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ENZYME HISTOCHEMISTRY OF INDUCED HETEROTROPIC BONE FORMATION IN GUINEA-PIGS

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Summary—In an experimental bone induction model in guinea-pigs, large amounts of heterotopic osteoid and bone were found after 21 days. A semiquantitative intrasectional analysis of oxidative and hydrolytic enzyme activities in the reaction zones showed that matrix-producing cells as well as dentine-resorbing cells had acid phosphatase activity. Leucine aminopeptidase activity was lower in dentinoclasts and macrophages than in matrix-producing cells, which may indicate a function of this enzyme in the production of bone matrix. A substrate-specific ATPase, as well as non-specific alkaline phosphatase and acid phosphatase were active in matrix-producing cells. A close relationship between resorbing cells and matrix-producing cells suggested an inductive mechanism during heterotropic bone formation.

INTRODUCTION

A bone induction model in guinea-pigs, using pieces of demineralized lyophilised dentine as implants, forms bone readily and is suitable for studies on the mechanism of matrix production and dentine resorption (Bang, 1973; Nilsen, 1977). Transmission electron microscopy (TEM) showed 3 main reactions to the implants, (1) resorptive zones; (2) matrix-producing zones having osteogenic and chondrogenic potentials, and (3) fibroblastic zones (Nilsen, 1977).

Studies of enzyme activities in areas of hard tissue formation suggest that certain catalytic processes are important in matrix formation and mineralization as well as in resorption. Thus, high activities of non-specific alkaline phosphatase and ATPase have been recorded in cells associated with the formation of mineralized tissues (Robison, 1923; Göthlin and Ericsson, 1973; Magnusson and Linde, 1974). Activities of acid phosphatase and leucine aminopeptidase, however, have been detected both at sites of formation and, in greater abundance, at sites of resorption (Lipp, 1959; Wergedal and Baylink, 1969; Silberman and Frommer, 1974; Thyberg, Nilsson and Friberg, 1975).

Our principal aim was to study the activities of these hydrolytic enzymes in the resorptive, osteoblastic and fibroblastic zones of the bone induction model. The distribution of oxidative enzyme activities representative of the major energy-producing pathways was studied. Histochemical lipid stains were included to reveal the distribution of unstained hydrophobic lipids which might be related to fatty degeneration and of phospholipids which may induce apatite nucleation (Takazoe, Vogel and Ennever, 1970; Odutuga Prout and Hoare, 1975).

MATERIAL AND METHODS

Five randomly-bred guinea-pigs were fed a standard mixed diet (Norwegian standard for guinea-pigs and rabbits, Statens Institute for Folkehelse, Oslo, Norway) supplemented with swedes, hay and water *ad libitum.* During pentobarbital anaesthesia, dentine implants from four demineralized and lyophilised allogenic teeth were implanted intramuscularly as described by Bang (1973). After 21 days, the animals were killed with ether and the implants with some surrounding tissue were excised and divided into 2 groups. One sample was directly frozen in isopentane (Kebo, Sweden), pre-chilled to about -140°C with liquid nitrogen. The other sample was placed in ice-cold Histocon® (Histo-Lab, Gøteborg, Sweden) for 6 h before being frozen in the same manner. The tissue was sectioned (at 7 μm) in a cryostat (System Dittes-Duspiva, Heidelberg, Germany) and efforts to obtain serial sections were partially successful. Sections for morphological comparison were stained with haematoxylin and eosin and by the van Gieson technique. Phospholipids and free fatty acids were stained by the osmium tetroxide alpha-naphthylamine (OTAN) and the acid haematin techniques (Adams, 1965).

Enzyme histochemical evaluation was made after incubating tissue sections to show the following enzyme activities: $NADH_2$ and $NADPH_2$- diaphorase (Chayen, Bitensky and Butcher, 1973) glucose-6-phosphate dehydrogenase (Altman, 1968), non-specific alkaline phosphatase (azo-dye method, Burstone, 1958); ATPase, lead method at pH 7,2 (Wachstein and Meisel, 1957), calcium-cobalt method at pH 9,4 (Padykula and Herman, 1955); acid phosphatase (azo-dye and lead methods (Barka and Anderson, 1965) and leucine aminopeptidase (LAP) (Nachlas *et al.*, 1960). In addition to incubation with ATP as substrate, Na-β-glycerophosphate was used when the ATPase methods were employed. Incubations were also performed in the presence of 0.5 nM levamisole (Borgers, 1973) with either ATP or Na-β-glycerophosphate as substrate. Also, with these two substrates, some sections were treated for 15 min in 10 per cent aqueous Na_2 EDTA (pH 7.2) at $+4$°C prior to incubation. Quantitation of the enzyme activity between the reaction zones was confined to intra-sectional

Table 1. Enzyme activities recorded histochemically with various enzyme substrates

Enzyme	Recorded enzyme activity in						
	Osteoblasts	Osteoid Osteocytes	Dentinoclasts	Fibroblasts	Dentine tubules	Blood vessel walls	Striated muscle fibres
Alkaline phosphatase	+++	+++	−	−	+++	+	−
ATPase	+	+	++*	+	+	+++	+++
Acid phosphatase	+	++	+++	−	++	−	−
LAP	++	+++	+	+	+	+++	−
$NADH_2$	+++	+++	++	+	+	+	+
$NADPH_2$	++	+++	++	+	+	+	+
G-6-P	++	++	++	++	++	++	++

* Lead method only.
+++ Intense staining. ++ Moderate staining. + Weak staining. − Negligible or no staining.

evaluation. As controls for all the enzyme histochemical methods, incubations were carried out with the substrate omitted from the relevant media. Sections treated in distilled water at 90°C for 10 min were also incubated in the complete media. Dentinoclasts are defined here as multinucleate, dentine-resorbing giant cells, morphologically indistinguishable from osteoclasts (Nilsen, 1977).

RESULTS

The implants gave a high yield of osteoid (Fig. 1) and some bone formation as well as cartilage. The completely demineralized dentine had become remineralized in part after implantation and such areas were not associated with new bone formation. There were many regions of resorption by multinucleated cells. There were no histochemical differences (Table 1) between the material frozen directly and the material stored in Histocon® for 6 h before freezing.

High activities of $NADH_2$- and $NADPH_2$-diaphorase and of glucose-6-phosphate dehydrogenase were located throughout the implantation areas (Fig. 2). Osteoblasts, osteocytes, chondroblasts and dentinoclasts stained strongly, the $NADPH_2$-diaphorase activity of the chondrocytes and deeply seated osteoid osteocytes (Fig. 2) being especially prominent. Dentinal tubules related to osteoblastic zones contained cytoplasmic processes with a high oxidative enzyme activity. The absence of neutral fat and the negative controls confirmed that the reaction product lay in cellular projections in the dentinal tubules. The azo dye method for non-specific alkaline phosphatase gave essentially the same distribution of reaction product as did the ATPase methods with glycerophosphatase as substrate. However, the mineralized areas were not stained by the former method and the staining reactions obtained with the ATPase method at pH 7.2 were less intense than at pH 9.4. Intense activity of non-specific alkaline phosphatase was recorded in osteoblasts, osteocytes, chondroblasts and chondrocytes and in cell processes in dental tubules (Fig. 3). Little or no activity was seen in dentinoclasts (Fig. 3), cells of fibrous zones or striated muscle and there was no activity in the matrix of osteoid.

There was evidence of a substrate-specific ATPase in osteoblasts and chondroblasts as well as in osteocytes, chondrocytes and fibroblasts. Controls using glycerophosphate as a substrate were completely inhibited by EDTA or levamisole which are inhibitors of alkaline phosphatase. Blood vessel walls and striated muscle fibres showed intense ATPase activity both at pH 7.2 and pH 9.4. The osteoclasts were positive only at pH 7.2.

Strong acid phosphatase activity was recorded in the dentinoclasts, monocytes and macrophages of the resorption zones (Fig. 4) and sometimes also in the adjacent dentinal tubules (Fig. 4). The acid phosphatase activity in dentinal tubules in the matrix-producing areas, however, was less pronounced than that of non-specific alkaline phosphatase and that of the oxidative enzymes. Osteoblasts and chondroblasts reacted weakly, whereas osteoid osteocytes and chondrocytes reacted more strongly (Fig. 4). In the osteoid, the acid phosphatase reaction product was intracellular. Both dentinoclasts and macrophages exhibited close contact with cells showing an intense staining for alkaline phosphatase but low acid-phosphatase activity (Figs. 3 and 4). Strong positive reactions for leucine aminopeptidase were seen in osteoid osteocytes (Fig. 5), in cells near blood vessel walls and in scattered monocytic cells. Moderate activity was present in osteoblasts, some dentinoclasts and in cells of the fibrous capsule. In most dentinoclasts, however, the activity was weak (Fig. 5).

In the osteoid, a line indicating the presence of phospholipid was seen with the OTAN and the acid-haematin techniques near the mineralizing bone. Free fatty acids were found only in peripheral areas subjected to the trauma of dissection.

DISCUSSION

Our study confirms previous findings (Bang, 1973; Nilsen, 1977). The duration of the implantation period, 21 days, was selected as at that time all 3 reaction zones are present: resorption of dentine, matrix formation and a fibroblastic area, and the tissue can be sectioned without prior demineralization. The surrounding connective tissue and muscle, were also present, thus facilitating intra-sectional correlation and semiquantitation of the enzyme activities. Examination of serial, or nearly serial, sections also made possible comparisons between routine stains, substance-specific dyes and enzyme histochemistry. Histocon® gave a good preservation, confirming the findings of Heyden *et al.* (1972).

The intense activity of $NADH_2$ and $NADPH_2$-diaphorase in the implantation zones in addition to the moderate activity of glucose-6-phosphate dehydrogenase accords with the absence of degenerative areas in the ultrastructural study of Nilsen (1977) and is evidence of a functional citric cycle and pentose phosphate shunt around the implant.

With $NADH_2$- and $NADPH_2$-diaphorase methods, no marked difference in staining intensity between osteoblasts and dentinoclasts was detected. Fullmer (1966), found higher enzyme activity in osteoclasts than in osteoblasts. He, however, used the incubation period to get a certain formazan formation as a measure of the enzyme activity. The low glucose-6-phosphatase dehydrogenase activity in the dentinoclasts may be due to a low phagocytic activity (Rubenstein and Smith, 1962). Balogh and Hajek (1965) found a similar low activity of this enzyme in osteoclasts in fracture healing whereas the foreign body giant cells exhibited high activity. They also found increased activity in cartilage cells and proposed that this enzyme is important in enchondral ossification.

We found acid phosphatase in all regions of active resorption and in the osteoid and chondroid zones, thus confirming the observations on normal bone formation of Fullmer (1966) and Hammarstrøm, Hanker and Toverud (1971). Bühring (1974), however, did not report activity of acid phosphatase in osteoblasts or osteocytes in heterotopic bone formation. In bone, there is evidence of 2 forms of acid phosphatase (Wergedal, 1970; Hammarstrøm *et al.*, 1971) which could explain the presence of the enzyme in both resorption and formation. We interpret the reaction product in the tubules and channels in the dentine as enzyme activity within the cell processes and not as extracel-

lular activity. The presence of dehydrogenase activity in the same areas and absence of free fatty acids support this view.

Acid phosphatase is considered to be a lysosomal enzyme, although Göthlin and Ericsson (1973), using electron microscopy, and Hanker, Dixon and Smiley (1973), using light microscopy, demonstrated activity also in the Golgi region of osteoblasts and dentinoclasts. The function of the acid phosphatase in mineralization of bone appears to be to reduce the proteoglycan content of the matrix (Baylink, Wergedal and Thompson, 1972); this might explain the increased activity in the innermost part of the osteoid. The increased activity in the cartilaginous areas may then be explained by the higher proteoglycan content of forming cartilage. The mineralizing front in the bone, visualized by staining for phospholipids, contained acid phosphatase activity but not at a high level. Both dentinoclasts and macrophages exhibited an intense activity of acid phosphatase in accord with the findings of Wergedal and Baylink (1969) and Lucht (1971).

The dentinoclasts showed only a moderate to weak LAP activity in contrast to the intense staining of osteoclasts reported in murine bone (Lipp, 1959) and periodontal ligament of the rat (Gibson, 1974; Hasselgren and Hammarstrøm, 1976). The dentinoclasts ultrastructurally had a lysosomal system resembling that of osteoclasts (Nilsen, 1977).

In osteoid and forming cartilage, there was high intracellular LAP activity but no osteocytic osteolysis (Bélanger, 1969). Gibson (1974) found increased activity both in young osteocytes uninvolved in resorption and in osteocytes taking part in bone resorption, whereas Lipp (1959) observed increased activity only in osteocytes thought to have resorptive potential. Hasselgren and Hammarstrøm (1976) showed high LAP activity in cementoblasts but no staining in osteoblasts. The high activity in matrix-producing cells was in keeping with the results of Gibson (1974) and Silberman and Frommer (1974) who proposed that LAP in osteoid is involved in precollagen formation. Martin (1972) found that the α-chains of collagen were 20 per cent shorter than those of precollagen, possibly by action of an aminopeptidase. Our study suggests that LAP may be involved in matrix production as well as in hard tissue resorption.

Non-specific alkaline phosphatase may play a role in biological mineralization. ATPase also may take part in the formation of mineralized tissues (Severson, Tonna and Pavlec, 1968; Heyden and From, 1970; Magnusson and Linde, 1974). Fleisch and Neuman (1961) suggested that ATP, the most important energy- and phosphate-containing intracellular component produced by metabolically-active cells, is one of the phosphates that inhibits mineralization.

Hydrolysis of ATP by several enzymes makes the histochemical identification of ATPase difficult. Enzyme inhibition studies, however, have revealed inhibitors of non-specific alkaline phosphatase (Borgers, 1973; Linde and Magnusson, 1975).

Our finding of activity of a substrate-specific ATPase in osteocytes and osteoblasts agrees with those of Magnusson and Linde (1975) and Linde and Magnusson (1974) who studied normal bone and dentine formation in mice. One function of the non-specific alkaline phosphatase is possibly to split phosphate compounds which inhibit mineralization (Russel, Bisaz and Fleisch, 1969), whereas the substrate-specific ATP may help to transport Ca^{2+} to the mineralization sites (Granstrøm and Linde, 1976).

Inorganic pyrophosphatases, in addition to non-specific alkaline phosphatase, may be involved in mineralization (Alcock, 1972; Larsson 1974), or they may be identical to non-specific alkaline phosphatases (Wøltgens, Bonting and Bijvoet, 1970; Linde and Magnusson, 1975; Lerheim, Linde and Goldie, 1975).

We found non-specific alkaline phosphatase activity in the cells in close contact to resorbing cells which Nilsen (1977) proposed may have a function in the mechanism of bone induction. Huggins and Urist (1970) and Bühring (1974) pointed out that alkaline phosphatase is involved in the induction of heterotopic bone formation.

We believe that the processes involved in mineralization of heterotopic bone are the same as in normal bone formation and that our model is suitable for further studies of bone formation.

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Plate 1.

Fig. 1. Osteoid (O) near to implanted demineralized allogenic dentine (D). Resorption lacunae (arrows). Haematoxylin-eosin × 168

Fig. 2. $NADPH_2$-diaphorase in the area corresponding to Fig. 1 with intense staining of the innermost osteoid (O). Dentine (D) and resorption lacunae (arrows). × 168

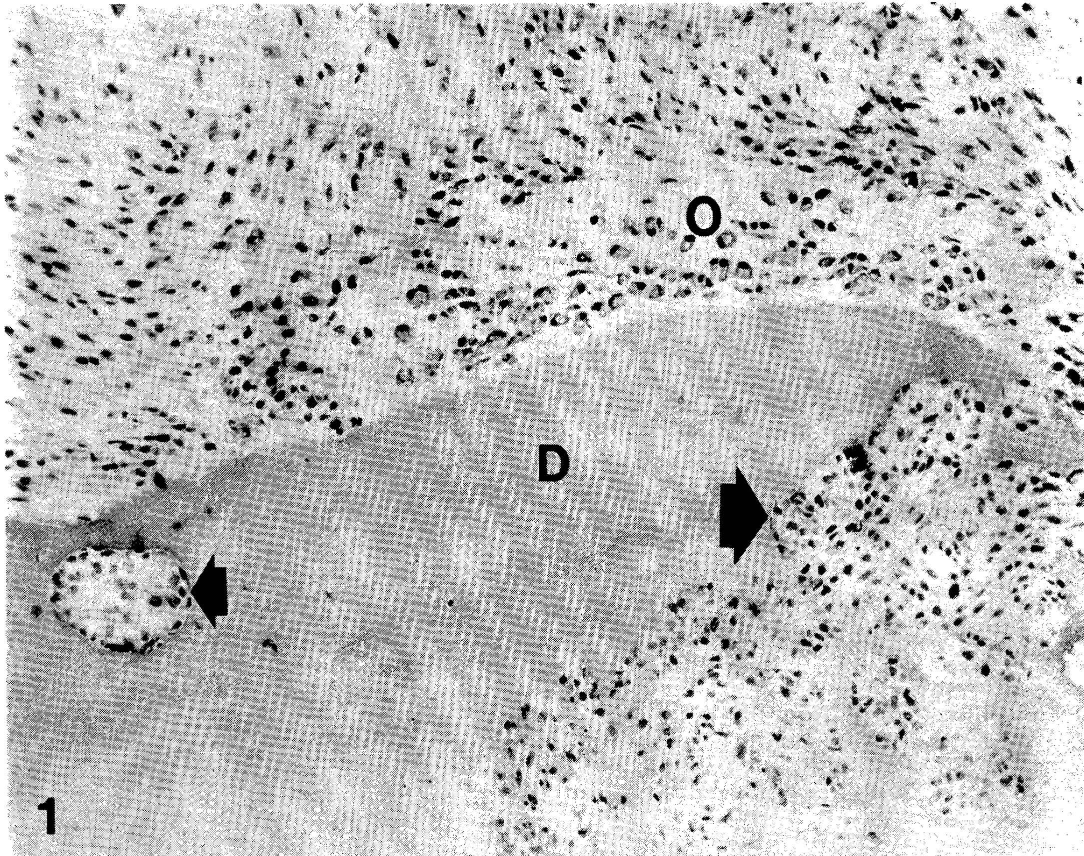
Plate 2.

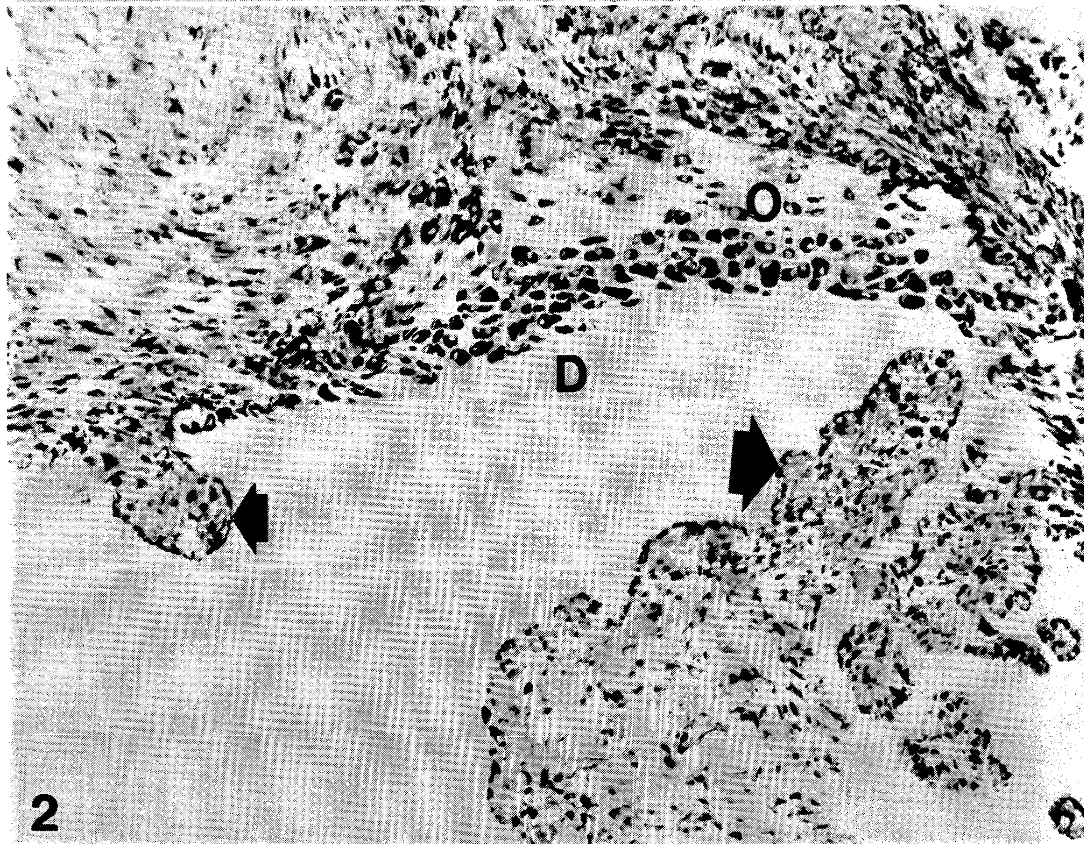
Fig. 3. Non-specific alkaline phosphatase, azo-dye method. Cells in the osteoid (O) with intense staining and only scattered cells with the same intensity in the resorptive area (arrow). Note activity in the dentinal tubules. D, Dentine. Similar area to Figs 4 and 5. × 360

Fig. 4. Acid phosphatase, azo-dye method. Intense staining in the dentinoclasts (arrow) and moderate activity in the osteoid (O). D, Dentine. × 360

Plate 3.

Fig. 5. Leucine aminopeptidase. Dentinoclasts (arrow) with weak staining. Intense staining of osteoid osteocytes O). D, Dentine. × 450





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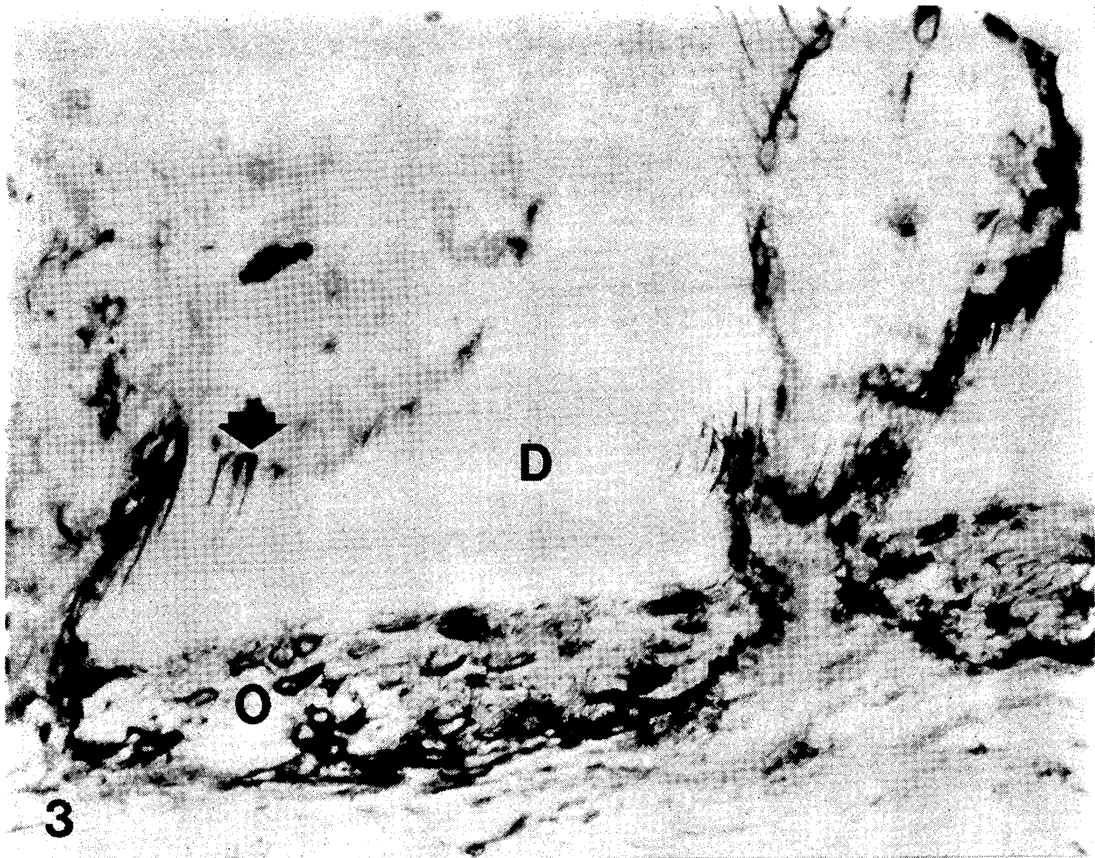

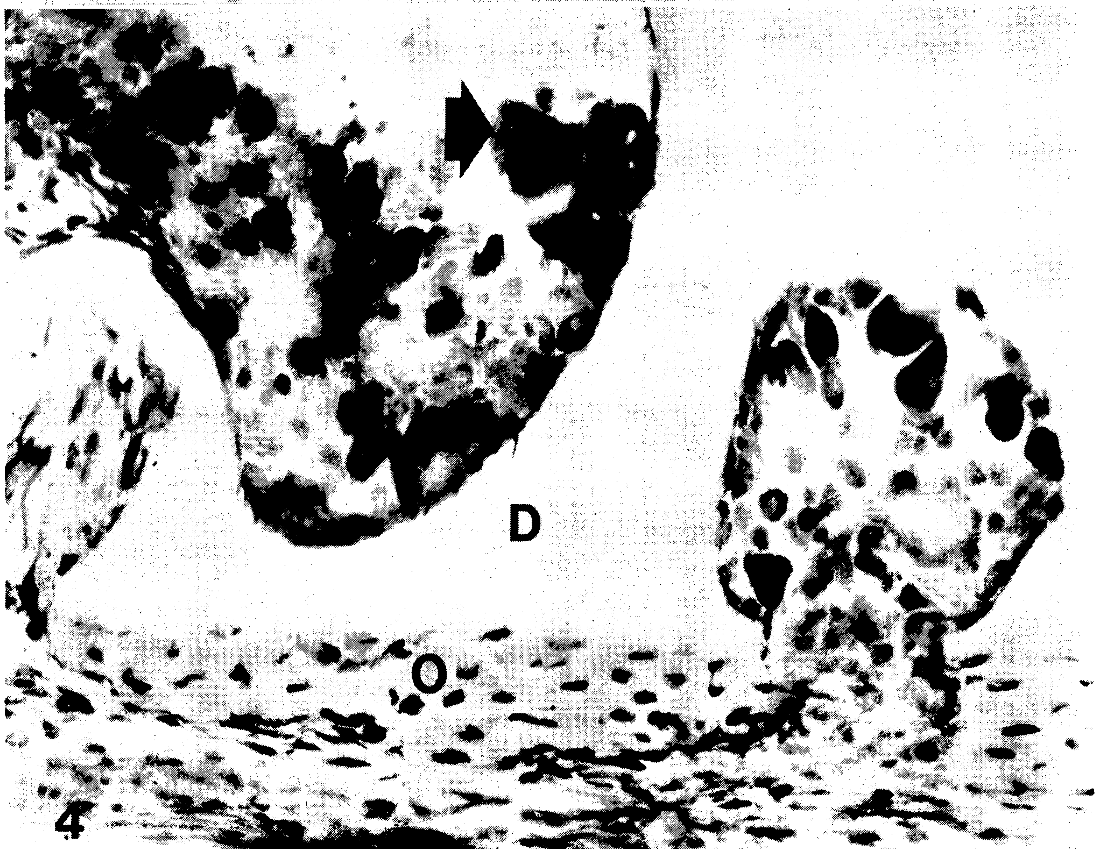

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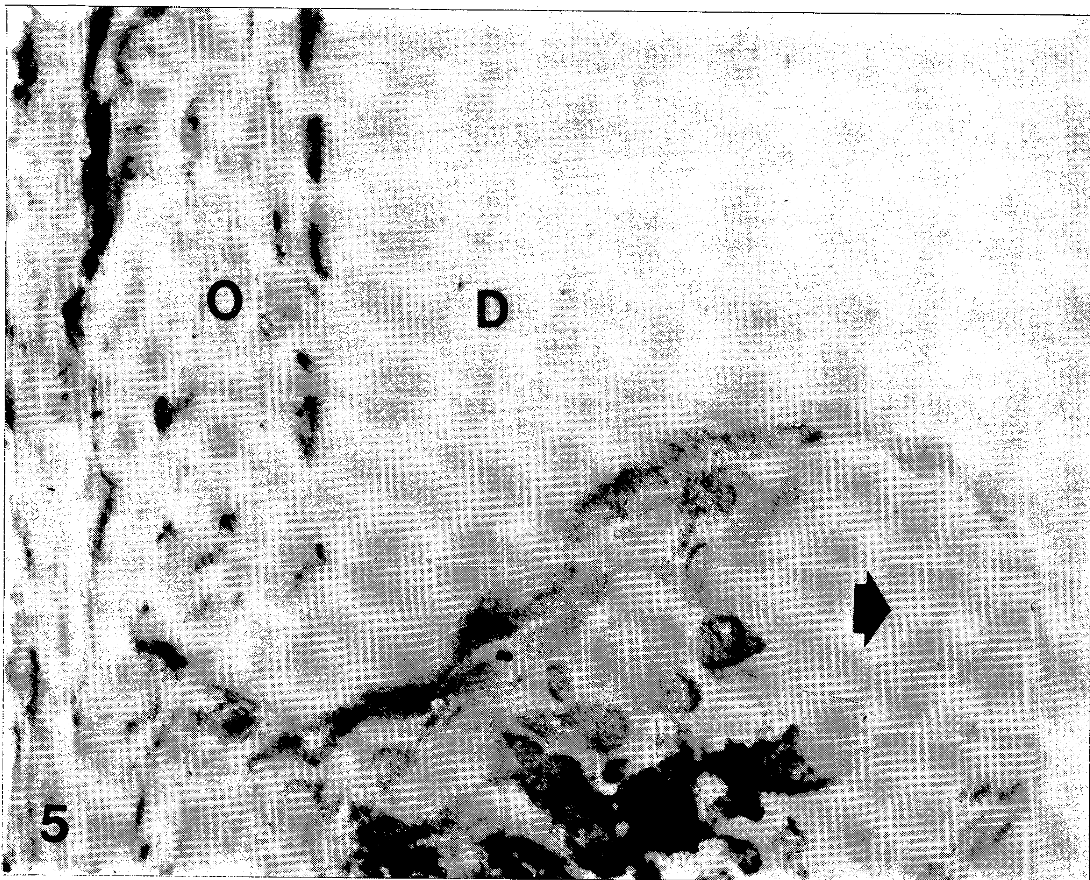

Plate 3.