A histochemical study of arylaminopeptidases and alkaline phosphatases in sound and carious human teeth

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Fresh, undecalcified sections (of 6—20 μm) from intact or carious human teeth as well as teeth with different types of pulpal inflammation were cut in a heavy duty microtome at —20° C. Scotch tape was used to stabilize the teeth during sectioning. The localization of arylaminopeptidases (EC 3.4.1.) and alkaline phosphatase (EC 3.1.3.1.) was demonstrated histochemically using an azo dye principle. The teeth were also examined by microradiography. No arylaminopeptidase activity was normally observed in intact teeth by the histochemical method. In carious teeth, however, this enzyme activity could be localized in the dentinal tubules of the carious lesion, whereas sound dentin, predentin and pulp did not reveal any activity. The inflamed part of the pulp was observed to reveal marked arylaminopeptidase activity. Alkaline phosphatase was observed to be localized mainly in the predentin layer and in the subodontoblastic layer of the pulp and additionally in the cementum and in the remaining parts of the periodontal membrane in intact and carious teeth. This activity disappeared from the odontoblast and subodontoblast zones at exposure due to caries and was observed to precede the inflamed part of the pulp with partial pulpal inflammations. The borderline between sound and carious dentin was detected by microradiography. On the basis of their distribution arylaminopeptidases were considered as »attacking enzymes», whereas alkaline phosphatases were considered as »response enzymes» in the carious process and pulpal inflammations.

Key-words: Histocytochemistry; microradiography; peptide hydrolases; alkaline phosphatase; tooth; dental caries; dental pulp diseases.

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Previous histo- and biochemical studies have revealed the occurrence of about 30 individual enzymes in human carious dentin, excavated *in situ* or obtained from extracted teeth (*Larmas, Mäkinen & Scheinin*, 1968; *Larmas*, 1968, 1972a, b, c, d, e; *Mäkinen, Larmas & Scheinin*, 1969; *Mäkinen*, 1970; *Larmas & Mäkinen*, 1972). These enzyme activities were considered as markers of primary and/or secondary catabolic processes in the dentin undergoing destruction during caries. On the other hand, the occurrence of certain enzymes in the predentin layer of the tooth was thought to indicate that the enzymes involved may also play a role in the normal turnover metabolism of dentin and/or in the response mechanisms of the tissue against carious attack.

Recent improvements in technical apparatus motivated reinvestigation of the occurrence of some of the enzymes in

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whole human tooth. Arylaminopeptidases were selected because: (1) they might be an indicator of protein catabolism, (2) there are controversial opinions concerning their occurrence in developing dentin (see *Hammarström et al.*, 1971; *Larmas & Larmas* 1974), and (3) aminopeptidase B-like enzyme activity might play a role in the preinflammatory peptide formation (*Hopsu et al.*, 1966). Alkaline phosphatase-like enzyme activity was selected because: (1) there is general agreement that there is alkaline phosphatase activity in dentinogenesis, and that the enzyme is present in the pulp cells of the subodontoblastic layer and in developing odontoblasts (*Yoshiki & Kurahashi*, 1971), (2) it was one of the few enzymes demonstrable in the pedentin under a carious lesion (*Larmas*, 1968), (3) this enzyme activity could thus be considered as one marker of the normal turnover reactions in dentin, and perhaps, as one marker of the response mechanisms of dentin during caries.

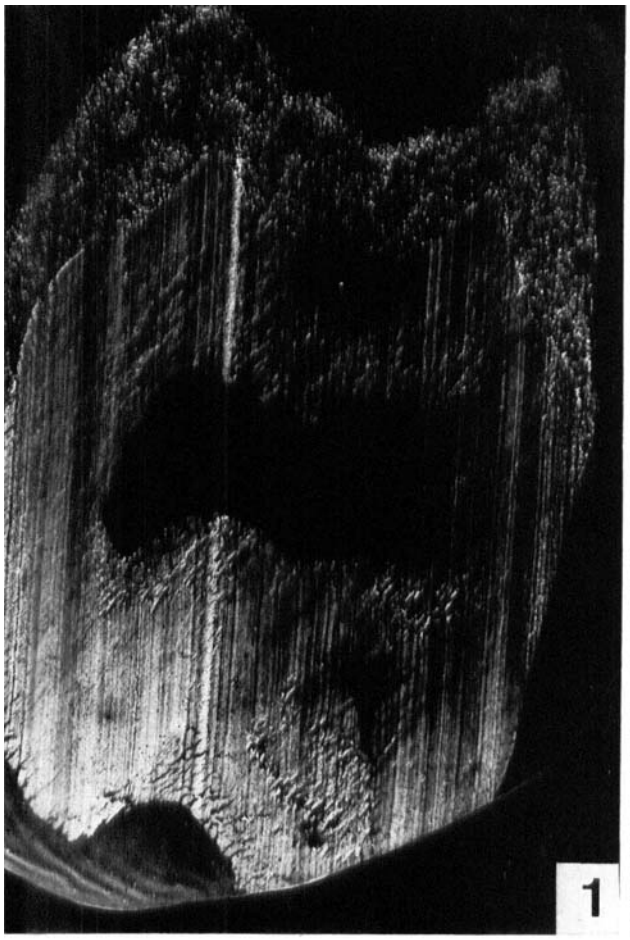
MATERIAL AND METHODS

Teeth. The teeth were rinsed rapidly with cold tap water immediately after extraction and frozen with liquid nitrogen or solid carbon dioxide. Altogether 26 human teeth (with and without carious lesions) and with pulpal inflammation (from acute pulpitis to gangrene) were used.

Sectioning of the teeth. A microtome (Heavy Duty Microtome, Modell K, R. Jung AG, Heidelberg) was placed in a commercial freezer of 550 litre capacity and in order to control the temperature, humidity and air circulation, a thermohydrometric apparatus (Wallac/LKB, Turku) using the LiCl-solution method was employed (design *Lindgren*, 1970). The frozen teeth were imbedded in a 20 % solution of CM-cellulose in water, which was then deep-frozen. A temperature of -18° — -20°C and relative humidity or 20 % — 30 % was found to be optimal for this purpose. The teeth were stabilized during the sectioning with adhesive tape (Microtome tape, 3 M Manufactoring, St. Paul, Minn.) and thereafter the sections were allowed to dry at -20°C. Sections of 6—20 μm were cut.

Microradiography. The sections on the adhesive tapes were pressed firmly against the emulsion of the photographic plates (Scientia, Agfa-Gevaert, Leverkusen or High Resolution Plates, Eastman Kodak Company, Rochester). The microradiography was carried out by using a camera design *Rönning*, 1962) in an x-ray diffraction unit, model Philips P V 1012/10 with a copper target. The target film distance was 30 cm, exposure time 5—7 days at 5—7 kV and 7—8 mA.

Enzyme assay. The azo-coupling principle was selected for the histochemical demonstration of the enzymes mainly because the tissue itself did not contain factors which interfered markedly with the coupling reagent, as stated earlier (*Larmas*, 1972d). The demonstration of arylaminopeptidase activity was carried out in principle according to *Nachlas, Crawford & Seligman* (1957), the modifications of the method being described in detail elsewhere (*Larmas & Mäkinen*, 1972; *Larmas*, 1972e). The substrates used were N-amino acyl 2-naphthylamines (2-NA) of the following L-amino acids: alanine, arginine, leucine and lysine. Fast Blue B (tetrazotized o-dianisidine, Light & Co, Colnbrook) was used in the coupling of the liberated 2-NA. The naphthol phosphate method of *Gomori* (1952) was followed in the determination of alkaline



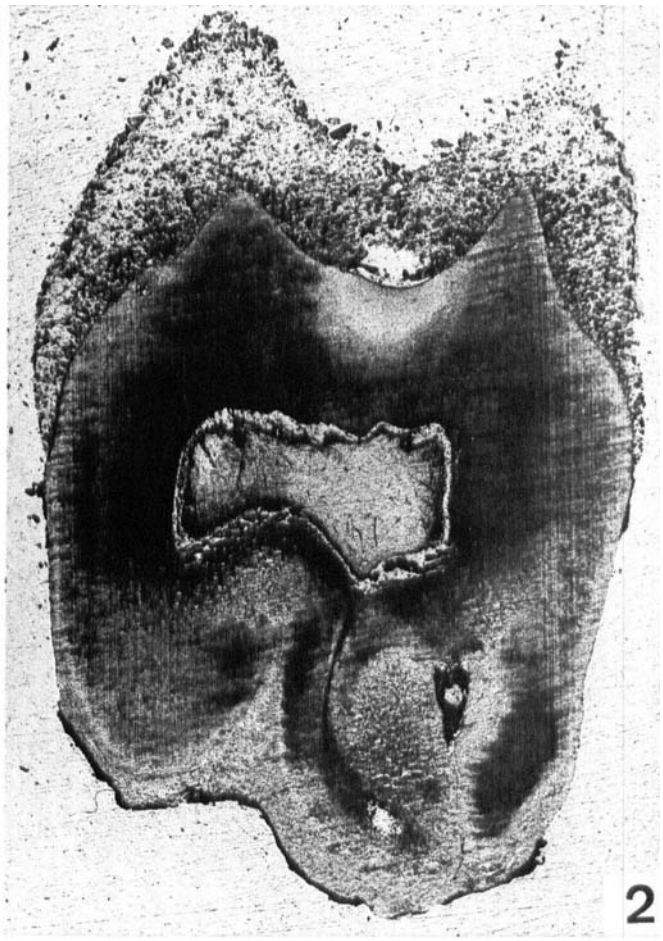


Fig. 1. A microradiogram of a 6 μm section from a human molar tooth with occlusal caries. Strength of current: 7 mA; voltage: 5 kV; time of exposure: 5 days. (9×)
Fig. 2. *Alkaline phosphatase.* An 8 μm section from the same tooth as in Fig. 1. Alkaline phosphatase activity is seen as a black zone in the predentin layer and in the subodontoblastic layer of the pulp, as well as in the cementum and in the remaining parts of the periodontal ligament. Some enzyme activity is seen all over the pulp whereas the carious lesion and sound normal dentine do not reveal any activity. Method: *Gomori*, 1952; substrate: 1-napthyl acid phosphate (0.4 mg/ml), Fast Blue RR, pH 9.2. (9×)

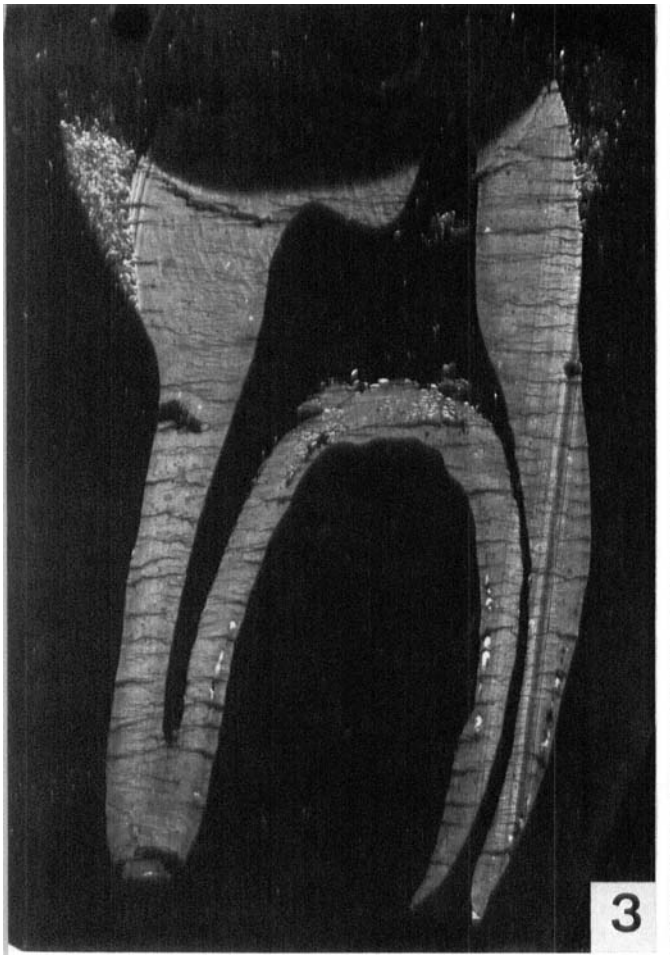
phosphatase activity. The substrate used was sodium 1-naphthyl phosphate (Mann Research Laboratories, New York) and diazonium salt Fast Blue RR (4-benzoyl-amino-2:5-dimethoxyaniline, G. T. Gurr Ltd, London).

All the enzyme activity determinations were performed in the unfixed sections with the adhesive tape attached and the controls described in detail elsewhere were included (*Larmas*, 1972e) i.e. tests to control the diffusion of the enzymes, and end products, the effect of the diazonium salts without the substrates and the avidity of the end products to certain sites of the tissue.

Other methods. Sections stained with hematoxylin and eosin and with the Gram method served for reference.

RESULTS

The apparatus was observed to be suitable for the sectioning of teeht including the soft pulpal tissue. The enamel was crushed during the sectioning, as was the case with the dentin, too. The sections



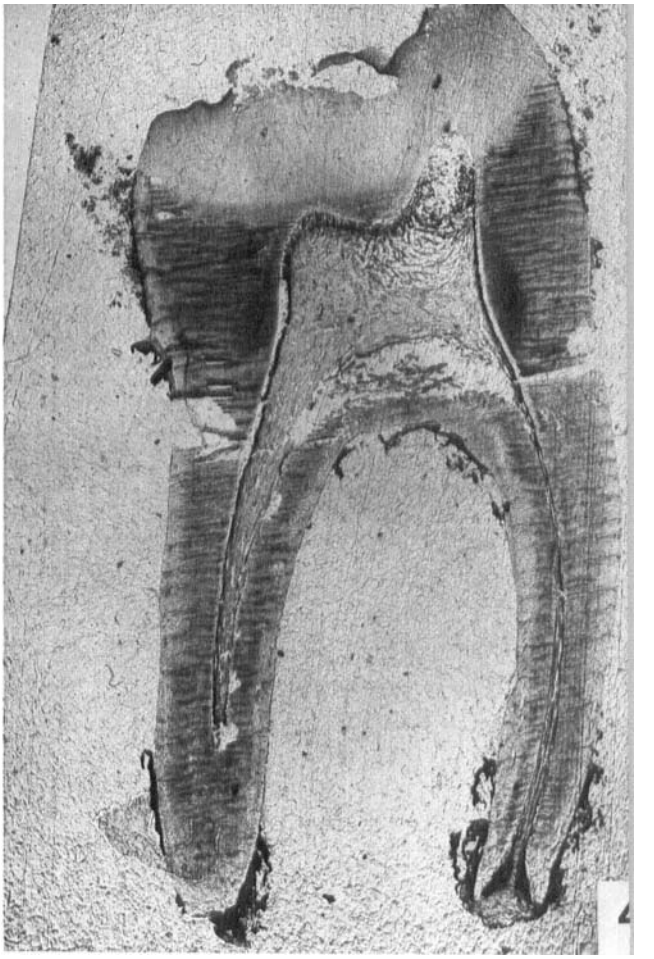


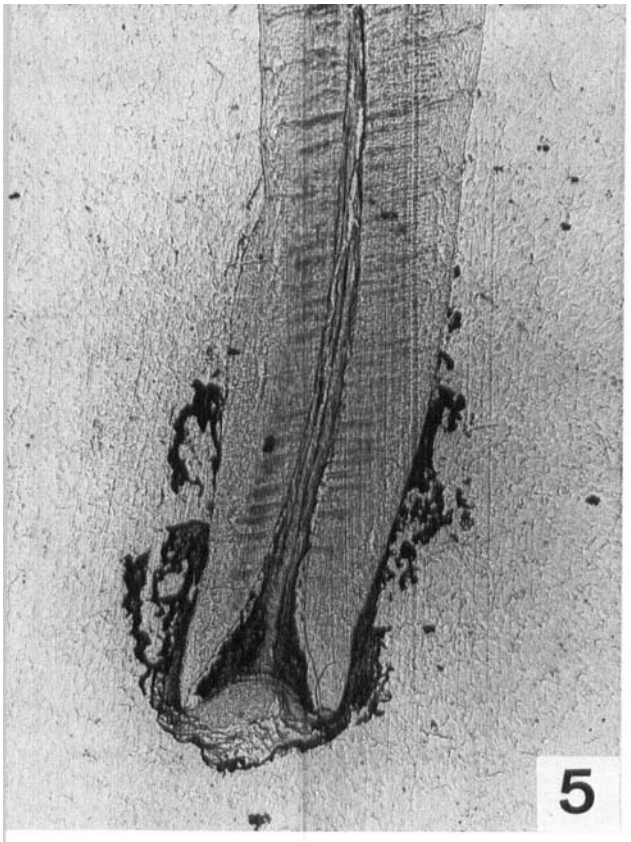
Fig. 3. A microradiogram of a 10 μm section of a young molar tooth with an advanced occlusal caries lesion and pulpal perforation. Strength of current: 8 mA; voltage: 7 kV; time of exposure: 7 days. (9 ×)
Fig. 4. *Alkaline phosphatase.* An 8 μm section from the same tooth as in Fig. 3. The enzyme activity is seen as black zones in the predentin and cementum area. Assay conditions as in Fig. 2. (9 ×)

were, however, observed to be suitable for the purposes of the present study (Fig. 1).

By histochemical enzyme assay methods, no arylaminopeptidase activity could be demonstrated in any structures of the intact teeth. The alkaline phosphatase activity was observed mainly in the predentin layer and in the subodontoblastic layer of the pulp and additionally in the cementum and in the remaining parts of the periodontal membrane.

In sections from carious teeth (Fig. 1) the distribution of alkaline phosphatase was similar to that observed in sound teeth, whereas the carious lesion did not reveal any alkaline phosphatase activity (Fig. 2). The hydrolysis of all the arylaminopeptidase substrates studied was localized in the dentinal tubules of the carious lesion, whereas sound dentin, predentin and pulp did not reveal any enzyme activity.

When the carious process reached the pulp (Fig. 3), the distribution of alkaline phosphatase activity was in principle the same as described earlier (Fig. 4). In the apex region in the young molar the alkaline phosphatase activity zone is seen to surround the area in the apex (Fig. 5). In microradiography this area was seen to be almost unmineralized (Fig. 6). At



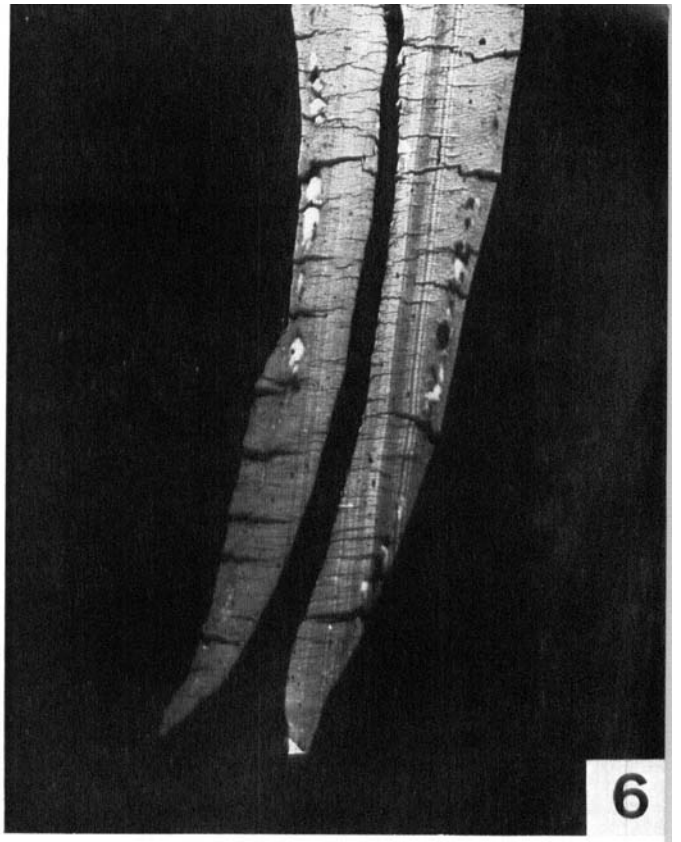
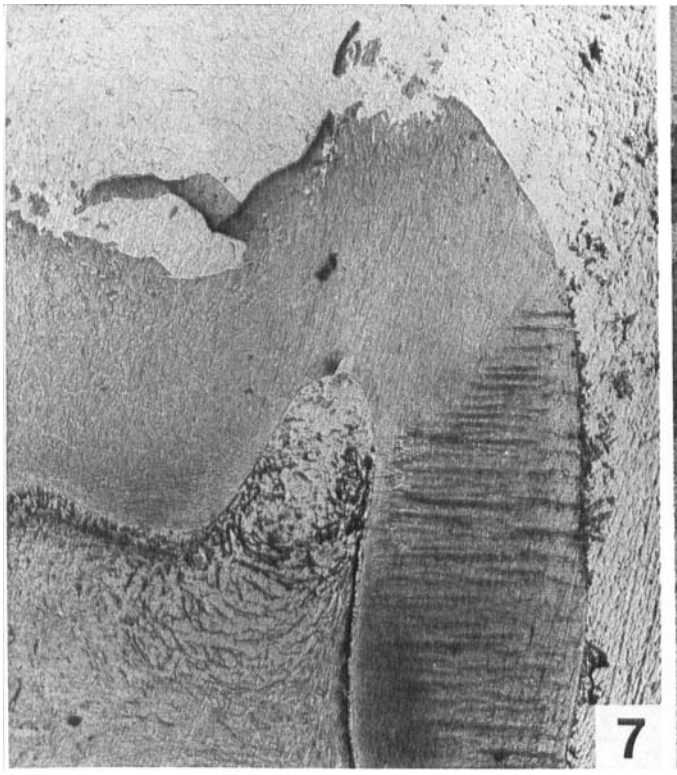


Fig. 5. *Alkaline phosphatase.* A noticeable alkaline phosphatase activity is seen in the mineralizing apex region. Assay conditions as in Fig. 2. (18 ×)
Fig. 6. A higher magnification of the microradiogram from the apex area seen in Fig. 3. revealed that this region was not fully mineralized. (18 ×)

the site of carious exposure of the pulp horn, however, alkaline phosphatase activity disappeared from the odontoblast and subodontoblast zones and a marked activity zone could be observed to surround the inflamed part of the pulp (Fig. 7). This region, on the other hand, was the only one to reveal arylaminopeptidase activity in pulpal tissue (Fig. 8). In this case alkaline phosphatase activity was also seen in the carious lesion. When the pulpal inflammation was classified as gangrene, strong arylaminopeptidase activity was seen all over the gangrenous tissue.

DISCUSSION

These studies are thought to further indicate that arylaminopeptidase activity is connected with the catabolic processes of carious attack. Further, it became evident that this activity is also connected with the pulpal inflammation. The observation that arylaminopeptidase activity was undemonstrable in healthy human pulp does not necessarily mean that there is no activity, because the hydrolysis of arylaminopeptidase substrates is biochemically demonstrable in human and swine (*Mäkinen, Brummer & Scheinin*, 1970) as well as bovine pulp (*Oya et al.*, 1972). The different results concerning the arylaminopeptidases between the biochemical and histochemical studies may simply be due to the fact that the activity involved in healthy pulp is so low or the enzymes so soluble that they are undemonstrable by histochemical means. Another possible explanation may be that in the bio-



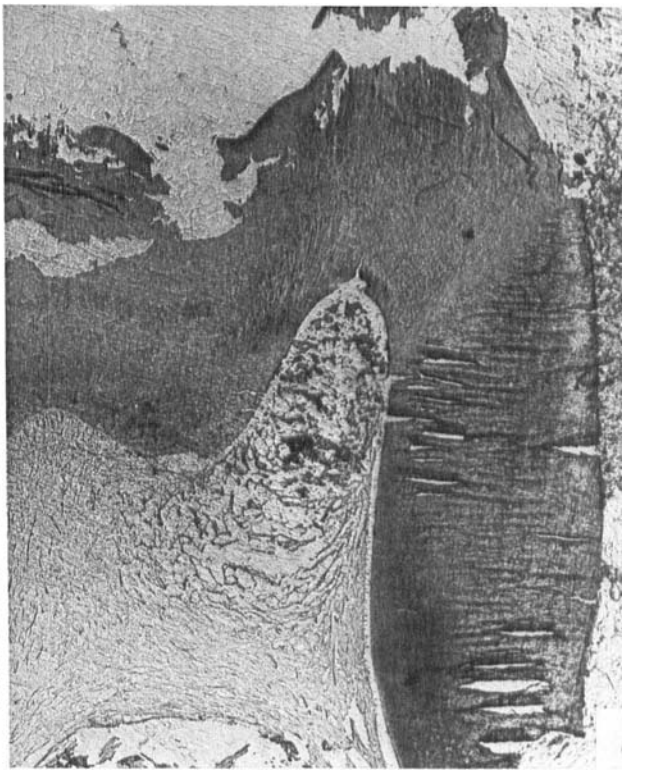


Fig. 7. *Alkaline phosphatase.* A higher magnification from the pulp horn area seen in Fig. 3. Note the disappearance of the alkaline phosphatase from the odontoblasts in the site of carious exposure. In this case alkaline phosphatase is seen to surround the inflamed part of the pulp as well as in the carious lesion. (18×)

Fig. 8. *Arylaminopeptidase.* The same area as seen in Fig. 7. Note that the enzyme activity is seen only in the inflamed part of the pulp. Method: *Nachlas et al.*, 1957; substrate *N*-L-arginyl-2-naphthylamine (0.2 mg/ml), Fast Blue B, pH 6.8. (18×)

chemical studies quoted above the enzymes were liberated from homogenized pulps of extracted teeth. Thus postmortem liberation of lysosomal enzymes may have occurred in these injured tissues. In the present study the teeth involved were dropped into liquid nitrogen immediately and thus severe postmortem changes were less probable, and the pulps involved were less traumatized. The possibility that arylaminopeptidases lost their activity due to the freezing for the histochemical purposes (*Mäkinen et al.*, 1970) seem to be less probable, because the sectioning and incubations were carried out on the day of tooth extraction, and samples stored at —20°C had lost their activity gradually to one third only after 14 days (*Mäkinen et al.*, 1969).

The histochemically demonstrable hydrolysis of N-L-arginyl-2-naphthylamine in inflamed pulp and the finding that aminopeptidase B-like enzyme activity is also biochemically demonstrable in human and swine dental pulp homogenates (*Mäkinen et al.*, 1970) further indicate that pulpal inflammation does not differ from any other inflamed tissue, and thus aminopeptidase B-like enzymes may be responsible for the liberation of mediators, like bradykinin, in the course of the inflammation process in the pulp (*Hopsu et al.*, 1966).

The microradiographic observations support the earlier findings that peptide hydrolases could be localized on the borderline between sound and carious dentin (*Larmas et al.*, 1968, *Larmas & Mäkinen*, 1972) and thus the histological picture of the carious process would indicate

that a kind of proteolysis occurs in the same region as other destructive processes in the carious attack (i.e. for example, the demineralization processes). However, by the present methods it was not possible to demonstrate which process happens first, demineralization or proteolysis, although both processes occur in the same region. The present findings would indicate that in pulpal inflammations arylaminopeptidase activity was displayed in the regions where severe pulpal inflammations occurred whereas alkaline phosphatase was observed outside that region. On the basis of the distribution and with the same limitations as stated above one could classify arylaminopeptidase activity as an »attacking enzyme», whereas alkaline phosphatase activity could be considered as »response enzyme» even in pulpal inflammations.

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