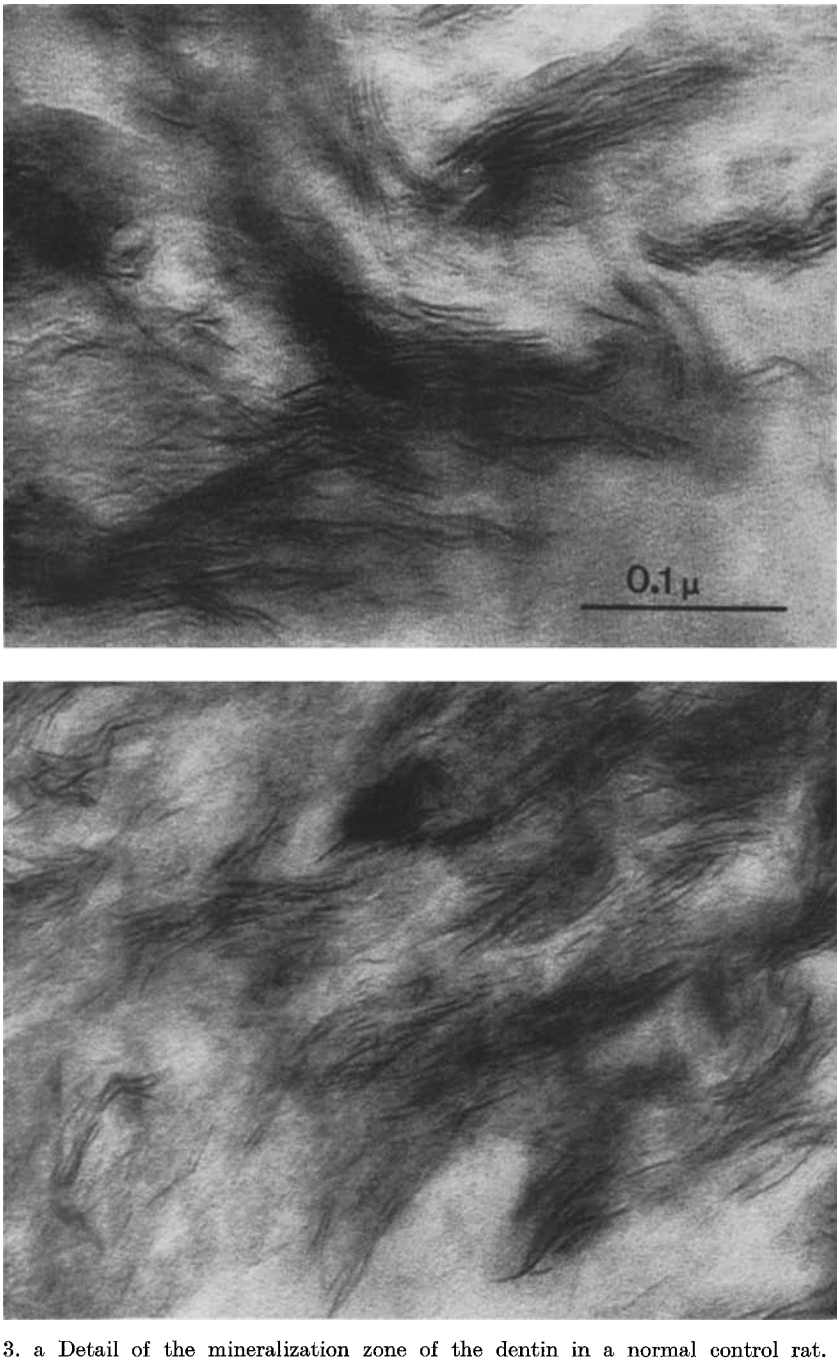


Fig. 2. Rats receiving 500 μg EHDP/kg/day exhibit decreased crystallisation in their dentin. In survey micrographs, a mineralization zone (*MZ*, roughly corresponding to the distance given by long arrows) is no longer distinguishable from the intertubular dentin. Throughout the dentin, a cross-striation is observed indicating decreased crystal growth in the intertubular dentin (short arrows). Uncontrasted, × 12000



a

b

Fig. 3. a Detail of the mineralization zone of the dentin in a normal control rat. The needle-like crystallites measure approximately 25 Å in width. Uncontrasted, × 300000. b Detail of the mineralization zone in an EHDP-treated rat. A smaller crystallite size than normal is observed; the crystallites hardly ever reach 20 Å in width in this zone. Uncontrasted, × 300000

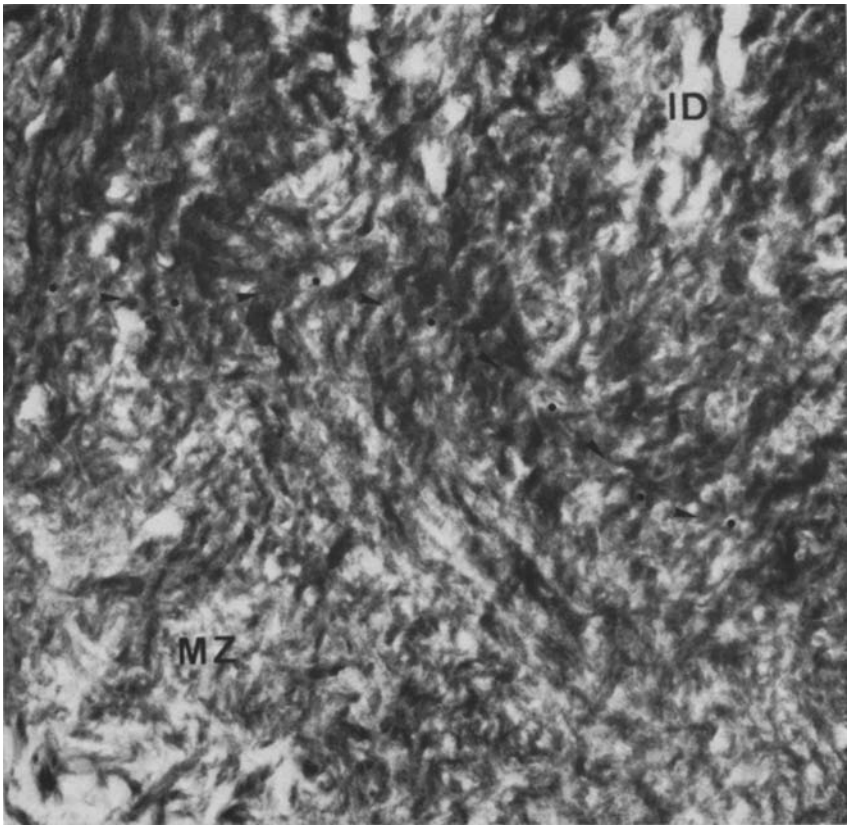


Fig. 4. The border between the mineralization zone *MZ* and the intertubular dentin *ID* is observed (dotted line) in normal rats. The cross-striated pattern within *MZ* disappears gradually because of crystal growth and is no longer observed in *ID* (*cf.* Fig. 2 which illustrates the effect of EHDP). Uncontrasted, × 35000

width in the intertubular dentin. Because of the dense mass of crystallites present in the intertubular dentin it was difficult to obtain good pictures properly illustrating these relatively small differences.

Part of the crystallite formation in dentinogenesis is intimately associated with collagen fibrils. Such fibrils are present in great amounts in predentin where their characteristic cross-striation is visible in contrasted sections. In the mineralization zone, due to a segmented deposition of calcium phosphate upon the fibrils, cross-striation is often observed also in unstained sections (Fig. 4). This striation is normally obscured in the intertubular dentin by lengthwise growth of the crystallites (Fig. 4), overbridging the "overlapping zone of the collagen" (Höhling *et al.*, 1971). In contrast, the EHDP-treated rats displayed cross-striation throughout the dentin including also the intertubular dentin (Fig. 2) which indicated inhibited growth of the deposited crystallites. Neither the width of the predentin nor the dentin zone was influenced by the doses of EHDP used in the present study.

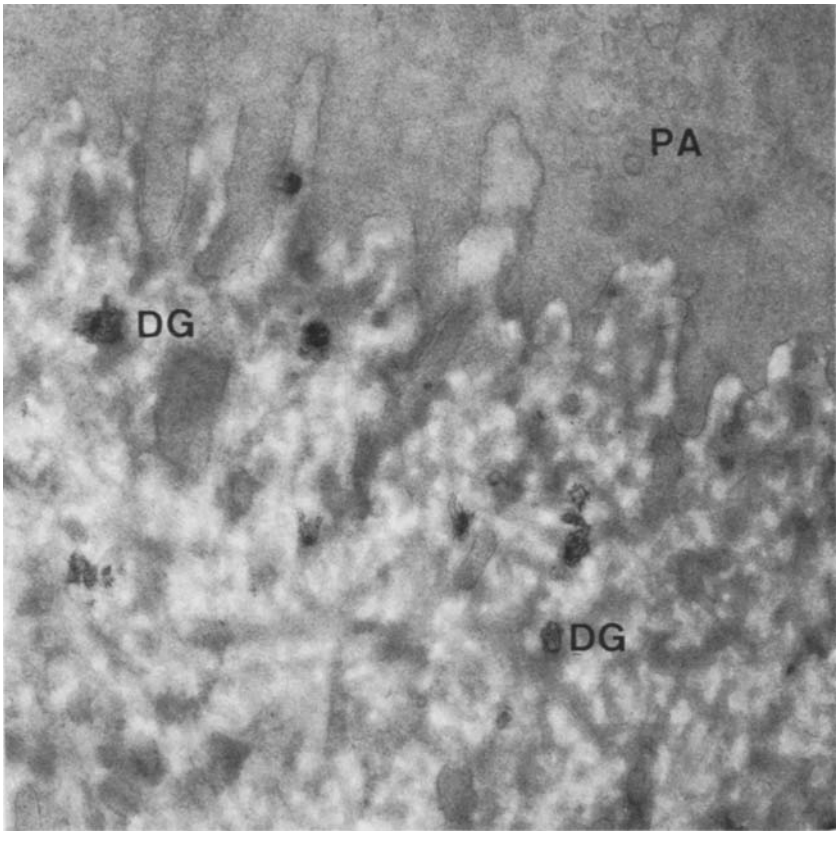
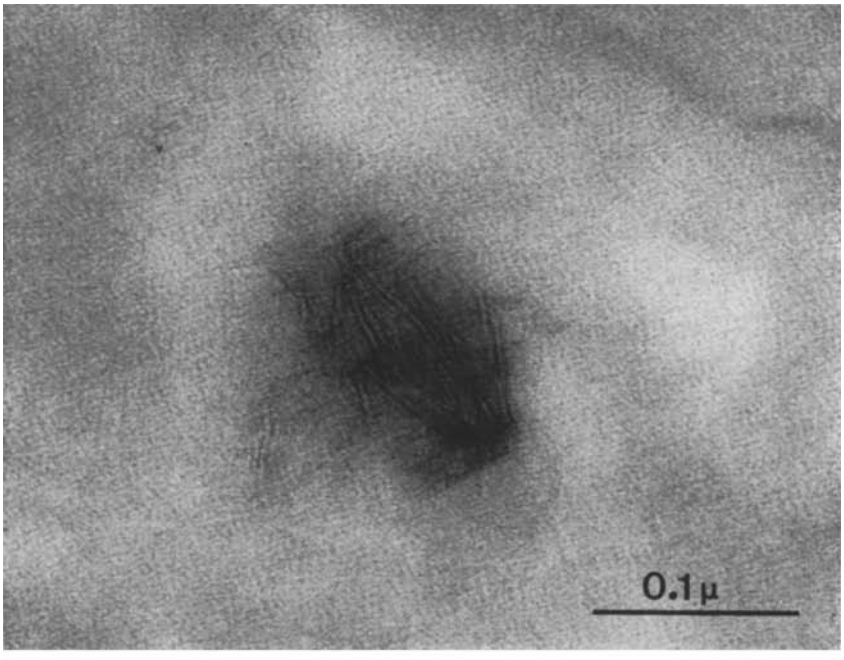


Fig. 5. Electron micrograph of the early stage in dentinogenesis. Globular bodies, or "dentinal globules" *DG* are present in the predentin matrix close to the preameloblastic cell layer *PA*. Needle-like crystallites are formed within these globules before apatite crystals are observed in the surrounding matrix. Uncontrasted, × 35000

The early calcification in dentin formation has recently been related to certain extracellular globular structures. Within these "dentinal globules" needle-like crystallites appear before mineral deposits are visible in the predentin matrix (Fig. 5). The crystallites have a paired appearance (Fig. 6a) with a central electron-lucid core. The pattern of these crystallites was disturbed in the EHDP-treated rats; they became thinner and did not exhibit the distinct paired appearance observed in the normal control rats (Fig. 6b). These observations were based upon studies of approximately 20 globules in each case.

Discussion

The exact mechanism by which diphosphonates exert their effects on the formation, growth and dissolution of hydroxyapatite crystals is not fully understood. An inhibition of heterogeneous nucleation and also of crystal growth has been



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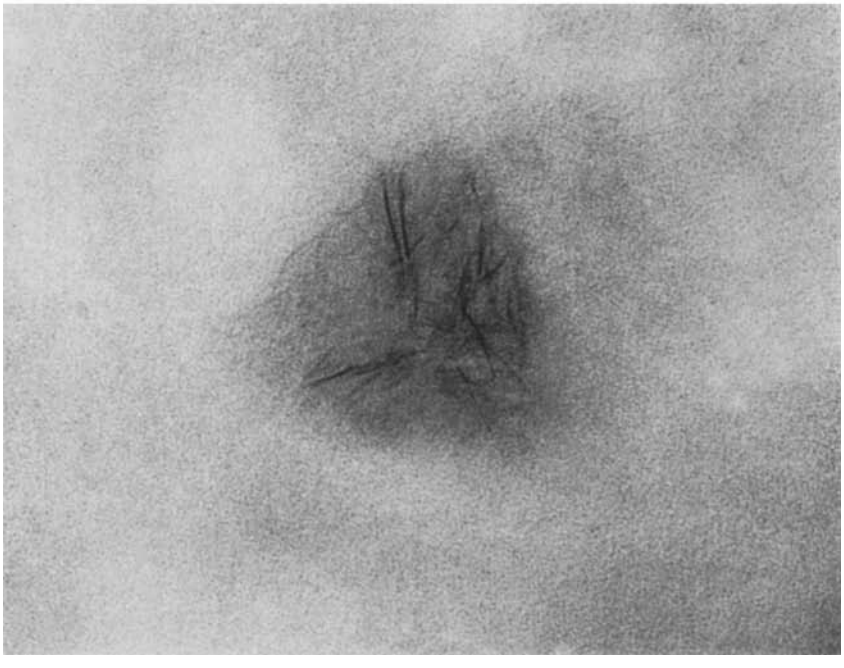

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Fig. 6a and b. "Dentinal globules" in normal and EHDP-treated rats. The paired configuration of crystallites observed in normal rats is not observed in EHDP-treated rats. In the latter, the crystallites are also thinner than normal. Uncontrasted, × 300000

suggested (Russell and Smith, 1972). EHDP has been found to reduce bone turnover rate at low doses while at higher doses it directly prevents the mineralization of bone matrix (Russell *et al.*, 1973). By adsorbing to the earliest formed crystals EHDP may act upon the mineralization step *per se* rather than cell metabolism and matrix production (Russell and Smith, 1973).

In the present investigation, an inhibition of the very initial crystal growth occurring in globules and in the mineralization zone of the dentin was found at doses of EHDP corresponding to 2.5 mg P/kg/day. Also the subsequent crystal growth occurring in the earlier formed intertubular dentin was inhibited in the rat. Unlike bone, dentin is not subjected to resorption under physiological conditions. Therefore, the effect of EHDP appears to be limited to prevention of crystal formation and growth in this calcifying system. With the low doses of EHDP used in the present study there was no apparent inhibition of the initial crystal formation, neither in the globules nor in the mineralization zone.

Apatite formation is considered to be preceded by the formation of an amorphous calcium phosphate (Posner, 1969). EHDP does not prevent the formation of amorphous salt *in vitro* (Francis *et al.*, 1969). In animals treated with higher doses of EHDP than those used in the present study increased osteoid tissue has been observed at the light microscopic level (Jowsey *et al.*, 1971; Russell *et al.*, 1973). A "decreased crystallisation" was proposed as a possible explanation of these changes. With high doses of EHDP (10 or 30 mg P/kg/day), a direct prevention of the full mineralization of new bone matrix was obtained in the rat (Russell *et al.*, 1973). Under these circumstances a complete inhibition of crystal formation might have occurred. At the low doses of EHDP used in the present investigation, the initial crystal formation was apparently not disturbed and the predentin zone showed the normal width. Therefore, the effect of EHDP at low doses appears to be limited to an inhibition only of crystal growth during predentin calcification.

In recent years, interest has focused upon various extracellularly located elements considered to play an active role in governing the precipitation of apatite (see e.g. Bonucci, 1971). "Pseudopods" or "globules" in cartilage (Bonuccci, 1970), "nodules" in bone (Bernard and Pease, 1969) and "crystal bodies" in dentin (e.g. Eisenmann and Glick, 1972) may contain the components necessary for apatite formation. Some of these elements are assumed to be produced by the cells and subsequently released to the surrounding matrix. In initial dentin formation, "dentinal globules" (Larsson and Bloom, 1973) have been considered to play an important role. However, their cellular origin has not yet been demonstrated. In this connection, it is of interest to note that the low doses of EHDP used in the present study did not inhibit globular formation but were found to change the normal crystallite pattern within the globules. The paired appearance of the crystallites found in normal rats might suggest the existence of nucleating factors in an electron-lucid central core. In addition to inhibiting the growth of the crystallites within the globules, EHDP might also interfere with these proposed nucleating sites, since the paired appearance of the crystallites almost completely disappeared in EHDP-treated rats.

In conclusion, the low doses of EHDP used in the present investigation were found to inhibit crystal growth in the mineralization zone and also within the

"dentinal globules". Furthermore, the normal arrangement of crystallites in the globules was disturbed. Preliminary observations on the effects of high doses of EHDP suggest that an almost complete inhibition of predentin calcification can be obtained.

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